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lncRNA RP5-916L7.2 correlates with advanced tumor stage, and promotes cells proliferation while inhibits cells apoptosis through targeting miR-328 and miR-939 in tongue squamous cell carcinoma

Yong Chen, Yanjun Guo*, Wei Yan

Department of Oral and Maxillofacial Surgery, Cangzhou Central Hospital, Cangzhou, China

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ABSTRACT

Background: This study aimed to investigate the correlation of lncRNA RP5-916L7.2 with tumor features of tongue squamous cell carcinoma (TSCC), and its effect on cells proliferation and apoptosis as well as its target miRNAs in TSCC cells.

Methods: 30 TSCC patients underwent surgery were consecutively enrolled, tumor tissue and paired adjacent tissue were obtained for lncRNAs determination. Blank mimic (NC(+)), lncRNA RP5-916L7.2 mimic (RP5-916L7.2(+)), blank inhibitor (NC(−)), lncRNA RP5-916L7.2 inhibitor (RP5-916L7.2(−)), lncRNA RP5-916L7.2 inhibitor/miR-328-5p inhibitor (RP5-916L7.2(−)/miR-328(−)) and lncRNA RP5-916L7.2 inhibitor/miR-939-5p inhibitor (RP5-916L7.2(−)/miR-939(−)) plasmids were transfected into Tca-8113 cells. qPCR assay, CCK-8 assay, AV/PI assay were performed to detect the miRNA/lncRNA expression, cells proliferation and cells apoptosis, respectively.

Results: lncRNA RP5-916L7.2 was increased in tumor tissue compared with paired adjacent tissue, and correlated with higher T stage, N stage as well as TNM stage in TSCC patients. In vitro experiments revealed that lncRNA RP5-916L7.2 promoted cells proliferation and repressed cells apoptosis in Tca-8113 cells. Subsequently, we selected top five potential target miRNAs of lncRNA RP5-916L7.2, and found that lncRNA RP5-916L7.2 reversely regulated the levels of miR-328-5p and miR-939-5p in Tca-8113 cells. Thus, we conducted rescue experiments, which showed that lncRNA RP5-916L7.2 enhanced cells proliferation and inhibited cells apoptosis through targeting miR-328-5p and miR-939-5p in Tca-8113 cells.

Conclusions: lncRNA RP5-916L7.2 was up regulated in tumor tissue and positively correlated with tumor stage, and promoted cells proliferation while inhibited cells apoptosis by targeting miR-328-5p and miR-939-5p in TSCC.

1. Introduction

Oral cancer, one of the most common head and neck cancers worldwide, presents with higher prevalence in Southeast Asia and an escalating incidence in several parts in Europe, which is resulted from an increase of tobacco use [1,2]. Tongue squamous cell carcinoma (TSCC), the most prevalent oral cancer, is generally accompanied by lymph node metastasis at the initial of diagnosis [3]. Despite that treatments of TSCC, including surgery, chemotherapy, radiotherapy, epidermal growth factor receptor (EGFR) blockers, etc., have broadly progressed, the survival of TSCC patients is still less satisfying compared with other types of oral cancers, which is resulted from a relatively rapid progression of the disease [4]. Thus, more effort is needed for exploring the underlying mechanisms and therapeutic targets of TSCC.

Long noncoding RNAs (lncRNAs), a novel class of RNAs without coding facility with length > 200 bases, often locate in nucleus and have higher tissue specificity compared with coding RNAs [5]. Mounting evidence from clinical studies and laboratory experiments have revealed that multiple lncRNAs function as pro-oncogenic or anti-oncogenic genes in cancer [6–8]. Emerging studies have revealed that lncRNAs promote or repress the progression of various cancers through sponging miRNAs [9–11]. However, very limited information is available concerning the specific regulatory function of lncRNAs in TSCC. One study elucidates that knockdown of lncRNA urothelial cancer-associated 1 (UCA1) has a notable effect on increasing cells apoptosis induced by cisplatin and the sensitivity of chemotherapy in TSCC cells, while another illustrates that lncRNA-EGFR enhances cells proliferation

* Corresponding author at: Department of Oral and Maxillofacial Surgery, Cangzhou Central Hospital, 16 West Xinhua Road, Cangzhou 061001, China.
E-mail address: yanjun_guooo@163.com (Y. Guo).

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and represses cells apoptosis through increasing EGFR expression levels [12,13]. Regarding the potentials of lncRNAs in TSCC, we reanalyzed the data from a previous study that assesses the lncRNAs expression profiles in oral squamous cell carcinoma (OSCC), and selected top five differentially expressed lncRNAs (DELs) as candidate lncRNAs in this present study, and found that lncRNA RP5-916L7.2 expression was up regulated in tumor tissue. Therefore, the aim of our study was to investigate the correlation of lncRNA RP5-916L7.2 expression with tumor features, and its effect on cells proliferation, cells apoptosis as well as its target miRNAs in TSCC.

2. Methods

2.1. Patients and samples

30 TSCC patients underwent surgery from July 2016 to Jun 2017 at Cangzhou Central Hospital were consecutively enrolled in this study. The inclusion criteria were: Diagnosed as primary TSCC according to clinical and pathological confirmation; about to receive surgery treatment; Age above 18 years. Patients with the following conditions were excluded: Secondary TSCC, previous oral surgery, complicated with hematological malignancies or severe infection. Tumor tissue and paired adjacent normal tissue were obtained during the surgery and stored in liquid nitrogen for lncRNAs determination. This study was approved by the Ethics Committee of Cangzhou Central Hospital, and all patients provided informed consents before enrollment.

2.2. Candidate lncRNAs selection and determination of their expressions in TSCC

Five top DELs between oral squamous cell cancer tissue and normal oral mucosa tissue were selected as candidate lncRNAs in this study as shown in Table 1 by analyzing a published lncRNA profiles data with accession number GSE84807 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84807). And the expressions of these lncRNAs in TSCC tumor tissue and paired adjacent normal tissue were determined by quantitative polymerase chain reaction (qPCR) assay.

2.3. Data collection

Patients' characteristics including age, gender, pathological grade and TNM stage were collected. Pathological grade was scored as well differentiation (G1), moderate differentiation (G2) and poor differentiation (G3), TNM stage was assessed according to AJCC 7.0 manual.

2.4. Target miRNAs prediction

Target miRNAs of these five candidate lncRNAs were predicted by using MiRanda, PITA and RNAhybrid methods, and potential miRNAs with standard free energy below −20 by the three methods were selected and the regulation network were presented in Fig. 3 [14–16].

2.5. Cells culture

TSCC cells (Tca-8113) were purchased from Cell Resource Center of Shanghai Institute of Life Sciences (Shanghai, China), and were cultured in 80% RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco, USA) in a humidified incubator under 95% air and 5% CO_2 condition at 37 °C. And the 293a cells were obtained from Cell Resource Center of Shanghai Institute of Life Sciences (Shanghai, China), and were cultured in 90% RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 10% FBS (Gibco, USA) in a humidified incubator under 95% air and 5% CO_2 condition at 37 °C.

