

Original Article

Up-regulation of the long non-coding RNA ANRIL indicates poor prognosis and promotes tumorigenesis in oral squamous cell carcinoma

Fen Liu^{1*}, Si Liu^{1*}, Linlin Chen¹, Zhenge Lei¹, Jingting Wu², Jianyong Wu^{1,3}

¹Department of Orthodontics, Affiliated Stomatological Hospital of Nanchang University, Nanchang, China;

²Department of Stomatology, Second Affiliated Hospital of Nanchang University, Nanchang, China; ³Department of Stomatology, Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China.

*Equal contributors.

Received February 5, 2017; Accepted April 17, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: Dysregulation of long non-coding RNAs (lncRNAs) plays a critical role in tumor progression. lncRNA antisense non-coding RNA in the INK4 locus (ANRIL) has been reported to promote tumor progression in numerous cancers. However, its expression and function in oral squamous cell carcinoma (OSCC) remain largely unclear. Herein, we investigated the expression and clinical significance of lncRNA ANRIL in human OSCC. The results showed that the expression of ANRIL was significantly increased in OSCC tissues and cell lines. In addition, high ANRIL expression was significantly associated with the tumor grade, TNM stage and distant metastasis ($P < 0.05$). Patients with high expression of ANRIL had poor overall survival as compared to those with low level ($P < 0.05$). Cox regression analysis showed that ANRIL could act as an independent prognostic factor in OSCC ($P < 0.05$). Functionally, *in vitro* assays showed that the suppression of ANRIL expression in OSCC cells significantly inhibited cell proliferation, migration, and invasion. These results indicated that ANRIL might play an important role in OSCC progression, and could serve as a novel prognostic biomarker and therapeutic target in OSCC.

Keywords: Oral squamous cell carcinoma, long non-coding RNAs, ANRIL, prognosis

Introduction

Oral squamous cell carcinoma (OSCC) accounts for approximately 90% of all malignant oral tumors [1]. Smoking, alcohol abuse, and betel quid chewing are the major risk factors of OSCC. Despite recent advances in diagnosis, surgery and chemotherapy strategies, the 5-year survival rate for OSCC patients remains very low [2]. Metastasis and recurrence are the main reasons of OSCC-induced mortality [3]. Therefore, the molecular mechanisms underlying OSCC tumorigenesis need to be elucidated in order to identify tumor-specific biomarkers and therapeutic targets for early diagnosis and treatment of the disease.

Long non-coding RNAs (lncRNAs) are a class of noncoding RNAs > 200 nucleotides, with limited protein-coding potential [4, 5]. Recent evidence indicates that lncRNAs play essential roles in many cellular and developmental pro-

cesses, including cell proliferation, apoptosis, and differentiation, as well as organ morphogenesis [6-8]. Abnormalities of lncRNAs have been implicated in the occurrence and development of various human cancers, including OSCC [9]. For instance HOTAIR [10], UCA1 [11], and MALAT1 [12], have been shown to contribute to the growth and metastasis of OSCC. Antisense non-coding RNA in the INK4 locus (ANRIL) is a 3.8 kb lncRNA transcribed from the *INK4B-ARF-INK4A* gene cluster in the opposite direction [13, 14]. It was identified as an oncogene in numerous tumors such as non-small-cell lung cancer [15], cervical cancer [16], gastric cancer [17], and hepatocellular carcinoma [18]. However, its expression pattern, clinical significance and functional roles in OSCC have not been investigated.

In the present study, we characterized the expression of lncRNA ANRIL in OSCC tissues and cell lines, and investigated its effects on

Table 1. Clinicopathological features and the expression of lncRNA ANRIL in OSCC patients

Parameters	Group	Total	lncRNA ANRIL		P value
			High	Low	
Gender	Male	65	37	28	0.669
	Female	51	27	24	
Age (years)	< 60	54	29	25	0.767
	≥ 60	62	35	27	
Tumor location	Tongue	45	26	19	0.452
	Floor of mouth	6	2	4	
	Buccal mucosa	25	15	10	
	Hard palate	8	4	4	
	Upper or lower gingival	32	17	15	
Tumor size	T1-T2	56	30	26	0.738
	T3-T4	60	34	26	
Tumor grade	G1	45	18	27	0.009
	G2/G3	71	46	25	
Distant metastasis	Yes	49	43	6	< 0.001
	No	67	21	46	
TNM Stage	I-II	43	18	25	0.027
	III-IV	73	46	27	

OSCC cell growth. To the best of our knowledge, this is the first study to provide novel insights into the role of ANRIL in the genesis and development of OSCC and as a potential therapeutic target in OSCC patients.

