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ORIGINAL RESEARCH

LincRNA-p21 leads to G1 arrest by p53 pathway in esophageal squamous cell carcinoma

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Background: Esophageal squamous cell carcinoma (ESCC) is the fourth most common cause of cancer death in China. Long noncoding RNAs have emerged as critical regulators in cancer. Long intergenic noncoding *RNA-p21*, a kind of Long noncoding RNAs, *LincRNA-p21* have been discussed dysregulated in several cancers, but its role in ESCC remains unknown. This study investigated the role of *LincRNA-p21* in ESCC.

Materials and methods: The *LincRNA-p21* expression level and its association with esophageal cancer was determined in 64 tumor tissues of esophageal squamous cell carcinoma patients and cells using quantitative real-time reverse transcription PCR. Fluorescence in situ hybridization of single-RNA molecular probes was used to determine subcellular localization of *LincRNA-p21*. CCK8 and EdU assays were used for proliferation assay, flow cytometry was performed for apoptosis and cell-cycle distribution, and 24-well Mill cell chamber was made for measuring the abilities of migration and invasion after transfected with lentivirus-expressing *LincRNA-p21* in EC109 cells. Then, quantitative real-time reverse transcription PCR and Western blot detected the expression of p21. Further, UC2288, an inhibitor of p21, was used to decrease the level of p21, and flow cytometry was used to detect cell cycle. Finally, screening for differential pathways from microarray analysis and expression of p53 and cyclin D were detected by Western blot.

Results: *LincRNA-p21* expression level was remarkably lower in tumor tissues versus nontumor tissues and lower in EC109 cells versus Het-1A cells. Statistical analysis found that *LincRNA-p21* might enhance the risk of ESCC. We observed that *LincRNA-p21* was expressed both in the nucleus and cytoplasm, and a larger proportion of *LincRNA-p21* was observed in the cytoplasm. The results demonstrated that upregulating the expression of *LincRNA-p21* could inhibit cell proliferation, migration, invasion, and the transition of cell cycle from G1 and promoted apoptosis of EC109. Then, we found that *LincRNA-p21* promotes the expression of p21. Decreasing the level of p21 revealed that cell-cycle arrest was restored. Pathway analysis found p53 pathway was downregulated, and upregulation of *LincRNA-p21* inhibited the expression of cyclin D.

Conclusion: Our study suggests that *LincRNA-p21* plays as a tumor inhibitor in ESCC development and *LincRNA-p21* might induce G1 arrest through p53 signal pathway.

Keywords: esophageal squamous-cell carcinoma, *LincRNA-p21*, G1 arrest, p53 pathway, Fluorescence in Situ Hybridization

Background

In recent years, cancer morbidity and mortality have continued to show growth trend. The 2014 China Health Statistics Yearbook reported that cancer ranked first in the death causes in which esophageal cancer ranked fourth cause of death. . Esophageal squamous cell carcinoma (ESCC) is the predominant histological type of esophageal carcinoma in the "Asian esophageal cancer belt",[1] and there is the highest incidence of ESCC in Huai'an, China. ESCC, causing serious health disorders, whose incidence rate is 62.91/10

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million people and mortality is 46.75/10 million people in Huai'an area. There is lack of typical clinical symptoms in early ESCC. Besides, patients are in advanced ESCC because of poor compliance during treatment with imaging and cytology, and 5-year survival rate is <15%. Therefore, it is particularly important to find accurately sensitive and early tumor biomarkers for diagnosing ESCC.

Long noncoding RNAs (lncRNAs) are a group of RNA with more than 200 nucleoties in length that seldom involved in protein coding but regulating gene expression in the form of RNA over various levels including epigenetic regulation, derivation regulation and post-transcriptional control.[2–5] An increasing number of evidence have revealed that lncRNA involved in magnanimous cellular processes ranging from embryonic stem cell pluripotency, cell apoptosis, cell-cycle regulation, and kinds of diseases, such as cancer.[6,7] lncRNA has been discerned in esophageal cancer including MALAT-1 (Metastasis-Associated in Lung Adenocarcinoma Transcript 1),[8] ESCCAL-1,[9] UCA1,[10] LncRNA FOXCUT,[11] PlncRNA1,[12] HOTAIR,[13–15] POU3f3,[16] HNF1A-AS1,[17] ANRIL,[18] and taurine-upregulated gene 1 (TUG1).[12]

LincRNA-p21 captured our attention because it was a transcription of the *p53*, which is also called TP53COR1 (tumor protein P53 pathway corepressor 1). *LincRNA-p21* was originally discovered in mice where it was a transcript of about 3 kb located proximal to the *p21/CDKN1a* gene which encodes the cell-cycle regulator.[19] Since our understanding of *LincRNA-p21* in esophageal, cancer biology is still in infancy. In the present study, we aimed to explore the link with *LincRNA-p21* and ESCC and lay the foundation for seeking molecular biomarkers of ESCC.

Materials and methods

Chemicals

Ambion® PARIS™ system (PARIS™ Instruction Manual, Thermo Fisher Scientific, Waltham, MA, USA), BCA Protein Assay Kit (Thermo Fisher Scientific), CCK-8 Cell Proliferation/Viability Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) and EdU Apollo Imaging Kit (RiboBio, Guangzhou, China), Cell Cycle (Nanjing KeyGEN, China), keyFluor 647-Annexin V/7-AAD Apoptosis Detection Kit (Nanjing KeyGEN, China), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, fetal bovine serum (Gibco, Waltham, MA, USA), *LincRNA-p21*-lentivirus and negative control (Shandong ViGene Biosciences, Shandong, China), Matrigel (BD, Franklin Lake, NJ, USA), nuclear/cytosol fractionation kit (Biovision, San Francisco Bay, CA, USA), PBS (Gibco), PrimeScript RT Master Mix Kit (Takara, Shiga, Japan), penicillin and streptomycin (Sigma-Aldrich), RNase-Free DNase I and Purification Kit (Tiangen, Beijing, China), Ribo™ lncRNA FISH Probe Mix and Ribo™ Fluorescent In Situ Hybridization Kit (RiboBio, Guangzhou, China), RPMI1640 (Hyclone, Logan, UT, USA), SYBR Premix Ex Taq II Kit (Takara, Kusatsu, Japan), TRIzol reagent (Invitrogen, USA), trypsin and streptomycin (Nanjing Sunshine, Nanjing, China) were used. All primers used for this study, including *LincRNA-p21, p21, β-actin*, and *U6*, were purchased from Nanjing GenScript Company (Nanjing, China).

Clinical samples and ethics statement

Sixty-four pairs of fresh-frozen tumor samples of ESCC tumors and adjacent normal tissues were collected from Thoracic Surgery of Jiangsu Huai'an First People's Hospital. The patients in these cases were diagnosed as primary ESCC in 2010. The eligibility criteria for the enrolled ESCC patients were: diagnosed as ESCC by pathology or endoscopy without radiotherapy or chemotherapy, Han residents have been living in Huai'an for >20 years. The study was conducted according to protocols approved by the Southeast University Affiliated Zhongda Hospital Ethics Committee, and written informed consent that was conducted in accordance with the Declaration of Helsinki was obtained from the participants.