2.6. Rescue experiments and related assays

Blank inhibitor (NC(−)), miR-328-5p inhibitor (miR-328(−)), miR-939-5p inhibitor (miR-939(−)), lncRNA RP5-916L7.2 inhibitor (RP5-916L7.2(−)), lncRNA RP5-916L7.2 inhibitor/miR-328-5p inhibitor (RP5-916L7.2(−)/miR-328(−)) and lncRNA RP5-916L7.2 inhibitor/miR-939-5p inhibitor (RP5-916L7.2(−)/miR-939(−)) plasmids were transfected into Tca-8113 cells. qPCR assay was performed at 24 h to determine the lncRNA RP5-916L7.2, miR-328-5p, miR-939-5p expressions, and CCK-8 assay was performed at 0 h, 24 h, 48 h and 72 h to assess Tca-8113 cells proliferation, while AV/PI assay was performed at 72 h to detect Tca-8113 cells apoptosis.

2.7. qPCR assay

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen, USA), and then 1 μg total RNA was applied for the synthesis of cDNA with PrimeScript™ RT reagent Kit (TAKARA, Japan). Subsequently, cDNA product was subjected to qPCR with SYBR Green kit (TaKaRa, Japan). The amplification of PCR was conducted in following conditions: 95 °C for 3 min, 40 cycles of 95 °C for 5 s, 61 °C for 10 s, and then 72 °C for 30s. The expressions of candidate lncRNAs and miRNAs were calculated using the $2^{-\Delta\Delta Ct}$ methods with U6 as the internal reference for miRNAs and phosphoglyceraldehyde dehydrogenase (GAPDH) as internal reference for lncRNAs. The primers used in this study was listed in Table 2.

2.8. CCK-8 assay

10 ul CCK-8 (Abcam, USA) and 90 ul medium were added to each plate, then Tca-8113 cells were incubated under 95% air and 5% CO2 at 37 °C. And optical density (OD) value was measured by microplate reader (BioTek, USA) for measurement of proliferation ability.

2.9. AV/PI assay

After being digested and washed, 2 ul AV (Invitrogen, USA) was added to each plate and the cells were placed in the darkness for 15 min followed by 1 ul PI (Invitrogen, USA) being added, the apoptosis rate was then detected by using flow cytometry (FCM) (Becton Dickinson, USA).

Table 1
Five candidate DELs derived from GSE84807.

Symbols	ID in microarray	ENSG	adj.p.Val	p.Value	t	B	logFC
CTD-2171N6.1	ASHGA5P033616	ENSG00000267013	2.05E-08	2.34E-12	−24.447	17.141	−3.456
RP11-59N23.1	ASHGA5P027877	ENSG00000255644	2.05E-08	2.45E-12	−24.361	17.111	−4.288
RP13-463N16.6	ASHGA5P045921	ENSG00000242147	1.22E-07	2.44E-11	−20.373	15.474	−3.576
RP5-916L7.2	ASHGA5P028370	ENSG00000256276	3.82E-07	1.22E-10	17.952	14.217	6.767
DSG1-AS1	ASHGA5P049985	ENSG00000266729	1.87E-06	7.49E-10	15.545	12.708	2.651

Data were derived from GSE84807 (accession number) on GEO Datasets (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84807).
DELs, differentially expressed lncRNAs.

Table 2
Primers of studied lncRNAs and miRNAs.

	Forward primer	Reverse primer
miR-328-5p	5′-ACACTCCAGCTGGGGGGGGGGCAGGAGGGGCT-3′	5′-TGTCGTGGAGTCGGCAATTC-3′
miR-939-5p	5′-ACACTCCAGCTGGGTGGGGAGCTGAGGCTCTG-3′	5′-TGTCGTGGAGTCGGCAATTC-3′
miR-1268b	5′-ACACTCCAGCTGGGCGGGCGTGGTGGTGGGGG-3′	5′-TGTCGTGGAGTCGGCAATTC-3′
miR-4656	5′-ACACTCCAGCTGGGTGGGCTGAGGGCAGGAGG-3′	5′-TGTCGTGGAGTCGGCAATTC-3′
miR-6795-5p	5′-ACACTCCAGCTGGGTGGGGGGACAGGATGAGA-3′	5′-TGTCGTGGAGTCGGCAATTC-3′
U6	5′-TCGCTTCGGCAGCACATATAC-3′	5′-ATGGAACGCTTCACGAATTTGC-3′
lncRNA CTD-2171N6.1	5′-GGATAGCATTCACGGTCATTAGC-3′	5′-CCTCTTCTCACTTCTCCAATCTCA-3′
lncRNA RP11-59N23.1	5′-CGGACGGCAATCACAGCAT-3′	5′-TTGGGTTTGGAGGGAGGATGA-3′
lncRNA RP13-463N16.6	5′-ATGGAAGAAGCAGCAGGAAACT-3′	5′-CGTTCACTGAGAGCAGAGGAG-3′
lncRNA RP5-916L7.2	5′-CCACAAGGAGATGAGGACACA-3′	5′-AGTTCTGGAGGCTGGAAGTG-3′
lncRNA DSG1-AS1	5′-TGTCATCCAGGTCTTCTCCAAT-3′	5′-CCGCCATCACTCACAGTCTAT-3′
GAPDH	5′-TGACCACAGTCCATGCCATCAC-3′	5′-GCCTGCTTCACCACCTTCTTGA-3′

miRNA, microRNA; lncRNA, long noncoding RNA; GAPDH, phosphoglyceraldehyde dehydrogenase.

2.10. Colony formation

First, the cells were prepared as follows (1) the cells were rinsed with PBS after culture, and then 0.25% trypsin was added, which were incubated at 37 °C for 5 min until the cells appeared round; (2) equal volume medium with 10% FBS was added subsequently, and the cells were detached by pipetting; (3) afterward, counting the cells using a hemocytometer (Isolab, German), and preparing desired seeding concentration, and then seeded the cells into dishes or 6-well plates. Then the cells were plated after treatment as follows (1) 500 cells were plated, serial dilutions with different numbers of cells were prepared and then the cells were incubated in a CO_2 incubator at 37 °C for 7 days until cells in control plates had formed colonies with substantially good size (50 cells per colony is the minimum). Subsequently, the medium was removed and the cells were rinsed with PBS twice and fixed with formaldehyde (Sigma, USA) for 15 min, after which the formaldehyde was removed and the cells were rinsed with PBS twice in the air. Finally, the cells were dyed by the crystal violet (Sigma, USA), and the relative colonies were counted under a stereomicroscope.