Materials and methods

Subjects

A total of 116 OSCC patients confirmed by pathological and clinical diagnoses at the Affiliated Stomatological Hospital of Nanchang University were enrolled between January 2013 and June 2015. This study was approved by the Ethics Committee of Affiliated Stomatological Hospital of Nanchang University, and written informed

consent was obtained from all patients. Tumor and adjacent normal tissues were obtained from the patients before they received any chemotherapy or radiotherapy. Clinicopathological characteristics are presented in **Table 1**. All tissue samples were stored in liquid nitrogen until they were utilized.

Cell lines and culture conditions

Human OSCC cell lines (Tca8113, SCC25, SCC4 and SCC9), and a human normal oral keratinocyte cell line (hNOK) were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma, MO, USA) containing 10% fetal bovine serum (FBS) (Hyclone, CA, USA), 100 U/ml penicillin sodium, and 100 mg/ml streptomycin. All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Small interfering RNA transfection

For gene knockdown, Tca8113 cells were seeded in 6-well plates to 100% confluency and transfected with siRNAs using Lipofectamine 2000 reagent (Invitrogen). The nucleotide sequence of siRNA for ANRIL was GGUCAUCU-

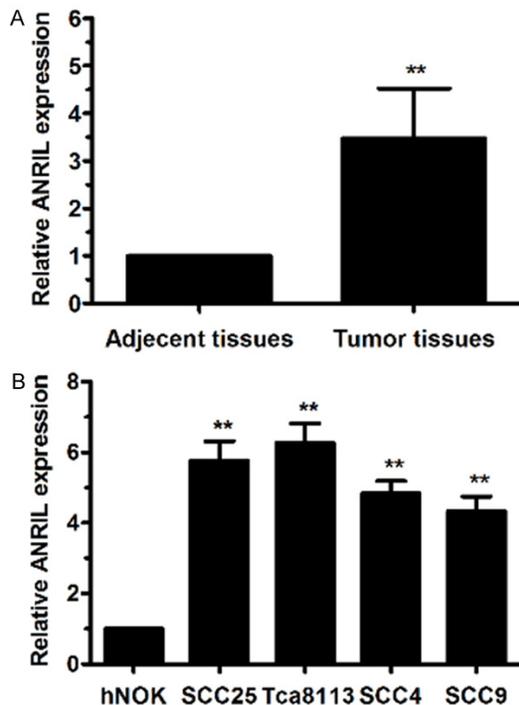


Figure 1. LncRNA ANRIL is up-regulated in OSCC tissues and cell lines. A. Relative expression of ANRIL in OSCC tissues (n=116) and adjacent normal tissues by qRT-PCR. B. Relative expression of ANRIL in oral cancer cell lines (SCC4, SCC9, SCC25, Tca8113) and normal oral keratinocyte cell line hNOK. **P < 0.01.

LncRNA ANRIL and oral cancer

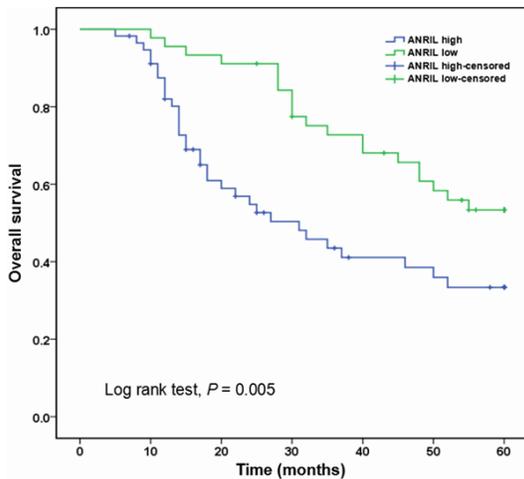


Figure 2. Kaplan-Meier curves for overall survival in patients with OSCC divided according to lncRNA ANRIL expression: Patients in high expression group had significantly poorer prognosis than those in low expression group ($P = 0.005$, log-rank test).

