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ORIGINAL ARTICLE

Low Expression LncRNA TUBA4B is a Poor Predictor of Prognosis and Regulates Cell Proliferation in Non-Small Cell Lung Cancer

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Abstract We aimed to unveil the clinical roles and biological function of lncRNA TUBA4B in on-small cell lung cancer (NSCLC). The relative expression level of TUBA4B was estimated by qPCR in 114 pairs of NSCLC and NT samples and the relation of TUBA4B to clinical data of NSCLC patients was analyzed. We found TUBA4B was lower expressed in NSCLC and five cell lines. The lower expression of TUBA4B was remarkably correlated with advanced TNM stage and lymph node metastasis and served as a predictor for overall survival of NSCLC. After overexpression of TUBA4B, cell proliferation ability of A549 and NCI-H1299 remarkably decreased. Our study ascertained low expression TUBA4B in NSCLC tissue, cell lines and is a poor predictor for prognosis and can regulate cell proliferation in NSCLC.

Keywords Non-small cell lung cancer · lncRNAs · TUBA4B · Prognosis

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Introduction

Lung cancer is the leading cause of cancer death worldwide, and its incidence continues to increase [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85 % of all lung cancers. Although there has been some progress in chemotherapy, radiation and surgery, NSCLC remain very aggressive and usually rapidly fatal [2]. The average 5-year survival of lung cancer is less than 15 % [3–6]. The mechanisms of NSCLC have not been elucidated.

Studies have shown that lncRNAs abnormally express in tumor cells or tissues and regulate coding gene expression. The altered expression of lncRNAs result in the development, invasion, and metastasis of many cancers with a series of mechanisms [7, 8]. The regulation of gene expression by lncRNAs at the epigenetic level, transcriptional and post-transcriptional level [9–11]. LncRNAs have been shown to be involved in the development and progression of lung cancer. However, lung cancer-associated lncRNAs are few as HOTAIR, H19, ANRIL, MALAT1 [12, 13], SCAL1 [14], AK126698 [15], and GAS6-AS1 [16], so it is very important to identify of additional lung cancer-associated lncRNAs and unveil their mechanism of action.

TUBA4B (Tubulin, Alpha 4b) is a pseudogene. Among its related pathways are development slit-robo signaling and EphB-EphrinB signaling. GO annotations related to this gene include GTP binding and structural constituent of cytoskeleton. We have found lncRNA-TUBA4B down-regulated in NSCLC by high-throughput microarray and real-time quantitative reverse transcription polymerase chain reaction (qPCR) method from our previous work. However, the clinical roles of TUBA4B are not well understood in NSCLC. In this study, the expression level of

TUBA4B was estimated by quantitative PCR in 114 pairs of NSCLC and NT samples and the relation of TUBA4B to clinical data of NSCLC patients was analyzed. We overexpressed TUBA4B basing on human lung adenocarcinoma A549 and NCI-H1299 cell line by lentivirus-mediated technology.

Materials and Methods

Patient Samples

The 114 NSCLC samples and corresponding NT samples were prospectively collected from patients of the First Affiliated Hospital of Wenzhou Medical University, China, from August 2013 to October 2015. The clinical data of these cases are shown in Table 1. The diagnosis of NSCLC was confirmed by histopathology. TNM clinical stage based on from the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) in 2002. The NSCLC and matched NT samples were snap-frozen in liquid nitrogen immediately after resection. We have followed prognosis of 89 NSCLC patients by telephone or other means, the longest follow-up time was 31 months. According to the expression level of TUBA4B, the survival data is divided into the high expression group and low expression group. This study was approved by the Institutional Ethics Review Board of the First Affiliated Hospital of Wenzhou Medical University, and all patients provided written informed consent for this study.

