Int J Clin Exp Pathol 2016;9(10):10803-10809
www.ijcep.com /ISSN:1936-2625/IJCEP0038080

Original Article

Long non-coding RNA ANRIL promotes tumorigenesis in glioma via MAPK signaling pathways

Xuchang Xu, Xuefeng Wan , Zhiren Zhang

Department of Neurosurgery, Zhumadian Central Hospital, Zhumadian 463000, Henan, China

Received August 17, 2016; Accepted August 26, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Long non-coding RNA has been proven to be involved in many biological processes in cancer tumorigenesis and progression. LncRNA ANRIL has various expression patterns in diverse human cancers. However, its clinical significance and function in glioma has not yet been elucidated. In the present study, we explored the expression of ANRIL in 33 paired glioma tissues and adjacent non-tumor tissues. We found that the expression of ANRIL was obviously upregulated in glioma tissue compared to adjacent non-tumor tissues and the detected level of ANRIL was associated with tumor grade. In addition, Kaplan-Meier analysis showed that high expression of ANRIL clearly predicted poorer overall survival (OS) in glioma patients. To explore the functional role of ANRIL, siRNA ANRIL and negative control siRNA were transfected into glioma cells. We found that decreased expression of ANRIL significantly inhibited cell proliferation in vitro. Further studies indicated that inhibition of ANRIL induced cell cycle arrest at the G1/S transition, and enhanced apoptosis of glioma cells. Finally, Western blot showed that ANRIL may exert its function by targeting the MAPK signaling pathways. In conclusion, our study suggested that ANRIL might act as an oncogenic lncRNA that promotes proliferation of glioma cells and could be regarded as a therapeutic target in human glioma.

Keywords: Glioma, ANRIL, long non-coding RNA, MAPK

Introduction

Glioma is one of the most common types of malignant tumor in central nervous system [1]. Despite recent advances in treatment of surgery, radiotherapy and chemotherapy, the prognosis of these patients is still poor [2]. Therefore, it is extremely vital for us to elucidate new mechanisms associated with glioma progression and establish potential therapeutic targets for the treatment of glioma.

In the light of recent transcriptomic studies, it has become apparent that the majority of the transcribed genome is non-coding [3]. Among these, there is a kind of non-coding RNA called long non-coding RNA (lncRNA >200 bp in length), which have been proven to regulate many biological processes from nuclear organization to epigenetic modification of post-transcriptional regulation and RNA splicing, especially in cancers [4, 5]. In previous studies, many lncRNAs have been reported to play crucial roles in glioma. For example, Hu et al showed that upregulation of lncRNA AB073614 predicted a poor prognosis in patients with glioma [6]. Liu et al suggested that knockdown of lncRNA SPRY4-IT1 suppressed glioma cell proliferation, metastasis and epithelial-mesenchymal transition [7]. Ke et al showed that knockdown of lncRNA HOTAIR inhibited malignant biological behaviors of human glioma cells via modulation of miR-326 [8]. However, there are little studies about the role of lncRNA ANRIL in glioma.

lncRNA ANRIL (CDKN2B antisense RNA 1) is a 3.8 kb lncRNA transcribed from the INK4B-ARF-INK4A gene cluster in the opposite direction [9]. Previous studies showed that ANRIL play important roles in tumor progression and development. For example, Lin et al found that increased expression of the lncRNA ANRIL promoted lung cancer cell metastasis and correlates with poor prognosis [10]. Zhang et al showed that lncRNA ANRIL indicated a poor prognosis of gastric cancer and promotes tumor growth by epigenetically silencing of miR-99a/

miR-449a [11]. Chen et al suggested that lncRNA ANRIL inhibited p15 INK4b through the TGFβ1 signaling pathway in human esophageal squamous cell carcinoma [12]. However, the relationship between lncRNA ANRIL and glioma is still largely unknown. The objective of our study was to detect the biological behaviors and molecular mechanisms of lncRNA ANRIL in glioma in order to seek a potential therapeutic target for glioma patients.