CAUUGCUCUAU. Sequences of non-target scramble controls (si-NC) were provided by Invitrogen. After transfection, the cells were harvested for RNA extraction and quantitative real-time PCR (qRT-PCR).

RNA extraction and qRT-PCR

Total RNA was isolated from tissue samples or cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription of 1 μ g total RNA using Reverse Transcription kit (Takara, China). The qRT-PCR was performed with SYBR Green assay (Takara, China). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. The PCR primers for ANRIL and GAPDH were as follows: ANRIL sense, 5'-TGCTATCCGCAATCAGG-3' and reverse, 5'-GGCCTCAGTGGCACATACC-3'; GAPDH sense, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTG-AAGACGCCAGTGG-3'. The data were analyzed by $2^{-\Delta\Delta CT}$ method. qRT-PCR was repeated in triplicate for each sample.

Cell proliferation assay

The cells were plated in 96-well plates at a density of 2×10^3 cells/well the day before transfection. Cell growth was assessed after the siRNA transient transfection for 24, 48, 72 and 96 h. A total of 10 ml WST-8 reagent from

Cell Counting kit-8 (Promega, CA, USA) was added to each well and incubated at 37°C for 2 h. The absorbance values were detected at 450 nm using SpectraMax M3 (Molecular Devices, California, USA).

Wound healing assay

Migration ability of the cells was measured using a wound healing assay. Tca8113 cells transfected with either si-ANRIL or si-NC were seeded into 6-well plates, incubated with their respective complete culture medium and grown to 100% confluency overnight. The monolayer was scratched and then incubated in fresh medium for 24 hours after which the width of the wound was measured. Three different locations were visualized and photographed with a phase-contrast inverted microscope (Leica, Solms, Germany).

Transwell invasion assay

Cell invasion assay was performed using 24-well transwells (8- μ m pore size, Corning Life Sciences) coated with 1 mg/mL Matrigel (BD Sciences). Tca8113 cells transfected with either si-ANRIL or si-NC were collected and resuspended in serum-free medium at a concentration of 1×10^5 cells/ml. The cells were seeded in the upper chamber of the wells in 200 μ l FBS-free medium, and the lower chambers were filled with 500 μ l 20% FBS medium. After incubation for 48 hours at 37°C in 5% CO₂, the cells on the filter surface were fixed with methanol, stained with 0.1% crystal violet, and photographed with a phase-contrast inverted microscope (Leica). The cells from at least five random microscopic fields ($\times 100$) were counted.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software (IBM, SPSS, Chicago, IL, USA). Student's t-test was used to estimate the different expression levels of ANRIL and the data are shown as mean \pm standard deviation (SD). The association between the clinical features and ANRIL expression was evaluated by chi-square method. Kaplan-Meier method with log-rank test was applied to analyze the overall survival of the OSCC patients, and univariate and multivariate Cox regression analysis were used to evaluate the prognostic value of ANRIL. All tests were two-tailed and $P < 0.05$ was considered statistical significance.

Results

Expression of lncRNA ANRIL is up-regulated in OSCC tissues

To investigate the role of ANRIL in OSCC, its expression levels in 116 OSCC tissues and 116 adjacent non-tumor tissues were detected by qRT-PCR. As shown in **Figure 1A**, expression of ANRIL was significantly up-regulated in OSCC tissues as compared to the adjacent non-tumor tissues ($P < 0.01$). These results suggested that abnormal ANRIL expression may be related to OSCC pathogenesis. Then, we assessed the expression of ANRIL in several OSCC cell lines and a human oral keratinocyte cell line hNOK by qRT-PCR. As shown in **Figure 1B**, OSCC cell lines exhibited a higher expression of ANRIL as compared to hNOK by qRT-PCR ($P < 0.01$).

Relationship between ANRIL expression level and the clinicopathological factors in OSCC patients

To evaluate the association between ANRIL expression and clinical features, the OSCC patients were divided into high and low expression groups based on their average expression of ANRIL. The chi-square test showed that the expression levels of ANRIL were associated with the TNM stage, tumor grade and distant metastasis ($P < 0.05$) (**Table 1**), but not with age, gender, tumor location and tumor size. Thus, ANRIL might have important implications for the progression of OSCC.