Cell culture and lentivirus transfection

The esophageal cancer cell line EC109 and human esophageal epithelial cell Het-1A were purchased from Shanghai Tiancheng Technology Co., Ltd. The EC cell lines EC109 with wild-type p53 and a human esophageal epithelial cell line Het-1A were cultured in complete medium containing 100 U/mL each of penicillin and streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO_2. Lentivirus-expressing *LincRNA-p21* and negative control were transfected into EC109 cells. First, cells were seeded in six-well plates, then transfected with 1×10^8 transducing units (TUs)/mL lentivirus (10 μL) and 5 μg/mL polybrene diluted by RPMI1640. Then, the medium was refreshed and incubation was continued for 24–96 hrs. Transfection efficiency was determined by a fluorescence microscope (FSX100 Oylmpus, Tokyo, Japan) and qRT-PCR. The infection efficiency needs to be >80% as monitored with GFP protein expression.

Total RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol/chloroform according to the manufacturer's instruction. After reverse transcription,

amplification was carried out in a total volume of 20 μL containing SYBR Green real-time PCR Master Mix. The PCR reaction included an initial "hot start" at 95°C for 30 s, followed by 40 cycles of amplification. Each cycle consisted of a denaturation step at 95°C for 5 s, annealing, and fluorescence signal acquisition at 60°C for 30 s. Primer sequences used are described in Table 1. Transcription levels were normalized against β-actin. Ct (cycle threshold) values were used to calculate the relative *LincRNA-p21* levels. $\Delta Ct = (Ct_{LincRNA\text{-}p21} - Ct_{\beta\text{-actin}})$, while $\Delta\Delta Ct = (Ct_{LincRNA\text{-}p21\ \text{in Tumor}} - Ct_{\beta\text{-actin in Tumor}}) - (Ct_{LincRNA\text{-}p21\ \text{in Normal}} - Ct_{\beta\text{-actin inNormal}})$. The fold change of *LincRNA-p21* levels in ESCC is $2^{-\Delta\Delta Ct}$.

Fluorescence in Situ Hybridization

To localize *LincRNA-p21* in the EC109 cell, the FISH assay was performed using the lncRNA FISH Probe and Fluorescent in Situ Hybridization Kit according to the manufacturer's guidelines. The probe cocktail included the *U6*/nuclear probe, *18S*/cytoplasm probe, and the *LincRNA-p21* probe (the nucleus is displayed through the DAPI, *LincRNA-p21* gene appears as a red signal). Fluorescence signals were captured by the OLYMPUS laser confocal microscope FV1000 (Olympus, Tokyo, Japan).

RNA separation of nuclear and cytoplasmic fractions

The Ambion® PARIS™ system is for the isolation of RNA from nuclear and cytoplasmic of EC109 cells. Tissue or cultured cells are first homogenized in ice-cold cell disruption buffer to prepare a total cell lysate. Since the homogenization is performed quickly on ice and in the presence of detergent, both protein and RNA can be purified directly from this lysate. For RNA isolation, a part of the total cell lysate is immediately mixed with an equal volume of lysis/binding solution. Total RNA is then purified from the mixture using an RNA binding glass fiber filter. After three rapid washing steps, high-quality RNA is eluted in a concentrated form. Then, total RNA from nuclear and cytoplasm were used for qRT-PCR.

Table 1 The primer sequences used in this study

Gene Name	Sequence
*LincRNA-p21**	5′-GGGTGGCTCACTCTTCTGGC-3′ (Forward)
	5′-TGGCCTTGCCCGGGCTTGTC-3′ (Reverse)
p21	5′-TGGACCTGTCACTGTCTTGT-3′ (Forward)
	5′-TCCTGTGGGCGGATTA-3′ (Reverse)
β-actin	5′- CCACTGGCATCGTGATGGA-3′ (Forward)
	5′- CGCTCGGTGAGGATCTTCAT-3′ (Reverse)
U6	5′-CTCGCTTCGGCAGCACA-3′ (Forward)
	5′-AACGCTTCACGAATTTGCGT-3′ (Reverse)

Notes: *Data from Huarte[19]

Cell proliferation assay

Cell proliferation assay was performed using the CCK-8 Cell Proliferation/Viability Assay Kit and EdU Apollo Imaging Kit as recommended in the manufacturer's protocol.

Cell-cycle analysis

EC109 transfected with *LincRNA-p21* and negative control were seeded in six-well plates and synchronized in serum-free medium for 24 hrs, then harvested and fixed in ice-cold 70% ethanol at 4°C overnight. The cells were washed with PBS and and then heated in 37°C water bath with 100 μL RNase A for 30 mins the next day. Finally, cells were stained with propidium iodide and analyzed by flow cytometry.

Apoptosis assay

LincRNA-p21-treated EC109 and negative control during logarithmic growth period were collected and detected using an Annexin V fluorescein isothiocyanate kit (APC/7-AAD) according to the protocol.

Migration and invasion assays

Cell invasion ability was measured by 24-well Mill cell chamber (corning 3422) containing a Matrigel-coated membrane, according to the protocol. After transfection, 5×10^5 EC109 cells in 100 μL RPMI1640 medium containing 1% fetal were seeded in the upper chamber. Then, 600 μL complete RPMI1640 medium was added to the lower chamber and incubatedfor at least 24 hrs, and noninvading cells were removed with a cotton swab and cells invading to the lower chamber were fixed with methanol, stained with 0.1% crystal violet, dried, and photographed. The number of invading cells was counted under the microscope (original magnification, ×200) in five representative fields and expressed as the average per field. The migration ability was performed in a similarity method without the Matrigel coating.

Assay for UC2288 inhibition

UC2288 were dissolved in 95% ethanol or DMSO and formulated into a 1 μmol/L stock solution, which was then diluted with RPMI1640 to the desired concentration. EC109 cells were seeded in six-well plates with complete

medium after upregulating *LincRNA-p21*. Then, the stock solution was added to 0, 0.1, 1, 3, 10, and 30 nmol/L, respectively. Finally, cell activity, qRT, and WB were performed after incubation for 24 hrs.

Western blot

The cells were lysed in RIPA buffer that mixed with protease inhibitors after wash with PBS. Then, BCA Protein Assay Kit was used to quantify the protein concentrations. Then, a total of 20 μg protein was transferred to polyvinylidene fluoride (PVDF) membranes after separation by 10% SDS-PAGE. Next, the PVDF membrane was closed using 5% skimmed milk powder at room temperature for 2 hrs, and membranes were incubated with rabbit monoclonal anti-human β-actin (1:1,000), rabbit monoclonal anti-human p21 (1:1,000), rabbit monoclonal anti-human p53 (1:1,000), rabbit monoclonal anti-human Cyclin D (1:1,000), and rabbit monoclonal anti-human GAPDH (1:1,000) overnight at 4°C. The second day, after washing the membranes, peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000) was applied to the cells for 1 hr at room temperature. Finally, the signals were detected with the SuperSignal West Femto Trial Kit.