Materials and methods

Tissue samples

A total of 33 glioma tissues were collected from surgical resections in Zhumadian Central Hospital. The adjacent non-tumor tissues, which are defined as normal in the results, were obtained 2 cm away from the glioma tissue. 3 patients had grade I (pilocytic astrocytoma), 8 patients had grade II (diffuse astrocytoma), 13 patients had grade III (anaplastic astrocytoma) and 9 patients had grade IV (primary brain glioblastoma), according to the 2007 World Health Organization's classification system. None of the patients received chemotherapy or radiotherapy before surgery. Tumor samples and normal tissues were rapidly frozen in liquid nitrogen and kept at -80°C until used. The study was approved by the Research Ethics Committee of Zhumadian Central Hospital, and written informed consent was obtained from all patients.

Cell lines

Human glioma cell lines (U87 and U251) were purchased from ATCC. All glioma cells were cultured at 37°C in a CO_2 incubator in Dulbecco's modified Eagle's media (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were used when they were in the logarithmic growth phase.

RNA isolation and quantitative qRT-PCR

Total RNA was extracted from tissues and cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The expression levels of ANRIL were determined by quantitative real-time polymerase chain reaction (qRT-PCR) using the SYBR® Green master mix (TaKaRa). The primers are as follows: lncRNA ANRIL 5'-GGGGTACCATGTCCGCTGCACTTT-3' and 5'-GCAAAGCTTACCCAACCTGGTAAGTGG-3'; GAPDH 5'-CGCTCTCTGCTCCTCCTGTTC-3' and 5'-ATCCGTTGACTCCGACCTTCAC-3'. The expression of lncRNA was normalized to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method.

Small interfering RNA transfection

Small interfering RNAs (siRNAs) specifically targeting ANRIL (si-ANRIL) and a scrambled negative control (si-NC) were provided by GenePharma. The sequences of si-ANRIL were as follows: si-ANRIL-1: 5'-GCAAGAAACATTGCTGCTAGC-3'; si-ANRIL-2: 5'-GCCCAATTATGCTGTGGTAAC-3'. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfected cells were harvested 48 h after transfection.

Cell proliferation assay

24 after transfection, cells were seeded in 96-well plates at 8000 cells per well. The MTT assay was used to determine cell viability at the point of 24, 48 and 72 h after seeding. The absorbance at 570 nm was measured using a Quant Universal Microplate Spectrophotometer (BioTek).

Colony formation assay

Cells were seeded in 6-well plates at a density of 100 cells/well. After incubation for 14 days, cells were washed twice with PBS, fixed with methanol and stained with crystal violet. The number of colonies containing >50 cells was counted under a microscope.

Cell cycle and apoptosis assays

For cell cycle analysis, transfected cells were cultured for 24 h and then treated with nocodazole (100 ng/ml) for 16-20 h, at last cells were collected and were fixed into 70% ethanol at 20°C for 24 h, stained with 50 μg/ml propidium iodide (Kaiji).

For apoptosis assay, cells were stained with 50 μg/ml propidium iodide and Annexin V-fluorescein isothiocyanate (Kaiji) and following the manufacturer's instructions. The results were analyzed using ModFit software (BD Bioscience).

Western blot assay

Western blot assay were performed using the following primary antibodies: p38, p-p38, JNK, p-JNK, ERK1/2, p-ERK1/2 and GAPDH (Abcam). Stimulated cells were lysed with RIPA buffer