Quantitative PCR

Total RNA was extracted from frozen NSCLC tissues by using TRIzol reagent (Invitrogen, USA). According to the manufacturer's instructions, Total RNA was reverse-transcribed into cDNA using an RT Reagent Kit (Shanghai Takara, China). TUBA4B and GAPDH mRNA expression in NSCLC tissues was measured by quantitative PCR by using SYBR Premix Ex Taq in ABI 7000 instrument. TUBA4B for sense primer: 5′-ATCAATCACCAGCCTCCC-3′, antisense primer: 5′-CCACCTCCTT-GTAATCCTTCT-3′.GAPDH for sense primer: 5′-TGACTTCAACA-GCGACACCCA-3′, antisense primer: 5′-CACCCTGTTGCTGTAGC-CAAA-3′. Total RNA (2 mg) was transcribed to cDNA. PCR was performed in a total reaction volume of 20 μl, including 10 μl of SYBR Premix (2×), 2 μl of cDNA

Table 1 The clinical features of 114 NSCLC patients and the relative expression levels of TUBA4B

Term	Case (n)	TUBA4B relative expression level	Kruskal-Wallis Test or Mann-Whitney U	P
Sex			235.60	0.554
Male	56	0.396(0.021–0.921)		
Female	58	0.421(0.018–0.947)		
TMN stage			58.623*	0.017
Ia	24	0.402(0.389–0.947)		
Ib	56	0.321(0.360–0.834)		
IIa	14	0.213(0.176–0.567)		
IIb	4	0.193(0.158–0.453)		
IIIa	16	0.120(0.018–0.215)		
Histological degree			3.457*	0.721
Poor	22	0.402(0.025–0.923)		
Poor-moderate	14	0.399(0.103–0.947)		
Moderate	34	0.369(0.065–0.901)		
Moderate-high	18	0.371(0.123–0.894)		
High	26	0.386(0.018–0.856)		
Lymph node metastasis			2.324	0.002
Yes	28	0.090(0.018–0.131)		
No	86	0.465(0.304–0.947)		
Smoking			223.30	0.237
Yes	40	0.392(0.018–0.947)		
No	74	0.417(0.021–0.923)		

*Kruskal-Wallis Test

template, 1 μl of PCR forward primer (10 mM), 1 μl of PCR reverse primer (10 mM), and 6 μl of double-distilled water. The quantitative real-time PCR reaction included an initial denaturation step of 10 min at 95 °C; 40 cycles of 5 s at 95 °C, 30 s at 60 °C; and a final extension step of 5 min at 72 °C. All experiments were performed in triplicate, and all samples were normalized to GAPDH. The median in each triplicate was used to calculate relative lncRNA concentrations ($^{\triangle}Ct$ = Ct median lncRNA-Ct median GAPDH), and $2^{-\triangle\triangle Ct}$ in expression were calculated [17].

Cell Culture

Five human NSCLC cell lines (SPCA-1, NCI-H1299, A549, NCI-H441, LTEP-a2) and BEAS-2B were all purchased from Cell Bank of the Chinese Academy of Sciences and were cultured with complete medium (containing 10 % fetal serum and 90 % RPMI1640) set at 37 °C, 5 % CO_2 and complete medium was changed at least once two day.

Lentivirus-Mediated Overexpression Vector Transfection

A549 and NCI-H1299 cell were transfected overexpression vector targeting TUBA4B as well as a negative control (Genechem, Shanghai, China). Transfection was accomplished by seeding 2×10^5 cells into a six-well plate, and after 24 h, the medium was aspirated and incubated with transfection complex according to the manufacturer's protocol. The A549 and NCI-H1299 cells were infected by Lentivirus for 72 h and the overexpression efficiency was detected by qPCR.

Cell Proliferation Assay

Cell viability was evaluated by Cell Counting Kit-8 (CCK-8, Corning Corporation, USA) abiding by the manufacturer's protocols. Briefly, 3000 cells were resuspended and seeded into a 96-well plate supplemented in the presence of 10 % FBS and cultured for a week. The next day, the TUBA4B overexpression cells was incubated with CCK-8 for 1 h and the absorbance was measured at 450 nm using a multifunctional microplate reader (Tecan) in the 1d, 3d, 5d, 7d. This experiment was done in quadruplicate cells.