KEGG PATHWAY analysis

The possible pathway analysis of the identified differentially expressed proteins was elucidated by the KEGG PATHWAY database (http://www.genome.jp/kegg/pathway.html)

Statistical analysis

Data analysis was performed by SPSS 20.0. Each condition has three replicates, and data were described as the mean ± SD. Student's *t*-test, paired *t*-test, and ANOVA were conducted to analyse quantitative variables. A Cox regression analysis was used to analyse the relationship between the risk of ESCC incidence and the level of *LincRNA-p21*. A *P*-value ≤0.05 was considered statistically significant.

Results

Demographic characteristics

Sixty-four cases of ESCC patients were finally included in the study. The average age of the patients was 60.47±7.47 years, ranging from 43 to 76 years. There were 13 of the 64 patients (20.30%) with lymph node metastasis (Table 2). As shown in Table 3, there is no significant difference in *LincRNA-p21* expression according to age. There is no significant difference in *LincRNA-p21* expression with clinical performance. However, the *LincRNA-p21* expression levels were not significantly different with some risk factors such as smoking and drinking.

Table 2 Clinical characteristics of 64 patients with ESCC

Parameters	Class	Frequency	Proportion (%)
Age	<60	29	45.30
	>60	35	54.70
Sex	male	41	64.10
	female	23	35.90
Tea	never	48	75.00
	occasionally	8	12.50
	Often	8	12.50
Smoking	light	25	39.10
	middle	3	4.70
	deep	36	56.30
Drinking	yes	20	31.20
	no	44	68.80
Hot food	yes	34	53.10
	no	30	46.90
Hard food	yes	22	34.40
	no	42	65.60
Tumor site	upper	11	17.20
	middle	38	59.40
	lower	14	21.90
Lymphnode metastasis	yes	13	20.30
	no	51	79.70

Abbreviation: ESCC, esophageal squamous cell carcinoma.

LincRNA-p21 was down-regulating

First, qRT-PCR was used to evaluate *LincRNA-p21* expression levels in four esophageal cancer cell line EC109 and a normal esophageal epithelial cell line Het-1A as well as in 64 tumor tissues and paired normal tissues.

As shown in Table 4 and Figure 1, *LincRNA-p21* levels in four EC cell lines were lower than those in Het-1A (Figure 1C, $P<0.001$). In Table 5 and Figure 1A and B, the expression level of *LincRNA-p21* depressed significantly ($P=0.005$) in tumor tissues, as described in our previous study.[20] To investigate the association between the expression of *LincRNA-p21* and the risk of esophageal cancer, the *LincRNA-p21* levels were analyzed by conditional logistic regression. As shown in Table 5, increased risks of developing esophageal cancer are connected with downregulation level of *LincRNA-p21* (OR=3.76, $P=0.014$), suggesting that *LincRNA-p21* may play a role as tumor suppressor gene in the development of esophageal carcinoma.

Table 3 The associations of *LincRNA-p21* expression with demographic and clinical characteristics

Parameters	Class	Expression pattern			*P*-value
		Low expression	Normal	Over expression	
Age	<60	17	8	4	0.561
	>60	17	8	10	
Tea	never	27	11	10	0.745
	occasionally	4	2	2	
	often	3	3	2	
Smoking	light	17	5	3	0.265
	middle	1	0	2	
	deep	16	11	9	
Drinking	no	24	10	10	0.148
	yes	10	6	4	
Hot food	no	15	9	6	0.333
	yes	19	7	8	
Hard food	no	21	11	10	0.319
	yes	13	5	4	
Tumor site	upper	26	13	0	1.000
	middle	8	2	3	
	lower	3	4	4	
Lymph node metastasis	no	23	8	20	0.472
	yes	9	4	0	

Table 4 Relative *LincRNA-p21* level in tested cells

Group	N	ΔCt(Mean ± SD)	Fold Change	*P*-value
Het-1A	3	18.11±0.00	1.000	
EC109	3	20.56±0.00	0.183	<0.05

Abbreviation: CT, cycle threshold.

Evaluation of transfection efficiency

qRT-PCR was used to detect the expression of *LincRNA-p21* after transfection. After transfection, the expression of *LincRNA-p21* was significantly higher than the control group (Figure 1D and E, $P<0.05$).

LincRNA-p21 localized in nucleus and cytoplasm of EC109

As shown in Figure 2, *18S* as cytoplasmic control transcript, *U6* as nuclear control transcript, and *LincRNA-p21* were tagged by Cy3 (red), and the nuclei were stained with DAPI (blue). The result of fluorescence in situ hybridization revealed that *LincRNA-p21* localized both in nucleus and in cytoplasm of EC109. Fluorescence intensity of *LincRNA-p21* in the cytoplasm was stronger. qRT-PCR results revealed the expression of *LincRNA-p21* mainly in the cytoplasm.

Function of *LincRNA-p21* in EC109

LincRNA-p21 inhibited proliferation and promotes apoptosis of EC109 As shown in Figure 3A, cells labeled in red after reaction of EdU (5-ethynyl-2′-deoxyuridine) and Apollo represent proliferative cells and cell nuclei stained with Hoechst 33342 (blue) represent the total cells. Cells were captured by FSX100 (Olympus) at 200× magnification; the percentage of proliferating cells was significantly decreased compared with the control group (*$P<0.05$). CCK8 cell proliferation assays also revealed that cell proliferation was inhibited (Figure 3B, *$P<0.05$). The result of cell apoptosis assays suggested that upregulation of *LincRNA-p21* promoted apoptosis in EC109 cells (Figure 3C, *$P<0.05$).

LincRNA-p21 redistributes cell cycle of EC109

Flow cytometry was used to detect the number of cells in each phase of the cell cycle. The result shows that *LincRNA-p21* induced G1/S cell-cycle arrest in EC109 cells. The number of cells in G1 phase was significantly increased from 51.76% to 59.63%, while cells of S and G2/M phase decreased from 30.66% to 24.79% and 17.57% to 15.56%, respectively (Figure 3D, *$P<0.05$).