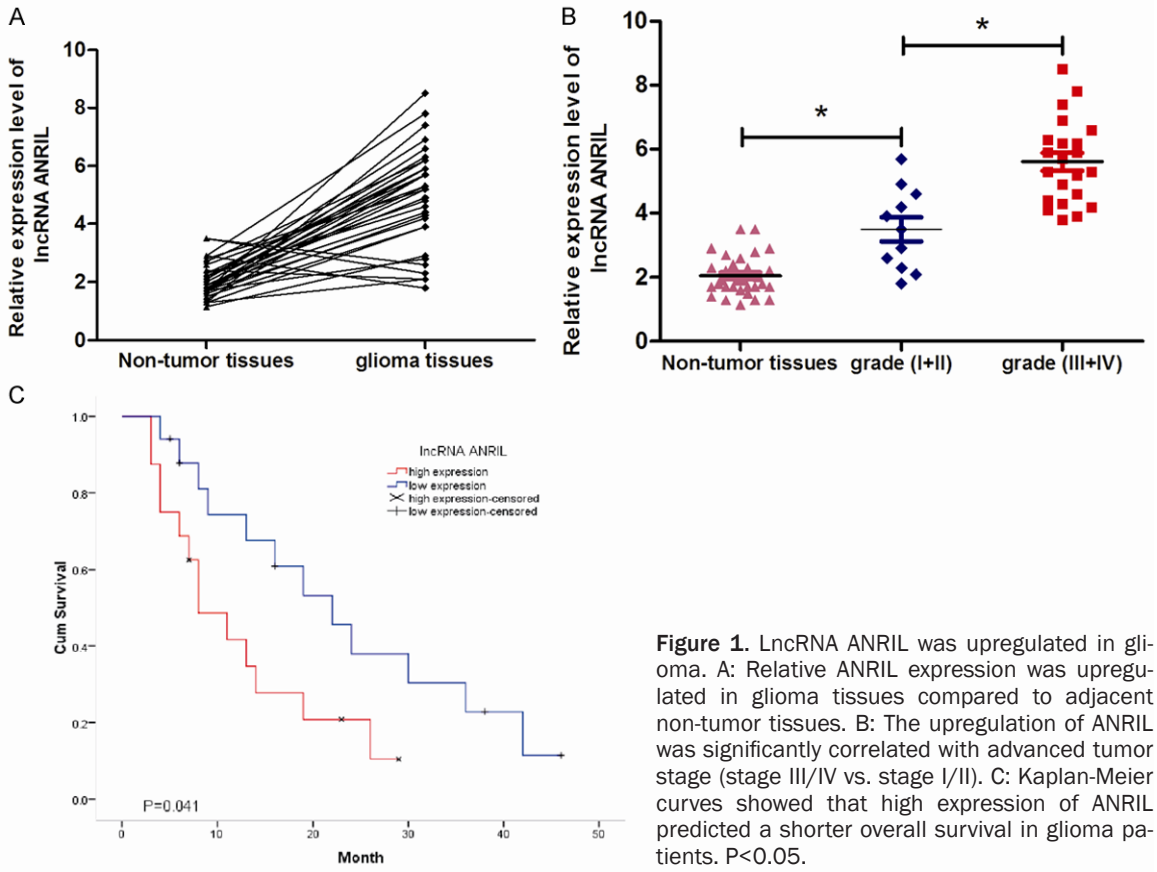

Figure 1. LncRNA ANRIL was upregulated in glioma. A: Relative ANRIL expression was upregulated in glioma tissues compared to adjacent non-tumor tissues. B: The upregulation of ANRIL was significantly correlated with advanced tumor stage (stage III/IV vs. stage I/II). C: Kaplan-Meier curves showed that high expression of ANRIL predicted a shorter overall survival in glioma patients. P<0.05.

containing 1% protease inhibitors (PMSF); cell protein lysates were separated on 10% SDS PAGE gels and transferred to PVDF membranes. The membranes were incubated with specific antibodies. Finally, ECL chromogenic substrates used in our experiment were quantified by densitometry.

Statistical analysis

All statistical analyses in our experiment were performed using SPSS 18.0 software. Data are shown as the mean ± SD. The differences between groups were analyzed by the Student's t-test, Wilcoxon test, or χ^2 test. The Kaplan-Meier method was performed for patients' overall survival analysis. All experiments were run in triplicate. P<0.05 was considered statistically significant difference.

Results

LncRNA ANRIL is up-regulated in glioma

To explore whether lncRNA ANRIL is dysregulated in glioma, qRT-PCR was used to determine the expression level of ANRIL. Among the 33 pairs of glioma tissues, the expression of ANRIL was significantly up-regulated in glioma tissues compared to the adjacent non-tumor tissues (**Figure 1A**, P<0.05). In addition, ANRIL overexpression was closely correlated with advanced tumor stage (III+IV vs. I+II, **Figure 1B**, P<0.05). We further determined whether ANRIL expression level was associated with the outcome of glioma patients. Kaplan-Meier curve indicated that high level of ANRIL was significantly associated with poor overall survival in glioma patients (**Figure 1C**, P<0.05). These results suggested that ANRIL might play a critical role in glioma progression.