2.11. Luciferase assay

Growth medium was removed from the cells, which were rinsed by PBS twice afterward. Then a minimal volume of 1 x CCLR (cell lysis reagent) was added to cover the cells, which were incubated for 5 min at room temperature. Subsequently, the attached cells were scraped free from the culture dish, which was transferred to a microcentrifuge tube. Afterward, the cells were centrifuged for 5 s at 12,000 rpm to pellet the cell debris, and then the supernatant (cell extract) was transferred to a new tube and the pelleted cell debris was discarded. Then 20 μl of cell extract was mixed with 100 μl Luciferase Assay Reagent at room temperature, and the mixture was placed in a luminometer.

2.12. FISH

The DNA oligo probes (Sangon, China) were put in 0 °C for 5–10 min after a 5 mins incubation with 75 °C water. Then the cells were grown to the exponential phase and were 80%–90% confluent before fixation with 4% formaldehyde (Sigma, USA) and permeabilization with 0.1% Triton X-100 (Sigma, USA), after which, the cells were washed by quench buffer for 10 mins and was then washed with PBS buffer thrice. Then the cells were hybridized with hybridization buffer with DNA oligo probes at 37 °C overnight, and the next day the cells were rinsed with 2× Saline-sodium citrate (SSC) (Sigma, USA) buffer by 4, 6-diamidino-2-phenylindole (DAPI) (Invitrogen, USA), and the images were obtained under a confocal microscope.

2.13. Statistics

Statistical analysis was performed by using SPSS 21.0 software (IBM, USA), and graphs were drawn using Graphpad Prism 5.01 software (GraphPad Software Inc., USA). Data were mainly presented as mean ± standard deviation, mean ± standard error, median (1/4–3/4 quantiles) or count (percentage). Comparison between two groups was determined by Wilcoxon rank sum test or *t*-test. Correlation was determined by Spearman test. $p < .05$ was considered significant.

3. Results

3.1. Characteristics of TSCC patients

The mean age was 51.1 ± 7.9 years in TSCC patients in our study, among whom there were 24 (80.0%) males and 6 (20.0%) females (Table 3). The numbers of patients with pathological grade G1, G2 and G3 were 4 (13.3%), 21 (70.0%) and 5 (16.7%), respectively. In addition, the numbers of patients at T1, T2 and T3 stage were 6 (20.0%), 15 (50.0%) and 9 (30.0%), respectively. And the numbers of patients at N0, N1 and N2 stage were 19 (63.3%), 10 (33.3%) and 1 (3.3), respectively. As for the TNM stage, 5 (16.7%), 12 (40.0%), 12 (40.0%) and 1 (3.3%) patient was at stage I, II, III and IV, respectively.

Table 3
Characteristics of TSCC patients.

Parameters	TSCC patients (N = 30)
Age (years)	51.1 ± 7.9
Gender (n/%)	
Male	24 (80.0)
Female	6 (20.0)
Pathological grade (n/%)	
G1	4 (13.3)
G2	21 (70.0)
G3	5 (16.7)
T stage (n/%)	
T1	6 (20.0)
T2	15 (50.0)
T3	9 (30.0)
N stage (n/%)	
N0	19 (63.3)
N1	10 (33.3)
N2	1 (3.3)
TNM stage (n/%)	
I	5 (16.7)
II	12 (40.0)
III	12 (40.0)
IV	1 (3.3)

TSCC, tongue squamous cell carcinoma.

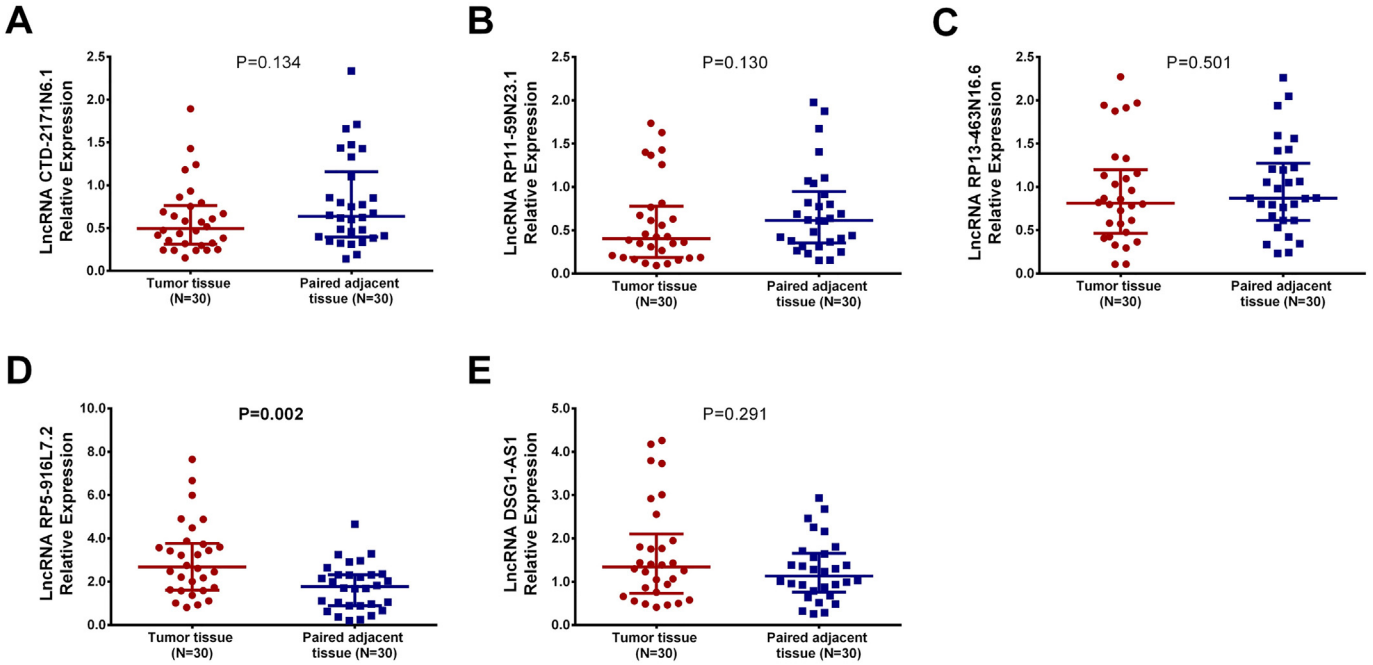

Fig. 1. Expressions of candidate lncRNAs in TSCC tumor tissue and paired adjacent tissue. The expression of lncRNA RP5-916L7.2 (D) was increased, while the levels of lncRNA CTD-2171N6.1 (A), lncRNA RP11-59N23.1 (B), lncRNA RP13-463N16.6 (C) or lncRNA DSG1-AS1 (E) in tumor tissue were of no difference in tumor tissue compared with paired adjacent tissue. Comparison between two groups was determined by Wilcoxon rank sum test. $p < .05$ was considered significant. lncRNA, long noncoding RNA; TSCC, tongue squamous cell carcinoma; NC, negative control.