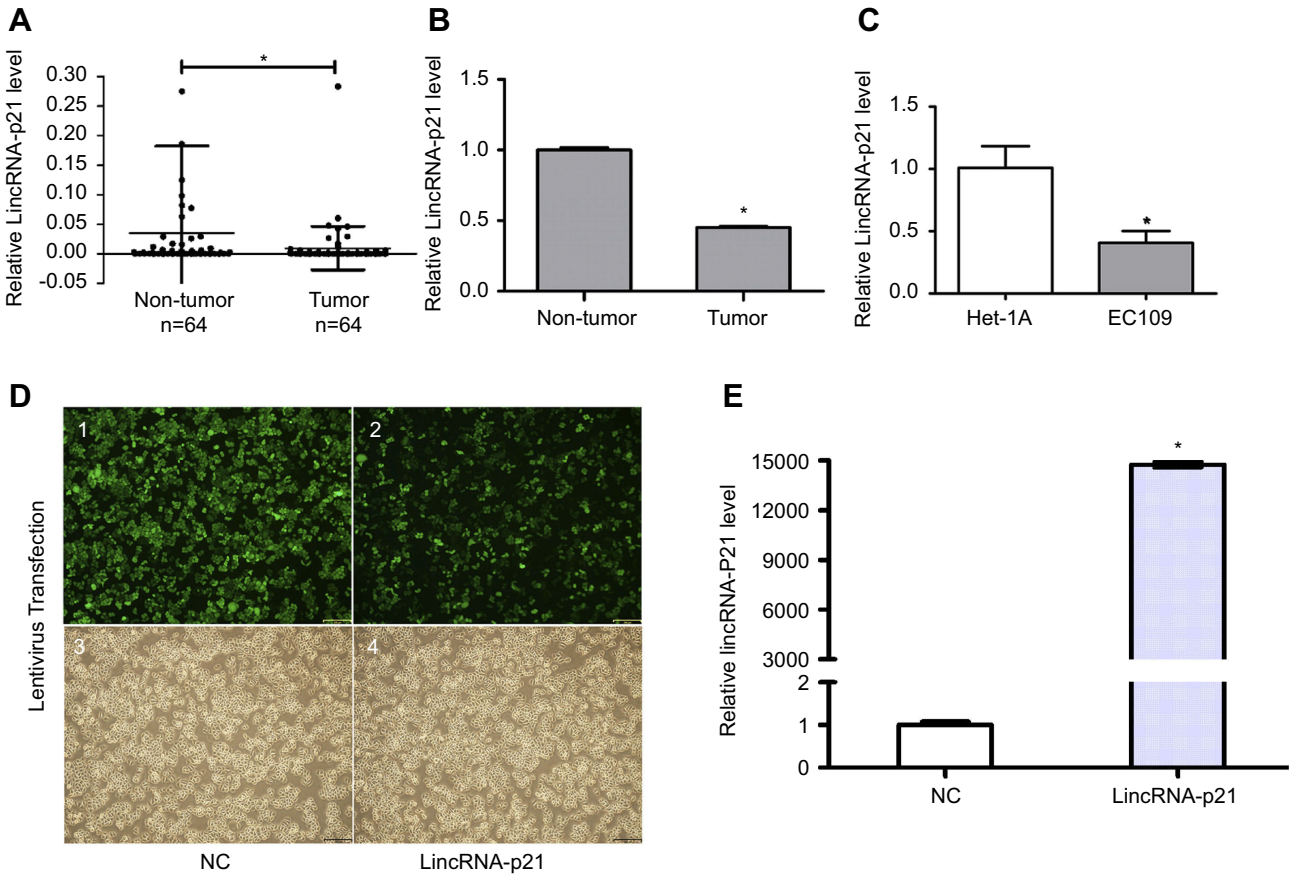

Figure 1 Expression of *LincRNA-p21*. To evaluate the expression of *LincRNA-p21* in ESCC tissues and cells, we carried out qRT-PCR test before and after application of lentivirus. Our experiments divulged that LincRNA-p21 was lower expressed in tumor tissues and EC109 cells (**A**–**C**). EC109 cells, after transfected with lentivirus, were captured by FSX100 (Olympus) at 200× magnification (**D**): 1 and 2, fluorescence; 3 and 4, bright field; 1 and 3, negative control; 2 and 4, virus-transfected with *LincRNA-p21*; *LincRNA-p21* was significantly increased after transfected with *LincRNA-p21*-targeting lentivirus (**E**). Data indicate mean ± SD, n=3. **P*<0.05.
Abbreviation: ESCC, esophageal squamous cell carcinoma.

Table 5 Relative *LincRNA-p21* level in tumor tissues and adjacent normal tissues

Group	N	ΔCt(Mean ± SD)	Fold Change	OR (95% CI)
Tumor	64	13.54±2.94	0.451*	3.76 (2.20, 9.18)#
Paired paracancer tissue	64	12.39±3.37	1.000	

Notes: *and #Significant difference compared to the Normal group (*P*<0.05).
Abbreviation: CT, cycle threshold.

LincRNA-p21 prohibit migration and invasion of EC109

Transwell chamber assay was employed to assess migration and invasion ability of *LincRNA-p21* on EC109 cells. With *LincRNA-p21* level up-regulated, the ability of EC109 cells to pass through the Matrigel membrane was weakened. The results showed that the migration and invasion ability of EC109 cells was significantly weakened than negative control group (Figure 3E and F, **P*<0.05).

The effect of LincRNA-p21 on expression of p21

In our previous studies, we have found that mRNA of *p21* downregulated both in EC cells and ESCC tissues.[20] Moreover, protein of p21 was lower in EC109 cells than Het-1A cells. However, expression of *p21* was upregulated both in mRNA and protein level after overexpression of *LincRNA-p21* (Figure 4, Table 6).

LincRNA-p21 impact on EC109 cells with inhibition of p21

To get rid of influence of p21, we used a kind of p21 attenuator, named UC2288, which could decrease p21 mRNA expression and attenuated p21 protein levels as shown in Figure 5A–C. Then, we detected the cell apoptosis and periodic distribution in EC109 cells overexpressing *LincRNA-p21*. The apoptosis of EC109 cells was still high. But the periodic distribution is

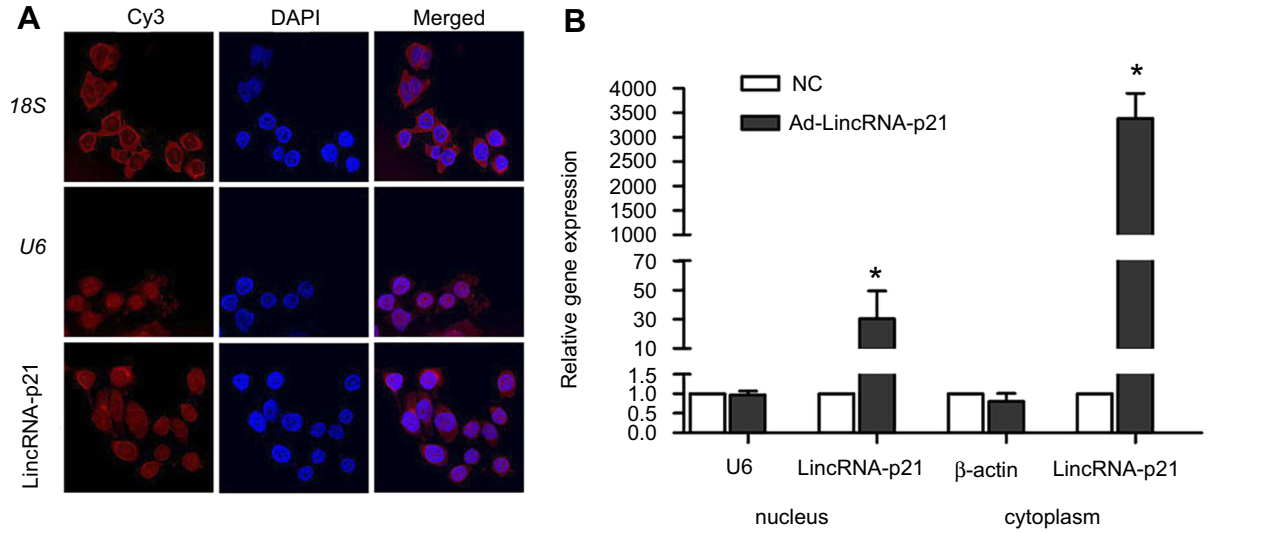

Figure 2 Fluorescence in situ hybridization for *LincRNA-p21* in EC109 cell. (**A**) Using Single Molecule lncRNA Fluorescent in Situ Hybridization (lncRNA-FISH) to fix a position on *LincRNA-p21*. As observed under a confocal microscope, *18S* (as cytoplasmic control), *U6* (as nuclear control) and *LincRNA-p21* were tagged by Cy3 (red), the nuclei were stained with DAPI (blue). (**B**) Expression of *LincRNA-p21* was detected by qRT-PCR after separation of nuclear and cytoplasmic fractions, using U6 as nuclear control transcript and actin as cytoplasmic control transcript. Data indicate mean ± SD, n=3. *P<0.05.