Knockdown of lncRNA ANRIL inhibits glioma cell proliferation

To explore the functional role of lncRNA ANRIL in glioma, the si-ANRIL was transfected into glioma cell lines. qRT-PCR assay revealed that ANRIL expression was significantly reduced in si-ANRIL transfected glioma cells (**Figure 2A**, P<0.05). In order to examine the proliferative ability of ANRIL, MTT assay was performed. As

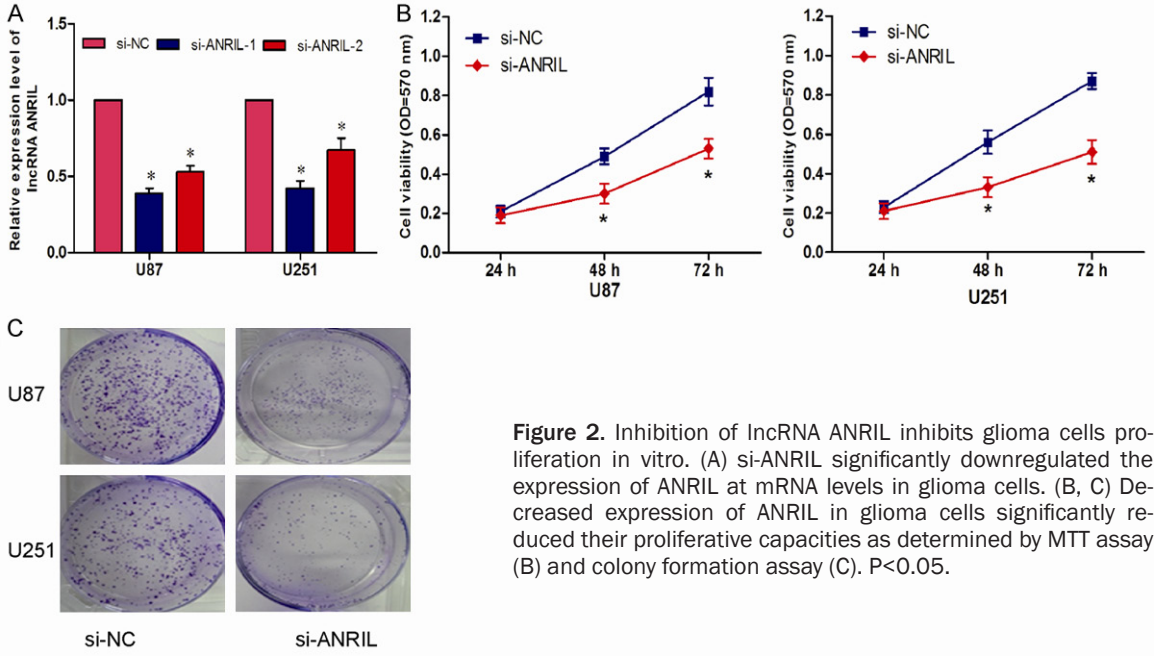

Figure 2. Inhibition of lncRNA ANRIL inhibits glioma cells proliferation in vitro. (A) si-ANRIL significantly downregulated the expression of ANRIL at mRNA levels in glioma cells. (B, C) Decreased expression of ANRIL in glioma cells significantly reduced their proliferative capacities as determined by MTT assay (B) and colony formation assay (C). P<0.05.