3.2. lncRNA RP5-916L7.2 was up-regulated in TSCC tumor tissue

The expressions of the five candidate lncRNAs in tumor tissue and paired adjacent tissue were detected by qPCR and compared by Wilcoxon rank sum test, and when Wilcoxon rank sum test showed a significant result ($P < .05$) of comparison of lncRNA expression in TSCC tissue compared with paired adjacent tissue, the lncRNA was defined as dysregulated lncRNA in TSCC.

As shown in Fig. 1, the expressions of five candidate lncRNAs in tumor tissue and paired adjacent tissue were analyzed by qPCR assay, which disclosed that the expression of lncRNA RP5-916L7.2 was increased in tumor tissue than paired adjacent tissue ($p = .002$) (Fig. 1D). However, the levels of lncRNA CTD-2171N6.1 ($p = .134$) (Fig. 1A), lncRNA RP11-59N23.1 ($p = .130$) (Fig. 1B), lncRNA RP13-463N16.6 ($p = .501$) (Fig. 1C) or lncRNA DSG1-AS1 ($p = .291$) (Fig. 1E) in tumor tissue were of no difference compared with paired adjacent tissue.

3.3. lncRNA RP5-916L7.2 negatively correlated with T, N and TNM stages

As listed in Table 4, lncRNA RP5-916L7.2 expression positively associated with T stage ($r = 0.450$, $p = .013$), N stage ($r = 0.411$, $p = .024$) and TNM ($r = 0.371$, $p = .043$) stage, while was not correlated with pathological grade ($r = 0.062$, $p = .745$). As to other candidate lncRNAs, lncRNA CTD-2171N6.1, lncRNA RP11-59N23.1, lncRNA RP13-463N16.6 and lncRNA DSG1-AS1 expressions were not correlated with pathological grade, T stage, N stage or TNM stage of TSCC (all $p > .05$). In view of that lncRNA RP5-916L7.2 was up regulated in tumor tissue and correlated with advanced stage of TSCC, we subsequently conducted in vitro experiments to explore its effect on cells proliferation and apoptosis as well as its target miRNAs in Tca-8113 cells.

3.4. lncRNA RP5-916L7.2 promoted proliferation while repressed apoptosis of TSCC cells

After transfection, the lncRNA RP5-916L7.2 expression was markedly higher in RP5-916L7.2 (+) group than NC (+) group ($p < .001$), while was reduced in RP5-916L7.2 (−) group compared with NC (−) group ($p < .001$) (Fig. 2A), indicating that the transfection was successful. The cells proliferation of Tca-8113 cells after transfection was evaluated by CCK-8 assay, which disclosed that the cells proliferation was increased in RP5-916L7.2 (+) group compared with NC (+) group ($p < .05$), while was decreased in RP5-916L7.2 (−) group than NC (−) group at 72 h after transfection ($p < .05$) (Fig. 2B). And the cells apoptosis rate assessed by AV/PI assay was reduced in RP5-916L7.2 (+) group than NC (+) group ($p < .05$), while was dramatically elevated in RP5-916L7.2 (−) group compared with NC (−) group at 72 h ($p < .001$) (Fig. 2C, Fig. 2D). In addition, the colony formation experiments revealed that the relative colony was increased in RP5916L7.2 (+) group compared with NC (+) group ($p < .001$), while was lower in RP5916L7.2 (−) group than that in NC (−) group ($p < .01$), indicating that lncRNA RP5-916L7.2 promoted cell proliferation of TSCC cells (Supplementary Fig. 1A, B).

Table 4
Correlation of five candidate lncRNAs expressions with tumor features of TSCC.

Parameters		Pathological grade	T stage	N stage	TNM stage
LncRNA CTD-2171N6.1	r	−0.183	−0.163	−0.057	−0.154
	p Value	0.334	0.389	0.764	0.416
LncRNA RP11-59N23.1	r	0.016	−0.065	−0.117	−0.062
	p Value	0.933	0.734	0.537	0.746
LncRNA RP13-463N16.6	r	−0.358	−0.060	−0.094	−0.090
	p Value	0.052	0.754	0.622	0.634
LncRNA RP5-916L7.2	r	0.062	**0.450**	**0.411**	**0.371**
	p Value	0.745	**0.013**	**0.024**	**0.043**
LncRNA DSG1-AS1	r	−0.034	0.099	0.324	0.270
	p Value	0.858	0.603	0.081	0.150

Correlation was determined by Spearman test. $p < .05$ was considered as significant. Bold font represented significant correlation with $p < .05$.
TSCC, tongue squamous cell carcinoma.