Statistical Methods

Differences in variables among groups were tested using the one-way ANOVA for the normal distribution or Kruskal–Wallis test for the non-normal distribution. A comparison between the two groups was performed by least significant difference (LSD) test or Student's t-test or Mann–Whitney U test. Survival analysis was performed using chi-square test. $P < 0.05$ was considered to be statistically significant.

Results

The Expression Level of TUBA4B in NSCLC and Adjacent Tissues and its Relationship with Clinical Data

According to Table 1, TUBA4B expression level of NSCLC is 0.413 (0.018–0.947) and significantly lower than its adjacent cancer tissues (Mann-Whitney U = 0.000, P = 0.000) (Fig.1). We showed that the TUBA4B level of NSCLC with lymph node metastasis was significantly lower than that of NSCLC without lymph node metastasis group (Mann-Whitney U = 2.324, P = 0.002). TUBA4B expression levels among different TMN stages were different (Kruskal-Wallis Test =58.623, P = 0.017). TUBA4B expression level of stage IIIa was significantly lower than that of stage Ia (Mann-Whitney U = 55.000, P = 0.000), stage Ib (Mann-Whitney U = 43.000, P = 0.000), stage IIa (Mann-Whitney U = 26.000, P = 0.003), stage IIb (Mann-Whitney U = 19.000, P = 0.010). The expression of TUBA4B was no relative to different pathological type (Kruskal-Wallis Test = 3.457, P = 0.721), the histology differentiation, (Kruskal-Wallis Test = 4.769, P = 0.682), the smoking (Mann-Whitney U = 223.30, P = 0.237), sex (Mann-Whitney U = 235.60, P = 0.554).

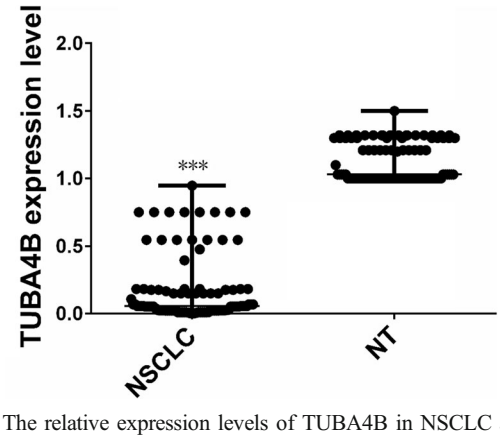

Fig. 1 The relative expression levels of TUBA4B in NSCLC and NT tissues. TUBA4B expression level of NSCLC is 0.413 (0.189–0.947) and significantly lower than its adjacent cancer tissues (Mann-Whitney U = 0.000, P = 0.000). *** $P < 0.001$

The Relation of TUBA4B Expression to the Prognosis of NSCLC

The overall survival time of NSCLC low expression TUBA4B group (median 12 months) was significantly lower than that of the high expression (median 28 months) (chi-square = 21.21, $P < 0.0001$), see Fig.2.

The Expression Level of TUBA4B from five NSCLC Cells

We detected the expression levels of TUBA4B from five NSCLC cell lines (including A549, NCI-H441, NCI-H1299, SPCA-1, LETP-a2) by qPCR. It shown that the expression levels of TUBA4B from A549(Mann-Whitney U = 42.000, $P = 0.000$), NCI-H441(Mann-Whitney U = 37.000, $P = 0.003$), NCI-H1299(Mann-Whitney U = 65.000, $P = 0.000$), SPCA-1(Mann-Whitney U = 47.000, $P = 0.000$), LETP-a2(Mann-Whitney U = 77.000, $P = 0.000$) were lowerly expressed, compared to normal human bronchial epithelial BEAS-2B cell line, and see Fig.3.