Overall survival analysis

In order to determine the prognostic significance of ANRIL in OSCC, the relationship between ANRIL levels and overall survival rates was examined by Kaplan-Meier analysis and log-rank test in 116 OSCC cases, which showed that high ANRIL expression predicted poor overall survival of OSCC ($P < 0.01$, **Figure 2**).

ANRIL expression was a potential independent prognostic marker for OSCC patients

Cox regression analysis was used to estimate the prognostic value of ANRIL. Univariate analysis showed that the levels of ANRIL were significantly associated with poor prognosis in OSCC patients ($P < 0.01$). Multivariate analysis

showed that ANRIL was an independent factor for OSCC prognosis ($P < 0.01$). These results are summarized in **Table 2**.

Knockdown of ANRIL inhibited growth of Tca8113 cells in vitro

To further investigate the role of ANRIL in OSCC cells, si-ANRIL was designed and transfected into Tca8113 cells to determine its effect on cell growth *in vitro*. As shown in **Figure 3A**, si-ANRIL transfected cells showed a significantly decreased mRNA expression level of ANRIL as compared to the control group ($P < 0.05$). CCK-8 assay revealed that ANRIL knockdown obviously suppressed the proliferation rate of Tca8113 cells at 48, 72 and 96 h ($P < 0.05$, **Figure 3B**). Furthermore, to analyze the role of ANRIL in cell migration and invasion, wound healing and transwell invasion assays were performed with Tca8113 cells. Wound healing assays showed that the migration ability of Tca8113 cells transfected with si-ANRIL was significantly decreased as compared to the control group ($P < 0.05$, **Figure 3C**). Transwell invasion assay revealed that the invasion capacity of Tca8113 cells transfected with si-ANRIL was notably down-regulated as compared to the control group ($P < 0.05$, **Figure 3D**). Taken together, these data demonstrated that down-regulation of ANRIL could inhibit OSCC cell growth and metastasis.

Discussion

Despite great progress in early diagnosis, surgical techniques, and chemotherapy, the prognosis of patients with OSCC remains unsatisfactory [19]. Therefore, a better understanding of the molecular events associated with OSCC is critical to improve the clinical strategies and outcomes of patients. Recently, many lncRNAs have been reported to play significant regulatory roles in human diseases [20]. Various OSCC-associated lncRNAs, and their clinical significance and functions have been identified. For example, Liu et al. reported that lncRNA HOTAIR was up-regulated and associated with poorer overall survival of OSCC patients [10]. Liang et al. showed that lncRNA TUG1 could promote OSCC cell growth, proliferation and invasion by targeting Wnt/ β -catenin signaling [21]. Zhou et al. showed that lncRNA MALAT1 was up-regulated and associated with poor prognosis of OSCC, and promoted tumor growth and metastasis *in vitro* by

Table 2. Cox regression analysis for prognosis in OSCC patients

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
Gender	1.307	0.581-2.365	0.623	-	-	-
Age	0.983	0.662-2.405	0.714	-	-	-
Tumor location	1.595	0.813-2.849	0.446	-	-	-
Tumor size	1.002	0.701-2.148	0.674	-	-	-
Tumor grade	2.960	1.615-5.487	0.007	2.975	1.529-6.094	< 0.001
Distant metastasis	3.855	1.836-7.304	< 0.001	3.240	1.671-6.853	< 0.001
TNM Stage	2.389	1.325-5.718	0.016	2.586	1.303-5.483	0.008
ANRIL	3.014	1.532-6.155	0.003	2.942	1.485-5.974	< 0.001

‘-’: Indicated no available data.