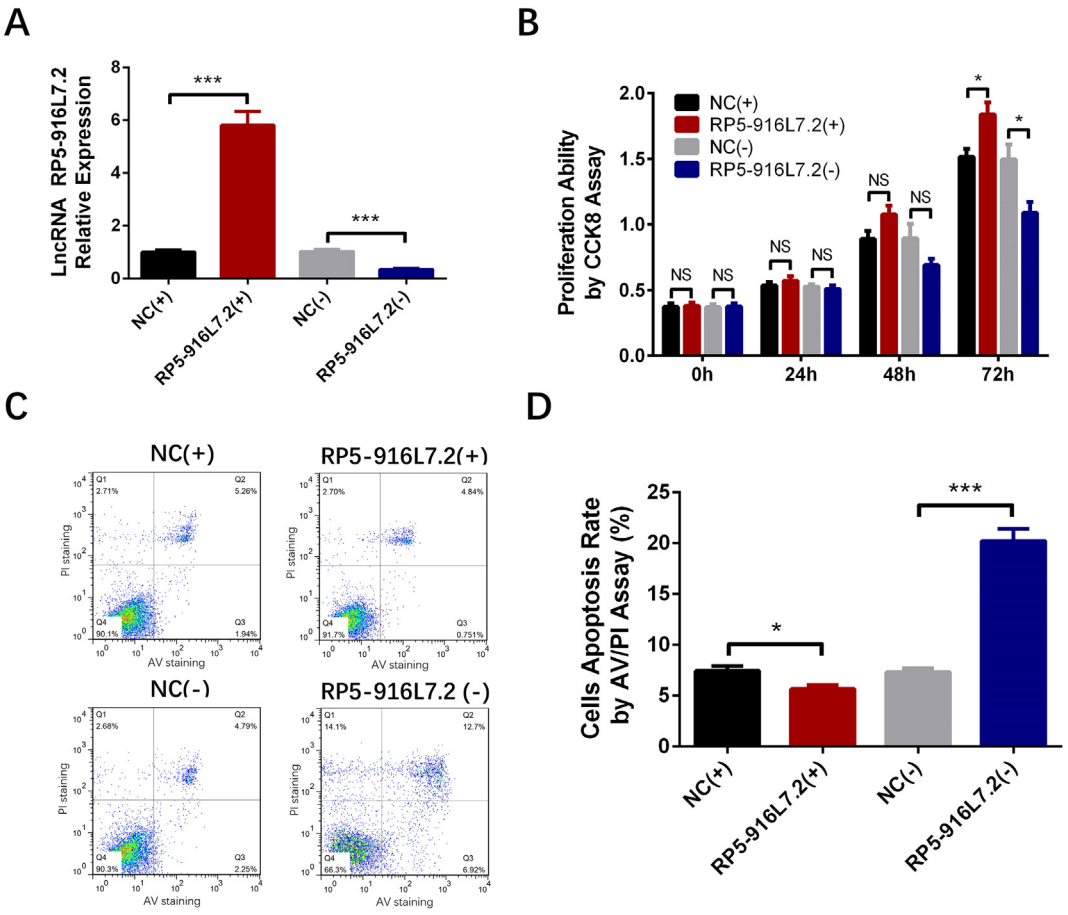

Fig. 2. Cells proliferation and apoptosis after RP5-916L7.2 plasmids transfection. The transfection of lncRNA RP5-916L7.2 plasmids was successful (A). And lncRNA RP5-916L7.2 promoted cells proliferation (B) while inhibited cells apoptosis (C, D) in Tca-8113 cells. Comparison between two groups was determined by *t*-test. $p < .05$ was considered significant. lncRNA, long noncoding RNA; NC, negative control. $^{*}p < .05$; $^{***}p < .001$.

3.5. lncRNA RP5-916L7.2 regulated miR-328-5p and miR-939-5p expressions in TSCC cells

The target miRNAs of these five candidate lncRNAs were predicted by using MiRanda, PITA and RNAhybrid methods. Then the top five candidate targeting miRNAs expressions in Tca-8113 cells were assessed by qPCR assay, which showed that after transfection, the expression of miR-328-5p was reduced in lncRNA RP5-916L7.2 (+) group compared with NC (+) group ($p < .05$), while was increased in lncRNA RP5-916L7.2 (−) group than NC (−) group ($p < .01$) (Fig. 4A). In addition, the miR-939-5p expression was also decreased in lncRNA RP5-916L7.2 (+) group than NC (+) group ($p < .05$), and was increased in lncRNA RP5-916L7.2 (−) group compared with NC (−) group ($p < .05$) (Fig. 4B). As to other potential targeting miRNAs, the results displayed that the expressions of miR-1268b (Fig. 4C), miR-4656 (Fig. 4D) and miR-6795-5p (Fig. 4E) did not vary between lncRNA RP5-916L7.2 (+) group and NC (+) group, or between lncRNA RP5-916L7.2 (−) group and NC (−) group (all $p > .05$).

3.6. LncRNA RP5-916L7.2 regulated miR-328-5p and miR-939-5p expressions in Tca-8113 cells in the rescue experiment

As presented in Fig. 5, the rescue experiment was conducted, and the results exhibited that the lncRNA RP5-916L7.2 expression did not alter in miR-328 (−) group ($p > .05$) or miR-939 (−) group ($p > .05$) compared with NC (−) group, and the expression of lncRNA RP5-916L7.2 in RP5-916L7.2 (−)/miR-328 (−) group ($p > .05$) or RP5-916L7.2 (−)/miR-939 (−) group ($p > .05$) was similar to RP5-916L7.2 (−) group (Fig. 5A). However, an increase of miR-328-5p expression was found in RP5-916L7.2 (−) group compared with NC (−) group ($p < .05$), and a decrease of miR-328-5p expression was observed in RP5-916L7.2 (−)/miR-328 (−) group compared to RP5-916L7.2 (−) group ($p < .05$) (Fig. 5B). As for miR-939-5p, similar results were observed, which displayed that the expression of miR-939-5p was up regulated in RP5-916L7.2 (−) group than that in the NC (−) group ($p < .01$), and was down regulated in RP5-916L7.2 (−)/miR-939 (−) group compared with RP5-916L7.2 (−) group ($p < .05$) (Fig. 5C). These results suggested that lncRNA RP5-916L7.2 reversely mediated the expressions of miRNA −328-5p and miR-939-5p in Tca-8113 cells, however, miR-328-5p or miR-939-5p could not regulate the lncRNA RP5-916L7.2 level in Tca-8113 cells. In addition, the luciferase assay displayed the miRNA binding site and the Mut site of miR-328-5p (Supplementary Fig. 2A) and miR-939-5p (Supplementary Fig. 2B), and the relative luciferase activity was lower in miR-328 group compared with miR-NC group ($p < .01$) (WT) (Supplementary Fig. 2C), and was also decreased in miR-939 group than that in miR-NC group ($p < .01$) (WT) (Supplementary Fig. 2D) in 293a cells. These data indicated that miR-328-5p and miR-939-5p were binding to lncRNA RP5-916L7.2. And the FISH assay also showed that lncRNA RP5-916L7.2 was located in the cytoplasm of 293a cells (Supplementary Fig. 3).

3.7. lncRNA RP5-916L7.2 promoted proliferation while inhibited apoptosis through targeting miR-328-5p and miR-939-5p in Tca-8113 cells

Cells proliferation was enhanced in miR-328 (−) group ($p < .05$) and miR-939 (−) group ($p < .05$) compared with NC (−) group,