TUBA4B can Regulate Ability of Cell Proliferation

In the Fig.4, the OD450nm of different A549 and NCI-H1299 groups gradually increased with the change of time. Compared with the 1d, the OD450nm of the 3d ($P < 0.05$, $P < 0.05$, $P < 0.05$), the 5d ($P < 0.001$, $P < 0.001$, $P < 0.01$), the 7 d ($P < 0.001$, $P < 0.001$, $P < 0.001$) were significantly increased. Compared with appropriate days of control group and NC group, the OD450nm of 1d, 3d in TUBA4B overexpression group were no statistically significant difference ($P > 0.05$), while that of the 5d ($P < 0.05$) and the 7d ($P < 0.01$) significantly reduced, it indicates that cell proliferation ability of A549 and NCI-H1299 was significantly reduced after TUBA4B overexpressed.

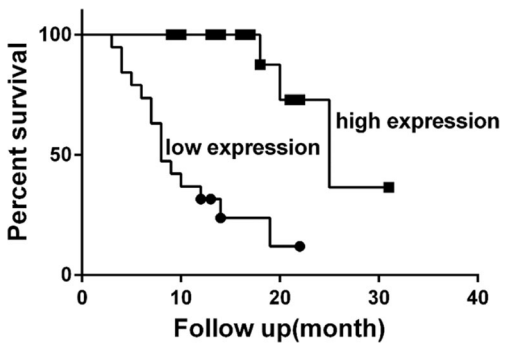

Fig. 2 The relation of TUBA4B expression to prognosis in NSCLC patients. The overall survival time of NSCLC low expression TUBA4B group was significantly lower than that of the high expression

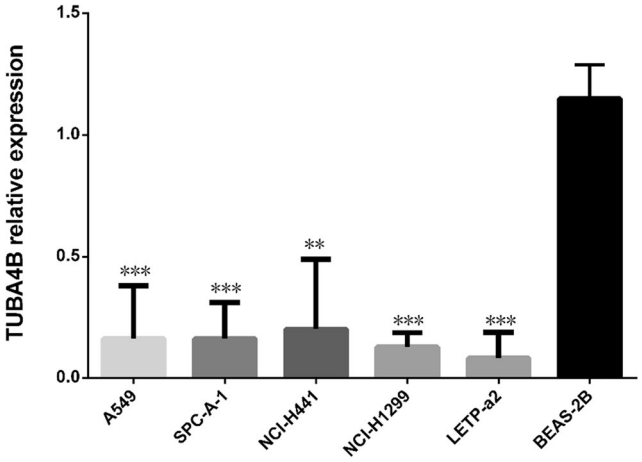

Fig. 3 The expression levles of TUBA4B in five NSCLC cell lines. It shown that the expression levels of TUBA4B from A549, NCI-H441, NCI-H1299, SPCA-1, LETP-a2 cells were lowly expressed, compared to normal human bronchial epithelial BEAS-2B cell line. ** $P < 0.01$, *** $P < 0.001$

Discussion

LncRNAs play an important role in many biological processes, including X-chromosome inactivation, gene imprinting, and so on [18, 19] and also control gene expression and accelerate the development and progression in cancers [8, 20]. Promoters bind to many transcription factors with some mechanisms such as chromosomal rearrangements and transfer elements [21]. A important function of lncRNAs can change the expression of nearby encoding genes by affecting the process of transcription [22] or directly playing an enhancer-like role [23, 24].

In this study, we uncovered the potential clinical role of TUBA4B in the pathogenesis of NSCLC. We found TUBA4B was lower expressed in NSCLC by qPCR. The expression TUBA4B in lymph node metastasis of NSCLC group was significantly lower than NSCLC without lymph node metastasis group, and it was relative to TMN stage, while it was no relative to degree of tissue differentiation, gender, age, smoking and so on. Survival analysis showed that survival time of high expression TUBA4B was significantly longer than low TUBA4B level in NSCLC patients. These results hinted that TUBA4B was tumor suppressors and abnormally expression in NSCLC.