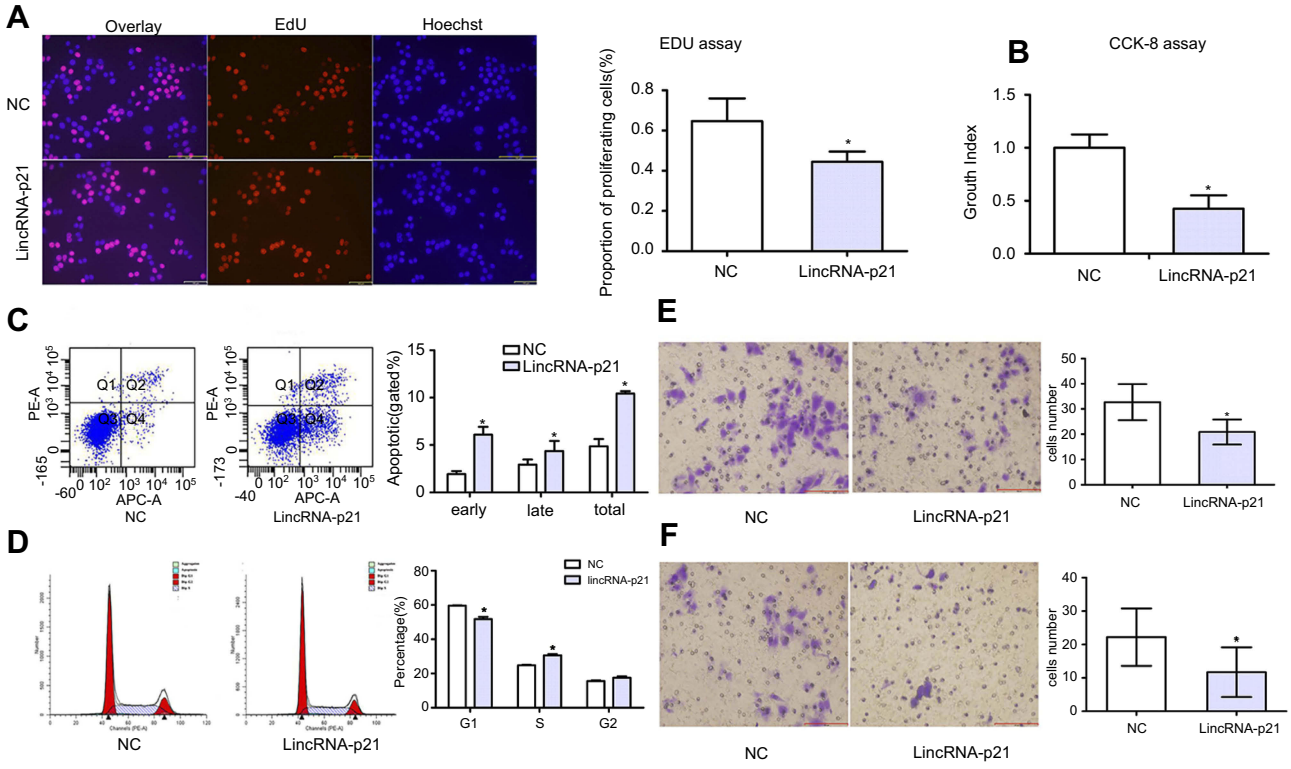

Figure 3 Function of *LincRNA-p21* in EC109 cells. The cell proliferation activity was evaluated using EdU-incorporation assays and CCk-8 assay. Cells labeled in red after reaction of EdU (5-ethynyl-2′-deoxyuridine) and Apollo represent proliferative cells., cell nuclei stained with Hoechst 33,342 (blue) represent the total cells (FSX100, 200× magnification), *LincRNA-p21* reduced activity of EC109 (**A** and **B**). Apoptosis rate and each phase of cell cycle were analyzed by flow cytometry (**C** and **D**). (**E**) The metastatic and infiltrated potential of EC09 cells was evaluated using the migration and invasion assay in vitro. The results indicated a significant decline in cells to pass through the Matrigel membrane after LincRNA-p21-knockin relative to the untreated control. Data indicate mean ± SD, n=3. *P<0.05.

completely different. As shown in Figure 5, the cells of G1 phase were decreased (from 56.14% to 47.55%, P<0.01); the proportion of S phase cells were elevated (from 29.28% to 39.63%, P<0.01); the cells of G2 phase were decreased with no statistically significant. (from 14.58% to 12.83%).

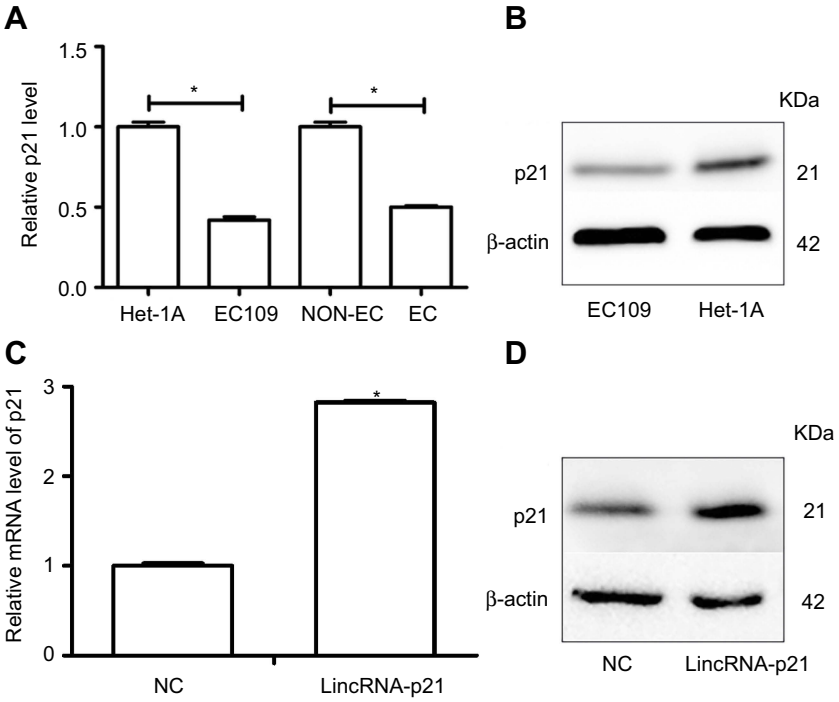

Figure 4 Expression of p21. Relative mRNA level of *p21* was determined using qRT-PCR and protein expression of p21 was determined using Western blot. We found p21 decreased in ESCC tissues and cells (**A** and **B**). mRNA of *p21* and protein of p21 were up-regulated after transfected with *LincRNA-p21*-targeting lentivirus (**C** and **D**). Data indicate mean ± SD, n=3. **P*<0.05.