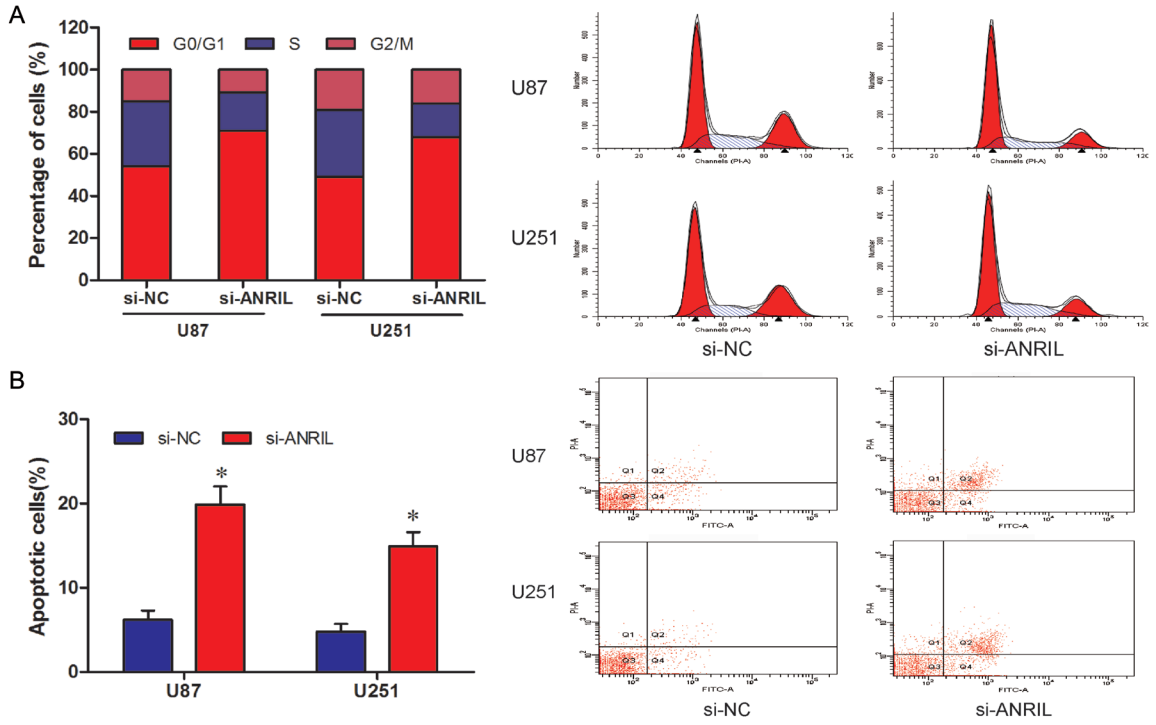

Figure 3. Inhibition of lncRNA ANRIL induces glioma cells cycle arrest and apoptosis. A: Cell cycle analysis showed that inhibition of ANRIL increased the percentage of G0/G1 phase cells and decreased the percentage of S phase cells. B: Cell apoptosis assay showed that inhibition of the ANRIL promoted glioma cells apoptosis. *P<0.05.

shown in **Figure 2B**, decreased expression of ANRIL significantly suppressed cell proliferation in glioma cells compared to the control group. Furthermore, colony formation assay was performed, and the result similarly indicated that clonogenic survival was significantly decreased in si-ANRIL transfected cells compared to the control group (**Figure 2C**, P<0.05).

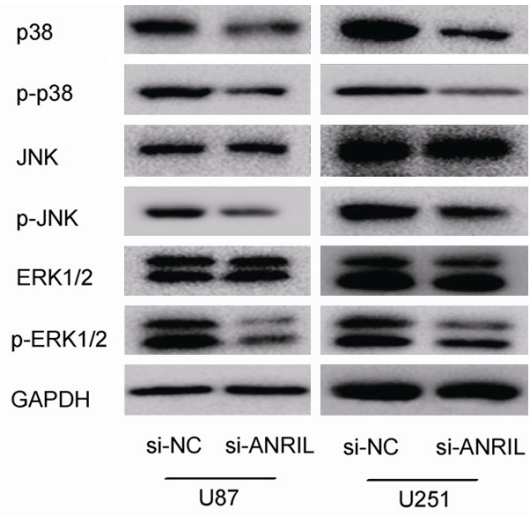

Figure 4. Inhibition of lncRNA ANRIL inactivates MAPK signaling pathways. The levels of ERK1/2, p-ERK1/2, p38, p-p38, JNK and p-JNK were examined by western blot analysis in si-ANRIL transfected glioma cells.