Compared to normal human bronchial epithelial BEAS-2B cell line, we detected the expression levels of TUBA4B from five NSCLC cell lines. It shown that the expression levels of TUBA4B were lowly expressed in five cells. We combine the TUBA4B expression of NSCLC tissue and cell lines, it suggested TUBA4B was a tumor suppressor lncRNA molecule. Since it TUBA4B appeared low

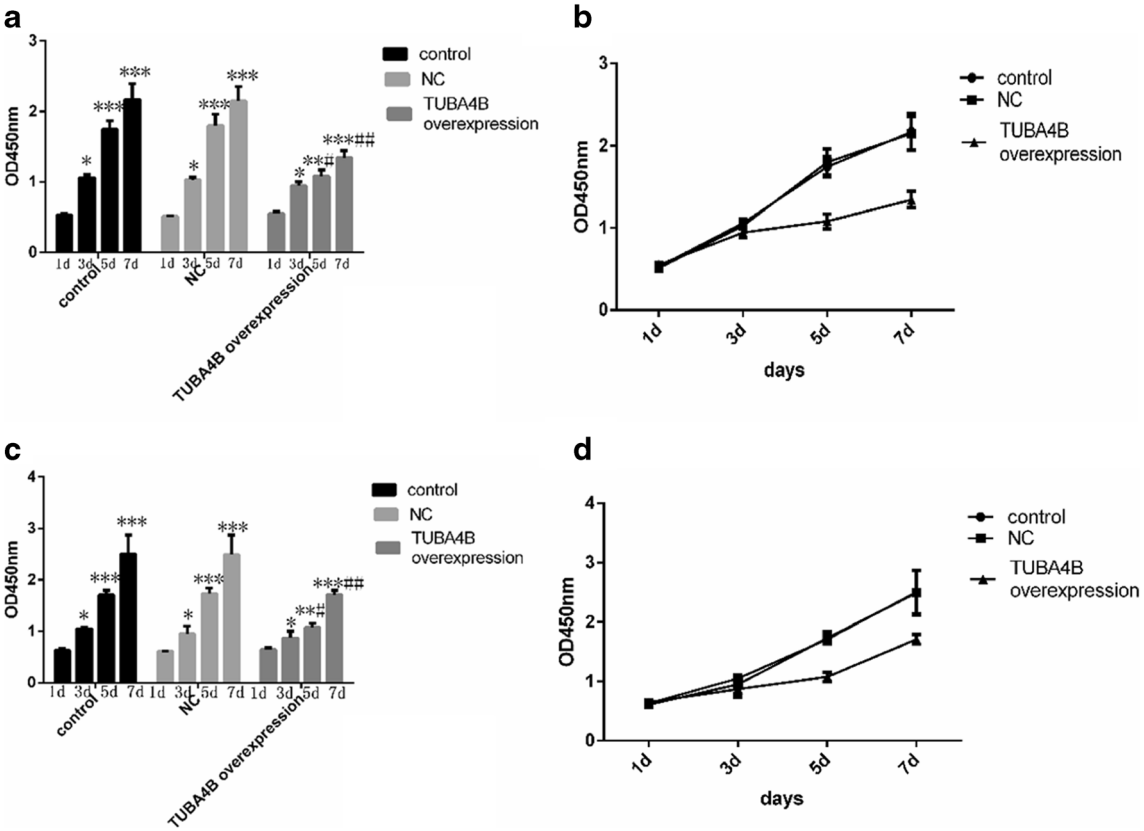

Fig. 4 The cell proliferation results of different A549 and NCI-H1299 groups. (**a**) The different curve of 3 days in different A549 groups,(B) The different figure of 3 days in different A549 groups.*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. #$P < 0.05$, ##$P < 0.01$, ### $P < 0.001$. (**c**) The different curve of 3 days in different NCI-H1299 groups,(**d**) The different figure of 3 days in different NCI-H1299 groups.*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. #$P < 0.05$, ##$P < 0.01$, ### $P < 0.001$

expressed, which led to its suppression function decline, thus promoted the development of NSCLC. In order to further study the mechanism of TUBA4B and we established TUBA4B overexpression of A549 and NCI-H1299 cell lines by lentivirus-mediated technology. After TUBA4B was overexpressed, cell proliferation ability of A549 and NCI-H1299 remarkably decreased, so TUBA4B can regulated cell proliferation of NSCLC cell. But we needs further study about how TUBA4B played biological functions and molecular mechanisms in NSCLC.