Table 6 Seventeen significant pathways from KEGG enrichment analysis

ID	Definition	Pathway name	Gene[a]	DEG[b]	*P*-value
hsa05322	Systemic lupus erythematosus	Autoimmune disease	138	14	0.000
hsa05330	Allograft rejection	Alloimmune response	39	7	0.000
hsa04940	Type I diabetes mellitus	Autoimmune disease	45	7	0.000
hsa04612	Antigen processing and presentation	Immune system	78	9	0.000
hsa05332	Graft-versus-host disease	Immune system	43	6	0.001
hsa05320	Autoimmune thyroid disease	Autoimmune disease	54	6	0.004
hsa04650	Natural killer cell mediated cytotoxicity	Immune system	137	10	0.006
hsa04060	Cytokine-cytokine receptor interaction	Inflammatory defenses	265	15	0.009
hsa03050	Proteasome	Immune responses, Stress, signaling, inflammatory	45	5	0.009
hsa04512	ECM-receptor interaction	Tissue and organ morphogenesis	85	7	0.011
hsa04380	Osteoclast differentiation	Immune receptors	128	9	0.011
hsa05416	Viral myocarditis	Cardiac disease	72	6	0.017
hsa05166	HTLV-I infection	Virus infection	267	14	0.020
hsa04145	Phagosome	Inflammation and infectious	155	9	0.033
hsa03440	Homologous recombination	Repair of DNA	28	3	0.045
hsa04115	p53 signaling pathway	Signal transduction	69	22	0.047
hsa05323	Rheumatoid arthritis	Alloimmune response	92	6	0.048

Notes: [a]The number of genes in the pathway. [b]The number of genes differentially expressed.

LinRNA-p21 result in G1 arrest via *p53* signaling in EC109 cells

We had found that there were 17 upregulated and 13 downregulated pathways involved in differentially expressed mRNAs which may be related to the occurrence and development of ESCC.[21] Upregulation pathways include protease pathways, homologous recombination pathways, and *p53* signaling pathways. Downregulation pathways include MAPK signaling pathway, prostate cancer pathway, etc. (Figure 6). We concentrated on the *P53*

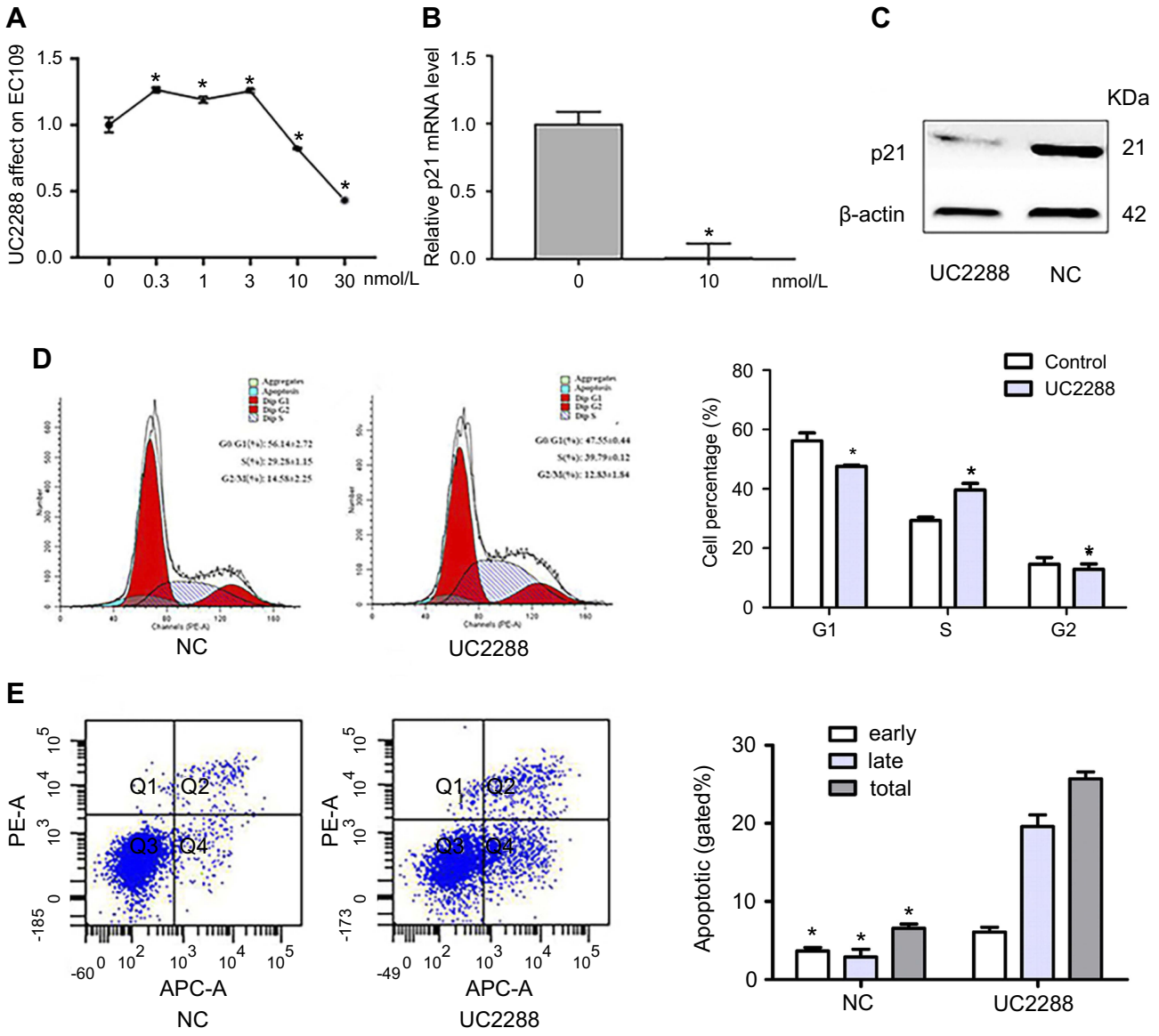

Figure 5 *LinRNA-p21* affected on EC109 cell after using UC2288. (**A**) EC50 of UC2288, determined by cell activity, was 10 noml/L; (**B** and **C**) The result of qRT-PCR and Western blot showed that mRMA and protein of p21 were decreased; (**D** and **E**) Flow cytometry assays was performed to tested cell cycle and apoptosis, G1/S arrest was recovered with p21 decreased. (**C**–**E**) The concentration of UC2288 is 10 noml/L. Data indicate mean ± SD, n=3. *P<0.05.

pathway is involved closely in ECSS. Particularly, $p14^{ARF}$, p53, CHK2, Reprimo, cyclin D, CDK4/6, CDK2, Cyclin B, Cdc2, Fas, Bid, Bax, PIGs, IGF, BAI-1, KAI, P48, p53R2, Sestnns, and IGF-BP3 were enriched in *p53* pathway (Figure 7).