Knockdown of lncRNA ANRIL induces glioma cell G1 phase arrest and apoptosis

To explore the potential mechanisms underlying cell growth, flow cytometry was used. Cell cycle distribution analysis revealed that inhibition of ANRIL resulted in a significant increased in the cellular population in G0/G1 phase but a sharp decrease in S phase (**Figure 3A**, $P<0.05$). Apoptosis analysis showed that silencing of ANRIL induced glioma cell apoptosis compared with control group (**Figure 3B**, $P<0.05$). These findings suggested that ANRIL promoted glioma cells proliferation by promoting cell cycle progression and inhibiting cell apoptosis.

lncRNA ANRIL activate MAPK signaling pathways in glioma cells

To further define the underlying molecular mechanism modulated by ANRIL in the growth of glioma cells, we focused on the Mitogen-activated protein kinase (MAPK) pathway, which including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, are crucial molecules involved in pathways associated with cancer pathogenesis [13]. Western blot analysis was used to detect the activation of MAPK signaling pathways in si-ANRIL transfected glioma cells. Our data showed that inhibition of ANRIL in glioma cells strongly reduced the levels of p-ERK1/2, p-p38 and p-JNK MAPK (**Figure 4**). Thus, we suggested that lncRNA ANRIL might promote glioma cell proliferation by regulating MAPK signaling pathway.

Discussion

The sequencing of the human genome revealed that the coding portion of the genome represents less than 2% of the genome. The remaining 98% of transcription products of the genome consists of non-coding RNA sequences, including microRNAs and lncRNAs [14]. Recently, numerous pieces of evidence showed that dysregulation in lncRNAs are proved to contribute in tumor development in many cancer types and can be used to develop as biomarkers and therapy target [15]. For example, Li et al showed that increased expression of lncRNA H19 promotes gastric cancer cell progression and correlated with poor prognosis [16]. Yang et al suggested that lncRNA HULC promoted colorectal carcinoma progression through epigenetically repressing NKD2 expression [17]. Li et al showed that overexpression of lncRNA HOTTIP increased chemoresistance of osteosarcoma cell by activating the Wnt/β-catenin pathway [18].

In the present study, we found that the expression of ANRIL was obviously upregulated in glioma tissue and the detected levels of ANRIL were associated with tumor grade. Kaplan-Meier analysis suggested that high expression of ANRIL clearly predicted poorer overall survival in glioma. In function assays, we found that inhibition of ANRIL significantly suppressed cell proliferation in vitro. Further studies revealed that inhibition of ANRIL induced cell cycle arrest at the G1/S transition, and enhanced apoptosis of glioma cells. These findings demonstrated that ANRIL might act as a tumor oncogene in glioma progression.

ERK/p38/JNK MAPK signaling pathways have been demonstrated to regulate a variety of cellular activities, including proliferation, differentiation, survival and death [19]. Recent studies suggested that MAPK signaling activation were involved in various human cancers. For example, Peng et al showed that lncRNA CCHE1 indicated a poor prognosis of hepatocellular carcinoma and promoted carcinogenesis via activation of the ERK/MAPK pathway [20]. Wu et al found that lncRNA MALAT1 promoted the proliferation and metastasis of gallbladder cancer cells by activating the ERK/MAPK pathway [21]. In this study, we showed that inhibition of ANRIL significantly suppressed p-ERK1/2, p-p38 and

p-JNK MAPK expression in glioma cells. These data indicated that tumor oncogenic effect of ANRIL was at least partly mediated by MAPK pathway in glioma cells, which expanded the function of MAPK signaling pathway in glioma progression.

In conclusion, the present study suggested that lncRNA ANRIL promoted cell proliferation via MAPK signaling in glioma. Our findings demonstrated that lncRNA ANRIL may become a promising new candidate for glioma prognosis and therapy.

Acknowledgements

The project was supported by the Technology Research and Development Project of Zhumadian (NO.20604).

Disclosure of conflict of interest

None.

Address correspondence to: Zhiren Zhang, Department of Neurosurgery, Zhumadian Central Hospital, No.747 West Zhonghua Road, Yicheng District, Zhumadian 463000, Henan, China. E-mail: yukun1967@163.com

References

[1] Louis DN, Khaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW and Kleihues P. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 2007; 114: 97-109.