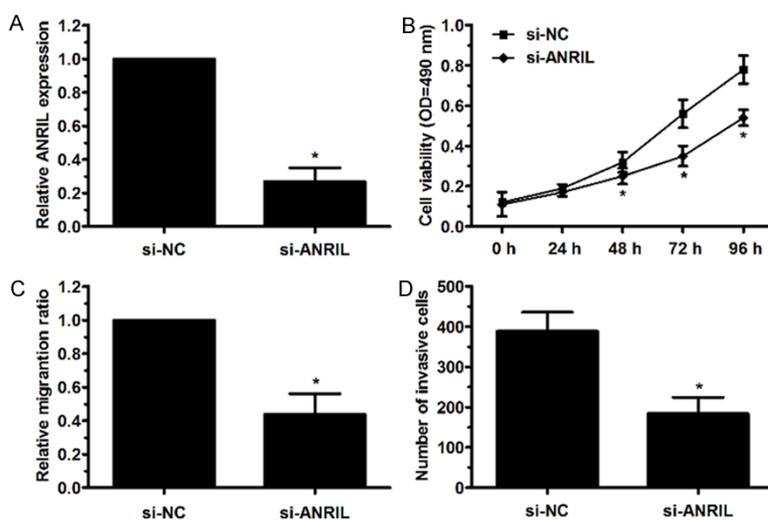


Figure 3. Knockdown of lncRNA ANRIL expression suppresses oral cancer cell proliferation, migration and invasion *in vitro*. A. qRT-PCR analysis of ANRIL expression following treatment of Tca8113 cells with siRNAs targeting ANRIL. B. MTT assay showing ANRIL knockdown inhibited cell proliferation of Tca8113 cells. C. Tca8113 cells transfected with si-ANRIL displayed significantly lower migration ability compared with those transfected with si-NC. D. Tca8113 cells transfected with si-ANRIL displayed significantly lower invasion ability compared with those transfected with si-NC. * $P < 0.05$.

inducing epithelial-mesenchymal transition (EMT) in OSCC [13]. These findings suggested that lncRNAs could serve as diagnostic and prognostic biomarkers in OSCC. However, lncRNAs are an emerging field, and most lncRNAs are not known to be involved in OSCC pathogenesis.

ANRIL was shown to be up-regulated and predicted poor prognosis of various cancers, and its knockdown arrested cell growth, inhibited invasion by affecting the EMT and promoted apoptosis in lung, breast and gastric cancers

[22]. However, to our knowledge, the role of lncRNA ANRIL in the carcinogenesis of OSCC remains unclear.

In the present study, the expression of ANRIL was found to be increased in OSCC tissues and cell lines as compared to adjacent non-tumor tissues and human normal oral keratinocyte cell line hNOK. The relative expression level of ANRIL was associated with TNM stage and distant metastasis of OSCC patients. Furthermore, the prognostic value of ANRIL by Kaplan-Meier and Cox regression analyses was evaluated, and patients with high ANRIL expression had poor overall survival. The multivariate analyses implied that high ANRIL expression was a potential independent prognostic factor for overall survival of OSCC. These findings highlight the clinical significance of ANRIL in patients with OSCC and imply a potentially important role for ANRIL in predicting the progression of OSCC. To further investigate the underlying mechanism of ANRIL in OSCC progression, we explored the effects of loss-of-function of ANRIL in OSCC cells. The results showed that knockdown of ANRIL significantly repressed the proliferation, migration and invasion of OSCC cells *in vitro*.

In summary, lncRNA ANRIL plays a vital role in the development and progression of OSCC. Moreover, ANRIL silencing in OSCC cells markedly suppressed cell proliferation, migration, and invasion. Taken together, these findings suggest an important role of ANRIL as a potential biomarker and therapeutic target in OSCC.

Disclosure of conflict of interest

None.

Address correspondence to: Jianyong Wu, Department of Stomatology, Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China. Tel: 86-21-25078999; Fax: 86-21-25078999; E-mail: yfyzk@qq.com