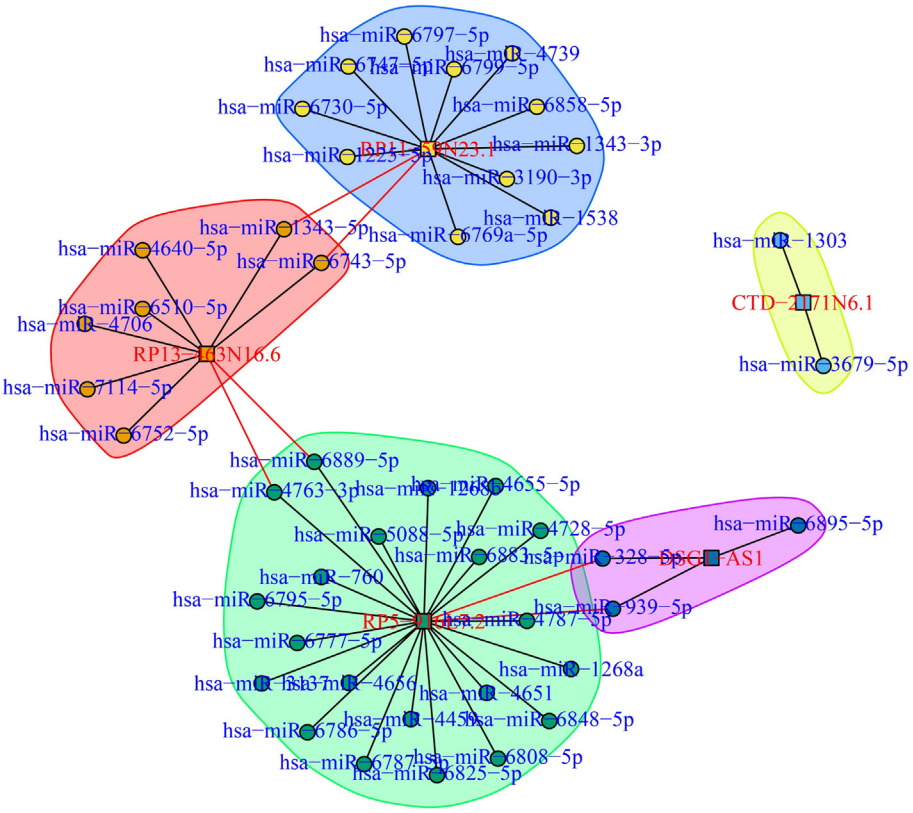

Fig. 3. miRNAs regulation network of five candidate lncRNAs in OSCC. MiRanda, PITA and RNAhybrid methods were used for predicting target miRNAs of these five candidate lncRNAs, and potential miRNAs with standard free energy below −20 by the three methods were selected. miRNA, microRNA; lncRNA, long noncoding RNA; OSCC, oral squamous cell carcinoma; NC, negative control.

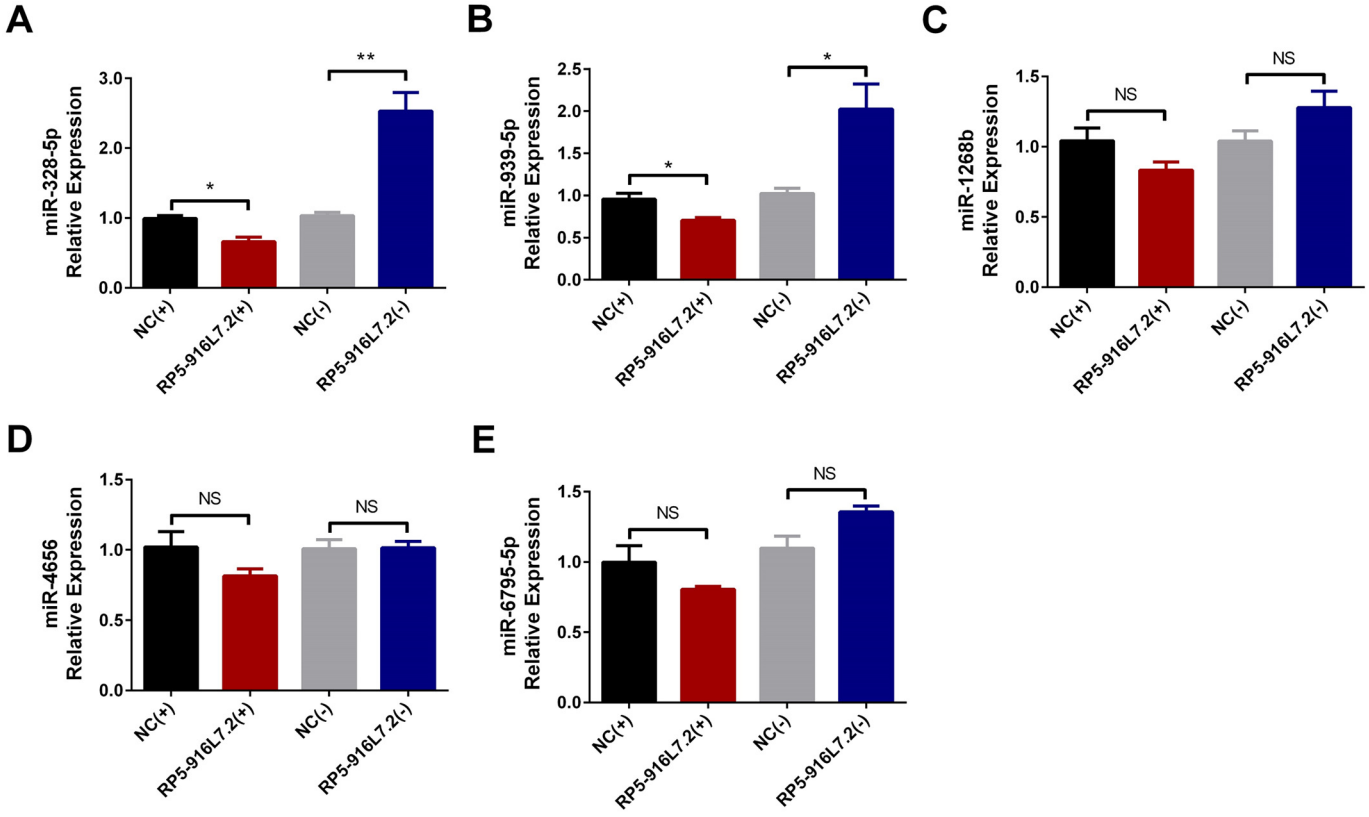

Fig. 4. Potential targeting miRNAs expressions after RP5-916L7.2 plasmids transfection. The expressions of miR-328-5p (A) and miR-939-5p (B) were both reversely regulated by lncRNA RP5-916L7.2 in Tca-8113 cells after RP5-916L7.2 plasmids transfection. However, the expressions of miR-1268b (C), miR-4656 (D) or miR-6795-5p (E) in Tca-8113 cells did not vary after RP5-916L7.2 plasmids transfection. Comparison between two groups was determined by *t*-test. $p < .05$ was considered significant. lncRNA, long noncoding RNA; miRNA, microRNA; NC, negative control. $^{*}p < .05$; $^{**}p < .01$.