[2] Schwartzbaum JA, Fisher JL, Aldape KD and Wrensch M. Epidemiology and molecular pathology of glioma. Nat Clin Pract Neurol 2006; 2: 494-503; quiz 491 p following 516.

[3] Mattick JS and Makunin IV. Non-coding RNA. Hum Mol Genet 2006; 15: R17-29.

[4] Mercer TR, Dinger ME and Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.

[5] Gutschner T and Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. RNA Biol 2012; 9: 703-719.

[6] Hu L, Lv QL, Chen SH, Sun B, Qu Q, Cheng L, Guo Y, Zhou HH and Fan L. Up-Regulation of Long Non-Coding RNA AB073614 Predicts a Poor Prognosis in Patients with Glioma. Int J Environ Res Public Health 2016; 13: 433.

[7] Liu H, Lv Z and Guo E. Knockdown of long non-coding RNA SPRY4-IT1 suppresses glioma cell proliferation, metastasis and epithelial-mesenchymal transition. Int J Clin Exp Pathol 2015; 8: 9140-9146.

[8] Ke J, Yao YL, Zheng J, Wang P, Liu YH, Ma J, Li Z, Liu XB, Li ZQ, Wang ZH and Xue YX. Knockdown of long non-coding RNA HOTAIR inhibits malignant biological behaviors of human glioma cells via modulation of miR-326. Oncotarget 2015; 6: 21934-21949.

[9] Yap KL, Li S, Munoz-Cabello AM, Raguz S, Zeng L, Mujtaba S, Gil J, Walsh MJ and Zhou MM. Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell 2010; 38: 662-674.

[10] Lin L, Gu ZT, Chen WH and Cao KJ. Increased expression of the long non-coding RNA ANRIL promotes lung cancer cell metastasis and correlates with poor prognosis. Diagn Pathol 2015; 10: 14.

[11] 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.

[12] Chen D, Zhang Z, Mao C, Zhou Y, Yu L, Yin Y, Wu S, Mou X and Zhu Y. ANRIL inhibits p15 INK4b through the TGFβ1 signaling pathway in human esophageal squamous cell carcinoma. Cell Immunol 2014; 289: 91-96.

[13] Johnson GL and Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002; 298: 1911-1912.

[14] Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, Ballew RM, Huson DH, Wortman JR, Zhang Q, Kodira CD, Zheng XH, Chen L, Skupski M, Subramanian G, Thomas PD, Zhang J, Gabor Miklos GL, Nelson C, Broder S, Clark AG, Nadeau J, McKusick VA, Zinder N, Levine AJ, Roberts RJ, Simon M, Slayman C, Hunkapiller M, Bolanos R, Delcher A, Dew I, Fasulo D, Flanigan M, Florea L, Halpern A, Hannenhalli S, Kravitz S, Levy S, Mobarry C, Reinert K, Remington K, Abu-Threideh J, Beasley E, Biddick K, Bonazzi V, Brandon R, Cargill M, Chandramouliswaran I, Charlab R, Chaturvedi K, Deng Z, Di Francesco V, Dunn P, Eilbeck K, Evangelista C, Gabrielian AE, Gan W, Ge W, Gong F, Gu Z, Guan P, Heiman TJ, Higgins ME, Ji RR, Ke Z, Ketchum KA, Lai Z, Lei Y, Li Z, Li J, Liang Y, Lin X, Lu F, Merkulov GV, Milshina N, Moore HM, Naik AK, Narayan VA, Neelam B, Nusskern D, Rusch DB, Salzberg S, Shao W, Shue B, Sun J, Wang Z, Wang A, Wang X, Wang J, Wei M, Wides R, Xiao C, Yan C, Yao A, Ye J, Zhan M, Zhang W, Zhang H, Zhao Q, Zheng L, Zhong F, Zhong W, Zhu S, Zhao S, Gilbert D, Baumhueter S, Spier G, Carter C, Cravchik A, Woodage T, Ali