Expression proteins were then examined by Western blot. Our result showed that p21 and p53 were significantly decreased in EC109 compared to Het-1A , while the Cyclin D was significantly increased in EC109. Upexpressing *LincRNA-p21* increased the expression of p21, reduced the expression of cyclin D, and had no effect on p53 protein (Figure 8). P21 and cyclin D regulate the cell cycle as the target genes for p53 signaling pathway. These results demonstrated that LincRNA-p21 was a corepressor of the p53 signaling pathway in EC109 cells.

Discussion

LincRNA-p21 is a regulatory factor of cell proliferation, apoptosis, and DNA damage response, which is stimulated by the *p53* tumor suppressor protein.[19,22,23] *LincRNA-p21* is implicated in the development and progression of human diseases, particularly in cancer, which has been reported to be associated with hepatocellular carcinoma,[24] colorectal cancer (CRC),[25,26] non-small cell lung cancer,[27,28] skin tumors, chronic

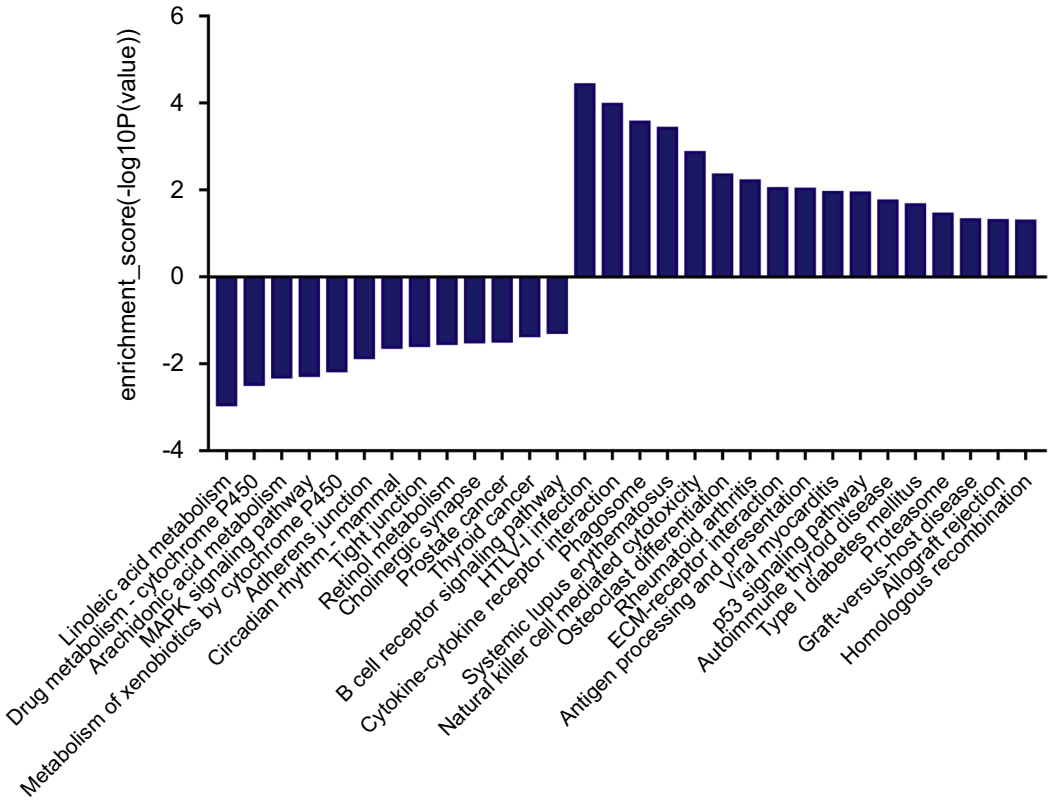

Figure 6 The histogram of KEGG pathway enrichment. The results of pathway analysis showed that there were 17 possible pathways involved in up-regulated differentially expressed mRNAs, and 13 possible pathways involved in down-regulated differentially expressed mRNAs ($P<0.05$).

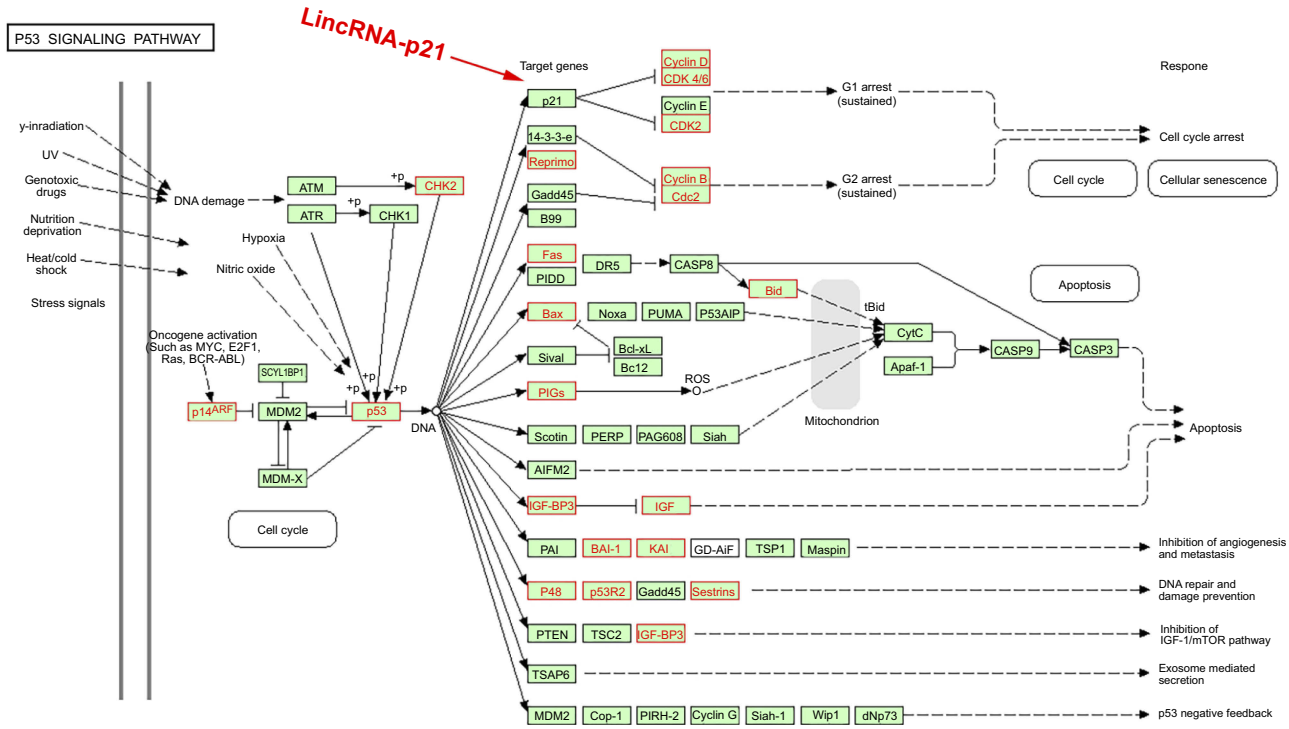

Figure 7 Differentially expressed genes in p53 signaling pathway. Red rectangles mean differentially expressed genes.