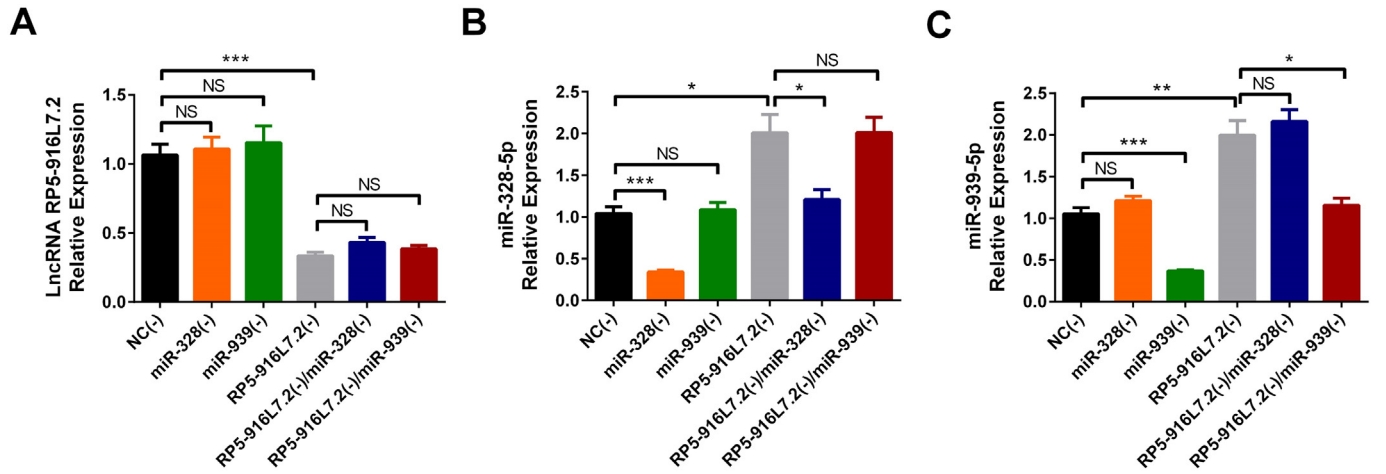

Fig. 5. lncRNA RP5-916L7.2, miR-328-5p and miR-939-5p expressions in rescue experiment. lncRNA RP5-916L7.2 reversely regulated the expressions of miR-328-5p (B) and miR-939-5p (C) in Tca-8113 cells, while miR-328-5p or miR-939-5p had no effect of the lncRNA RP5-916L7.2 level in Tca-8113 cells (A). Comparison between two groups was determined by *t*-test. $p < .05$ was considered significant. lncRNA, long noncoding RNA; miRNA, microRNA; NC, negative control. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

meanwhile, it was also increased in RP5-916L7.2 (−)/miR-328 (−) group ($p < .05$) and RP5-916L7.2 (−)/miR-939 (−) group ($p < .05$) than RP5-916L7.2 (−) group (Fig. 6A). As to cells apoptosis rate, it was declined in miR-328 (−) group ($p < .01$) and miR-939 (−) group ($p < .05$) compared to NC (−) group, similarly, it was also lower in RP5-916L7.2 (−)/miR-328 (−) group ($p < .05$) and RP5-916L7.2 (−)/miR-939 (−) group ($p < .05$) compared with RP5-916L7.2 (−) group (Fig. 6B, Fig. 6C). And the above results indicated that lncRNA RP5-916L7.2 enhanced cells proliferation and inhibited cells apoptosis through targeting miR-328-5p and miR-939-5p in Tca-8113 cells.

3.8. miR-328-5p and miR-939-5p expressions in tumor tissue, and their correlations with lncRNA PR5-916L7.2 expression as well as TSCC patients' clinical features

miR-328-5p ($p = .001$) (Supplementary Fig. 4A) and miR-939-5p ($p < .001$) (Supplementary Fig. 4B) were both downregulated in tumor tissue compared with paired adjacent tissue. miR-328-5p ($p = .004$) (Supplementary Fig. 4C) and miR-939-5p ($p = .002$) (Supplementary Fig. 4D) expressions were both negatively correlated with the lncRNA RP5-916L7.2 expression. In addition, miR-328-5p expression negatively associated with T stage ($p = .027$), N stage ($p = .007$) and TNM stage ($p = .011$), while miR-939-5p only negatively correlated with T stage ($p = .025$) in TSCC patients (Supplementary Table 1).

4. Discussion

In this study, we found that: (1) among the five candidate lncRNAs, lncRNA RP5-916L7.2 was up regulated in tumor tissue compared with paired adjacent tissue, and positively correlated with T stage, N stage as well as TNM stage. However, other candidate lncRNAs were not aberrantly expressed between tumor tissue and paired adjacent tissue or correlated with tumor features in TSCC patients; (2) moreover, in vitro experiments disclosed that lncRNA RP5-916L7.2 promoted cells proliferation and suppressed cells apoptosis of TSCC cells, and further rescue experiments revealed that lncRNA RP5-916L7.2 enhanced cells proliferation and inhibited cells apoptosis in TSCC cells through targeting miR-328-5p and miR-939-5p.

In this study, we selected five candidate lncRNAs from a previous data and then evaluated their expressions in TSCC tissues and paired adjacent tissues as well as its correlation with TSCC patients' clinical features, and found that lncRNA PR5-916L7.2 was up regulated in tumor tissue and positively associated with T stage, N stage and TNM stage in TSCC patients. Furthermore, our subsequent in vitro experiments revealed that lncRNA RP5-916L7.2 promoted cells proliferation while inhibits cells apoptosis in TSCC cells. To our best knowledge, the function of lncRNA RP5-916L7.2 in regulating tumorigenesis has not been reported yet, which is due to that the exploration of lncRNAs in the field of oncology is still in the primary stage and a large number of lncRNAs have not been explored including lncRNA RP5-916L7.2. Thus, there is no reported finding that could be possible evidence for supporting our results. However, considering that emerging studies illustrate that lncRNAs regulate cells functions via sponging miRNAs in various cancers, we subsequently performed bioinformatic analysis and selected top five potential target miRNAs of lncRNA RP5-916L7.2, and found that lncRNA RP5-916L7.2 reversely regulated the expressions of miR-328-5p and miR-939-5p in TSCC cells. Therefore, we conducted rescue experiments, which illuminated that lncRNA PR5-916L7.2 upregulated cells proliferation and downregulated cells apoptosis in TSCC cells through targeting miR-328-5p and miR-939-5p [22–24]. This novel and promising finding also provides a possible explanation to the pro-tumorigenesis role of lncRNA PR5-916L7.2 discovered in our study. Current findings by in vivo and in vitro studies suggest that lncRNA may be a crucial factor in tumorigenesis by triggering multiple oncogenic processes via regulating DNA, RNA or proteins [17–19]. Although evidence for detailed mechanism of lncRNAs in regulating progression of TSCC is extremely insufficient, there are still reports that have illuminated the regulatory functions of lncRNAs in multiple oral carcinomas, which mainly consists of TSCC. A cells experiment elucidates that lncRNA NEAT1 enhances cells proliferation and invasion through mediating miR-365/RGS20 in OSCC [20]. And down regulating lncRNA-HOTAIR notably suppresses the cells stemness and metastasis of oral carcinoma stem cells [21]. More importantly, in regard to tongue cancer, Sun T et al. report that lncRNA-EGFR advocates cells proliferation and represses cells apoptosis via elevating the level of EGFR in tongue cancer cells [13]. And knocking down lncRNA-UCA1 increases the chemosensitivity to cisplatin of TSCC cells [12].