References

- [1] Rosebush MS, Rao SK, Samant S, Gu W, Handorf CR, Pfeffer LM and Nosrat CA. Oral cancer: enduring characteristics and emerging trends. *J Tenn Dent Assoc* 2011; 91: 24-27; quiz 28-29.
- [2] Sasahira T, Kirita T and Kuniyasu H. Update of molecular pathobiology in oral cancer: a review. *Int J Clin Oncol* 2014; 19: 431-436.
- [3] Irani S. Distant metastasis from oral cancer: a review and molecular biologic aspects. *J Int Soc Prev Community Dent* 2016; 6: 265-271.
- [4] Ponting CP, Oliver PL and Reik W. Evolution and functions of long noncoding RNAs. *Cell* 2009; 136: 629-641.
- [5] Kornienko AE, Guenzl PM, Barlow DP and Pauler FM. Gene regulation by the act of long non-coding RNA transcription. *BMC Biol* 2013; 11: 59.
- [6] Huang Z, Luo Q, Yao F, Qing C, Ye J, Deng Y and Li J. Identification of differentially expressed long non-coding RNAs in polarized macrophages. *Sci Rep* 2016; 6: 19705.
- [7] Chen X, Liu L and Zhu W. Up-regulation of long non-coding RNA CCAT2 correlates with tumor metastasis and poor prognosis in cervical squamous cell cancer patients. *Int J Clin Exp Pathol* 2015; 8: 13261-13266.
- [8] Qiao C, Zhang Y, Jin L, Du X and Qiao Z. High expression of lncRNA AFAP1-AS1 promotes cell proliferation and invasion by inducing epithelial-to-mesenchymal transition in gastric cancer. *Int J Clin Exp Pathol* 2017; 10: 393-400.
- [9] Gomes CC, de Sousa SF, Calin GA and Gomez RS. The emerging role of long noncoding RNAs in oral cancer. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2017; 123: 235-241.
- [10] Liu H, Li Z, Wang C, Feng L, Huang H, Liu C and Li F. Expression of long non-coding RNA-HOTAIR in oral squamous cell carcinoma Tca8113 cells and its associated biological behavior. *Am J Transl Res* 2016; 8: 4726-4734.
- [11] Yang YT, Wang YF, Lai JY, Shen SY, Wang F, Kong J, Zhang W and Yang HY. Long non-coding RNA UCA1 contributes to the progression of oral squamous cell carcinoma by regulating the WNT/beta-catenin signaling pathway. *Cancer Sci* 2016; 107: 1581-1589.
- [12] Zhou X, Liu S, Cai G, Kong L, Zhang T, Ren Y, Wu Y, Mei M, Zhang L and Wang X. Long non coding RNA MALAT1 promotes tumor growth and metastasis by inducing epithelial-mesenchymal transition in oral squamous cell carcinoma. *Sci Rep* 2015; 5: 15972.
- [13] Li Z, Yu X and Shen J. ANRIL: a pivotal tumor suppressor long non-coding RNA in human cancers. *Tumour Biol* 2016; 37: 5657-5661.
- [14] Liu FT, Zhu PQ, Luo HL, Zhang Y, Hao TF, Xia GF, Zhu ZM and Qiu C. Long noncoding RNA ANRIL: a potential novel prognostic marker in cancer: a meta-analysis. *Minerva Med* 2016; 107: 77-83.
- [15] Lu Y, Zhou X, Xu L, Rong C, Shen C and Bian W. Long noncoding RNA ANRIL could be transactivated by c-Myc and promote tumor progression of non-small-cell lung cancer. *Onco Targets Ther* 2016; 9: 3077-3084.
- [16] Zhang D, Sun G, Zhang H, Tian J and Li Y. Long non-coding RNA ANRIL indicates a poor prognosis of cervical cancer and promotes carcinogenesis via PI3K/Akt pathways. *Biomed Pharmacother* 2017; 85: 511-516.
- [17] Zhang EB, Kong R, Yin DD, You LH, Sun M, Han L, Xu TP, Xia R, Yang JS, De W and Chen J. Long noncoding RNA ANRIL indicates a poor prognosis of gastric cancer and promotes tumor growth by epigenetically silencing of miR-99a/miR-449a. *Oncotarget* 2014; 5: 2276-2292.
- [18] Hua L, Wang CY, Yao KH, Chen JT, Zhang JJ and Ma WL. High expression of long non-coding RNA ANRIL is associated with poor prognosis in hepatocellular carcinoma. *Int J Clin Exp Pathol* 2015; 8: 3076-3082.
- [19] Sinevici N and O'Sullivan J. Oral cancer: deregulated molecular events and their use as biomarkers. *Oral Oncol* 2016; 61: 12-18.
- [20] Huarte M. The emerging role of lncRNAs in cancer. *Nat Med* 2015; 21: 1253-1261.
- [21] Liang S, Zhang S, Wang P, Yang C, Shang C, Yang J and Wang J. LncRNA, TUG1 regulates the oral squamous cell carcinoma progression possibly via interacting with Wnt/beta-catenin signaling. *Gene* 2017; 608: 49-57.
- [22] Aguilo F, Di Cecilia S and Walsh MJ. Long non-coding RNA ANRIL and polycomb in human cancers and cardiovascular disease. *Curr Top Microbiol Immunol* 2016; 394: 29-39.