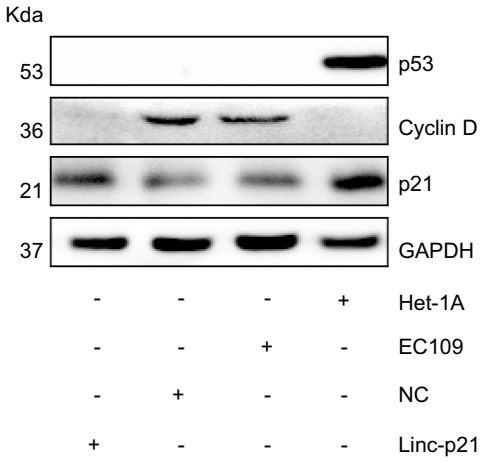

Figure 8 LincRNA-p21 affected on the expression of p53 and its downstream genes. Western blotting revealed a significant subsiding in Cyclin D protein expression and increasing in p21 protein expression in EC09 cells transfected with LincRNA-p21-targeting lentivirus.

lymphocytic leukemia (CLL),[29] and prostate cancer.[30] It has been reported that *LincRNA-p21* is emerging as either oncogenes or tumor suppressor genes. For example, the expression levels of *LincRNA-p21* are obviously reduced in CRC tissues and cell lines.[31] Yang Ning[32] also reported *LincRNA-p21* is downregulated in human hepatocellular carcinoma. In some other diseases, such as atherosclerosis,[33] acute respiratory distress syndrome,[34] and rheumatoid arthritis (RA),[35] the level of *LincRNA-p21* was also decreased. Above these evidence imply *LincRNA-p21* may play a role as tumor inhibitor. On the contrary, stabilization of HIF-1α by *LincRNA-p21* under hypoxia conditions implies that it could also play an oncogenic role.[36] In this work, we confirmed that *LincRNA-p21 level* was significantly downregulated in esophageal cancer cell line and tissues compared with normal tissue and cells, which was consistent with numerous previous studies performed.

Many papers mentioned that *LincRNA-p21* could be a good biomarker related to human diseases.[37] Wang[37] provided evidence that *LincRNA-p21* suppressed the tumorigenicity of cancer stem cells (CSCs) and may be potential therapeutic genes against CSCs. What's more, in the research of Isin et al,[29] it might be helpful to improve the diagnostic prediction of the malignant state for patients with prostate cancer by discriminating potential of exosomal *LincRNA-p21* levels. In our study, we find *LincRNA-p21* could be a risk factor of ESCC. Therefore, *LincRNA-p21* levels may be an auxiliary diagnostic factor for ESCC.

Single-molecule RNA FISH was executed to observe the location of *LincRNA-p21*, as we can see in Figure 2, *LincRNA-p21* localized in both nucleus and cytoplasm, mainly in the cytoplasm, which is consistent with Yoon et al's description.[22]

The biological function of *LincRNA-p21* in many diseases has been proposed, particularly in cancers. These roles are mediated by the epigenetic regulation, and transcriptional and posttranscriptional regulation.[38–40] Some lncRNA have been identified in ESCC, such as MALAT1, UCA1, Linc00152, CFLAR-AS1, and POU3F3.[41–43] However, no data are available between *LincRNA-p21* and ESCC so far.

There is discrepancy with previous functional studies, which have proposed *LincRNA-p21* is involved in metastasis and invasion,[44] metabolism and cell reprogramming,[40] cell activity,[45] and Warburg effect.[36] Huarte et al[19] revealed a role for *LincRNA-p21* in a p53-dependent apoptotic response following DNA damage, and transcriptional repression by *LincRNA-p21* is mediated through the physical association with hnRNP-K, which is required for proper genomic localization of hnRNP-K at repressed genes and regulation of p53-mediated apoptosis. We confirmed that the upregulating *LincRNA-p21* inhibits the ability of proliferation, migration, and invasion in EC109 cell; promotes apoptosis; and contributes G1/S arrest.

To explore the role of *LincRNA-p21* regulating cell-cycle block in ESCC, we had gotten inspiration from microarray results and KEGG pathway analysis which p53 signaling pathway plays a lot (Figures 6 and 7). p53 pathway is the classical cancer pathway which mediates cellular stress responses including DNA repair, cell-cycle arrest, and apoptosis.[46] As shown in our paper (Figure 6), p21 and cyclin D may be essential downstream effectors of p53 pathway in the regulation of a cell process, leading to growth arrest especially in G1 arrest, which had been reported by Suk et al.[47] p21 can be activated to come into play by either p53-independent[48] or p53-dependent[49] manner. In our study, overexpressing *LincRNA-p21* increased the level of p21 while had no effect on p53, which implies that *LincRNA-p21* could influence EC109 cell proliferation and cycle via enhancing p21 activity in a p53-independent manner.

However, our research was still not perfect, and we did not observe the effect of *LincRNA-p21* on EC109 cells after *LincRNA-p21* knockdown in LV-EC109 cells. Hence, there is a lot of work to be completed. Of course, the current work is very valuable.

To conclude, our current study validated that *LincRNA-p21* expression levels were decreased in human ESCC tissues compared with adjacent normal tissues. Further, our

experiments illustrated that upregulation of *LincRNA-p21* led to the inhibition of ESCC functions in cell proliferation, apoptosis, cycle, migration, and invasion, suggesting that *LincRNA-p21* is an important biomarker and treatment target for ESCC. In addition, *LincRNA-p21* impedes the progression of EC09 cells by promoting expression of p21 and inhibiting expression of cyclin D through p53-independent pathway. However, more sufficient evidence need to be supplemented.

Conclusion

In summary, the study demonstrated that aberrantly downregulated *LincRNA-p21* increases the risk of ESCC progressing. Acting as a tumor suppressor, *LincRNA-p21* inhibited cell functions such as proliferation, migration, invasion and disturbing cell cycle. *LincRNA-p21* plays as a tumor inhibitor in ESCC development and *LincRNA-p21* might induce G1 arrest through p53 signal pathway.

Abbreviation list

ESCC, esophageal squamous cell carcinoma; *LincRNA-p21*, large intergenic noncoding RNA-p21; qRT-PCR, quantitative real-time reverse transcription PCR; CDK, cyclin-dependent kinases; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum.

Ethics approval and consent to participate

The population study was approved by the institutional review board (IRB) of the Zhongda Hospital of Southeast University, and written informed consent that conducted in accordance with the Declaration of Helsinki was obtained from the participants.

Availability of data and materials

Data and materials will be shared upon request.

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Author contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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