As for lncRNA PR5-916L7.2, it is an intergenic gene that is located at chr12:125983702-126043634, which has 12 transcripts. However, no study of the regulatory role of lncRNA PR5-916L7.2 in tongue cancers has been reported. In addition, the five candidate lncRNAs in this study were selected from the dataset of a previous study, however there was only one lncRNA that was differentially expressed in TSCC tumor compared with paired adjacent tissue in this study, this inconsistency might derive from that: (1) the sample size in their study was smaller than ours, they used the samples of 6 OSCC patients, while the

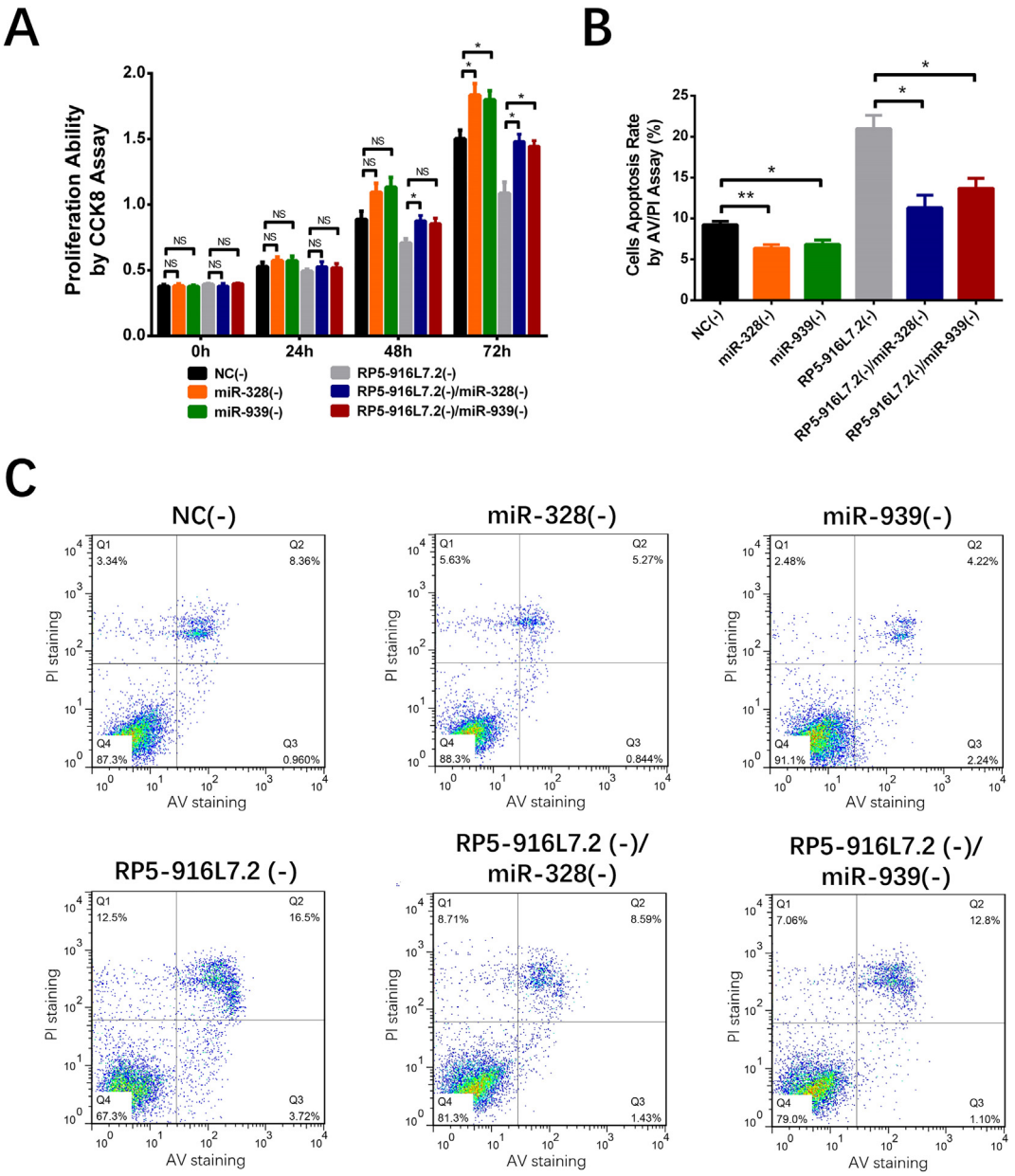

Fig. 6. Cells proliferation and apoptosis in rescue experiment. lncRNA RP5-916L7.2 promoted cells proliferation (A) while repressed cells apoptosis (B, C) through targeting miR-328-5p and miR-939-5p in Tca-8113 cells. Comparison between two groups was determined by *t*-test. $p < .05$ was considered significant. lncRNA, long noncoding RNA; miRNA, microRNA; NC, negative control. $^{*}p < .05$; $^{**}p < .01$.

five lncRNA expressions were detected in 30 TSCC patients in our study; (2) they included OSCC patients in their study, OSCC is a class of oral cancers that include the TSCC, the patients in our study were all TSCC patients but not OSCC patients, which could also contribute to this inconsistency.

As previously described, we found that lncRNA RP5-916L7.2 promoted TSCC cells proliferation and repressed cells apoptosis through targeting miR-328-5p and miR-939-5p. miR-328-5p, located on chromosome 16q22.1, has shown a promising anti-tumor effect in various cancers [22]. In breast cancer cells, miR-328-5p represses cells proliferation via targeting the receptor for advanced glycosylation end products [23]. And another study also illuminates that miR-328 is down regulated in recurrent gastric cancer patients and acts as anti-oncology gene by targeting CD44 [24]. miR-328 is also reported to suppress cells proliferation of cervical cancer cells by modulating transcription factor 7-like 2 gene expressions [25]. More interestingly, miR-328 is found to be capable of promoting cancer cells motility via modulating protein tyrosine phosphatase, receptor type, J at the post-transcription level in hepatocellular carcinoma [26]. In summary, mounting evidence suggest that miR-328 is a cancer suppressive gene through inhibiting cancer cells proliferation and enhancing cells apoptosis via multiple pathways. Whereas, the situation of miR-939 is more complicated due to that its role in tumor progression is bidirectional. A previous in vivo and in vitro experiment elucidates that through suppressing SLC34A2/Raf/MEK/ERK pathway, miR-939 increases the 5-fluorouracil-induced chemosensitivity of gastric cancer cells [27]. However, in the study of Di Modica M et al., they find that miR-939 reduces the VE-cadherin and damages barrier function of endothelial monolayers, consequently promoting the progression of breast cancer [28]. And miR-939 also boosts cells proliferation of ovarian cancer cells by inhibiting adenomatous polyposis coli 2 expression [29]. Those findings indicate that miR-939 plays a dual role in tumorigenesis, nonetheless, its tumor suppressive function observed by the previous study also provides possible explanation to our results. In view of the anti-tumor functions

of miR-328 and miR-939 found by the prior studies, our results could be explained [24–27,30].

In conclusion, lncRNA RP5-916L7.2 was up regulated in tumor tissue and positively correlated with tumor stage, and promoted cells proliferation while inhibited cells apoptosis by targeting miR-328-5p and miR-939-5p in TSCC.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2019.02.013.

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