F, An H, Awe A, Baldwin D, Baden H, Barnstead M, Barrow I, Beeson K, Busam D, Carver A, Center A, Cheng ML, Curry L, Danaher S, Davenport L, Desilets R, Dietz S, Dodson K, Doup L, Ferriera S, Garg N, Gluecksmann A, Hart B, Haynes J, Haynes C, Heiner C, Hladun S, Hostin D, Houck J, Howland T, Ibegwam C, Johnson J, Kalush F, Kline L, Koduru S, Love A, Mann F, May D, McCawley S, McIntosh T, McMullen I, Moy M, Moy L, Murphy B, Nelson K, Pfannkoch C, Pratts E, Puri V, Qureshi H, Reardon M, Rodriguez R, Rogers YH, Romblad D, Ruhfel B, Scott R, Sitter C, Smallwood M, Stewart E, Strong R, Suh E, Thomas R, Tint NN, Tse S, Vech C, Wang G, Wetter J, Williams S, Williams M, Windsor S, Winn-Deen E, Wolfe K, Zaveri J, Zaveri K, Abril JF, Guigo R, Campbell MJ, Sjolander KV, Karlak B, Kejariwal A, Mi H, Lazareva B, Hatton T, Narechania A, Diemer K, Muruganujan A, Guo N, Sato S, Bafna V, Istrail S, Lippert R, Schwartz R, Walenz B, Yooseph S, Allen D, Basu A, Baxendale J, Blick L, Caminha M, Carnes-Stine J, Caulk P, Chiang YH, Coyne M, Dahlke C, Mays A, Dombroski M, Donnelly M, Ely D, Esparham S, Fosler C, Gire H, Glanowski S, Glasser K, Glodek A, Gorokhov M, Graham K, Gropman B, Harris M, Heil J, Henderson S, Hoover J, Jennings D, Jordan C, Jordan J, Kasha J, Kagan L, Kraft C, Levitsky A, Lewis M, Liu X, Lopez J, Ma D, Majoros W, McDaniel J, Murphy S, Newman M, Nguyen T, Nguyen N, Nodell M, Pan S, Peck J, Peterson M, Rowe W, Sanders R, Scott J, Simpson M, Smith T, Sprague A, Stockwell T, Turner R, Venter E, Wang M, Wen M, Wu D, Wu M, Xia A, Zandieh A and Zhu X. The sequence of the human genome. Science 2001; 291: 1304-1351.

[15] Cheetham SW, Gruhl F, Mattick JS and Dinger ME. Long noncoding RNAs and the genetics of cancer. Br J Cancer 2013; 108: 2419-2425.

[16] Li H, Yu B, Li J, Su L, Yan M, Zhu Z and Liu B. Overexpression of lncRNA H19 enhances carcinogenesis and metastasis of gastric cancer. Oncotarget 2014; 5: 2318-2329.

[17] Yang XJ, Huang CQ, Peng CW, Hou JX and Liu JY. Long noncoding RNA HULC promotes colorectal carcinoma progression through epigenetically repressing NKD2 expression. Gene 2016; 592: 172-178.

[18] Li Z, Zhao L and Wang Q. Overexpression of long non-coding RNA HOTTIP increases chemoresistance of osteosarcoma cell by activating the Wnt/β-catenin pathway. Am J Transl Res 2016; 8: 2385-2393.

[19] Arbabi S and Maier RV. Mitogen-activated protein kinases. Crit Care Med 2002; 30: S74-S79.

[20] Peng W and Fan H. Long noncoding RNA CCHE1 indicates a poor prognosis of hepatocellular carcinoma and promotes carcinogenesis via activation of the ERK/MAPK pathway. Biomed Pharmacother 2016; 83: 450-455.

[21] Wu XS, Wang XA, Wu WG, Hu YP, Li ML, Ding Q, Weng H, Shu YJ, Liu TY, Jiang L, Cao Y, Bao RF, Mu JS, Tan ZJ, Tao F and Liu YB. MALAT1 promotes the proliferation and metastasis of gallbladder cancer cells by activating the ERK MAPK pathway. Cancer Biol Ther 2014; 15: 806-814.