To summarize, Our study ascertained the expression of TUBA4B down-regulated in NSCLC tissue, cell lines and is a poor predictor for prognosis and can regulate cell proliferation in NSCLC.

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Compliance with Ethical Standards

Conflict of Interest Statement These conflicts did not interfere with the conduct of this study. All other authors have no other conflict of interest to declare.

Reference

1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ. Cancer statistics, 2005. CA: a cancer journal for clinicians. 2005;55:10–30.
2. Gridelli C, Rossi A, Maione P (2003) Treatment of non-small-cell lung cancer: state of the art and development of new biologic agents. Oncogene 22:6629–6638
3. Stewart DJ (2010) Tumor and host factors that may limit efficacy of chemotherapy in non-small cell and small cell lung cancer. Crit Rev Oncol Hematol 75:173–234
4. Chen CH, Lai JM, Chou TY, Chen CY, Su LJ, Lee YC, Cheng TS, Hong YR, Chou CK, Whang-Peng J, Wu YC, Huang CY. VEGFA upregulates FLJ10540 and modulates migration and invasion of lung cancer via PI3K/AKT pathway. PLoS One 2009;4:e5052.

5. Ogawa E, Takenaka K, Katakura H, Adachi M, Otake Y, Toda Y, Kotani H, Manabe T, Wada H, Tanaka F (2008) Perimembrane aurora-a expression is a significant prognostic factor in correlation with proliferative activity in non-small-cell lung cancer (NSCLC). Ann Surg Oncol 15:547–554
6. Rachet B, Woods LM, Mitry E, Riga M, Cooper N, Quinn MJ, Steward J, Brenner H, Esteve J, Sullivan R, Coleman MP. Cancer survival in England and Wales at the end of the twentieth century. Br J Cancer 2008;99 Suppl 1:S2–10.
7. Fu X, Ravindranath L, Tran N, Petrovics G, Srivastava S (2006) Regulation of apoptosis by a prostate-specific and prostate cancer-associated noncoding gene, PCGEM1. DNA Cell Biol 25:135–141
8. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S, Chang HY (2010) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 464:1071–1076
9. Zhang H, Chen Z, Wang X, Huang Z, He Z, Chen Y. Long non-coding RNA: a new player in cancer. J Hematol Oncol 2013;6:37.
10. Hauptman N, Glavac D (2013) Long non-coding RNA in cancer. Int J Mol Sci 14:4655–4669
11. Chen G, Wang Z, Wang D, Qiu C, Liu M, Chen X, Zhang Q, Yan G, Cui Q (2013) LncRNADisease: a database for long-non-coding RNA-associated diseases. Nucleic Acids Res 41:D983–D986
12. Gibb EA, Brown CJ, Lam WL (2011) The functional role of long non-coding RNA in human carcinomas. Mol Cancer 10:–38
13. Ji P, Diederichs S, Wang W, Boing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, Thomas M, Berdel WE, Serve H, Muller-Tidow C (2003) MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene 22:8031–8041
14. Thai P, Statt S, Chen CH, Liang E, Campbell C, Wu R (2013) Characterization of a novel long noncoding RNA, SCAL1, induced by cigarette smoke and elevated in lung cancer cell lines. Am J Respir Cell Mol Biol 49:204–211
15. Yang Y, Li H, Hou S, Hu B, Liu J, Wang J (2013) The noncoding RNA expression profile and the effect of lncRNA AK126698 on cisplatin resistance in non-small-cell lung cancer cell. PLoS One 8:–e65309
16. Han L, Kong R, Yin DD, Zhang EB, TP X, De W, Shu YQ (2013) Low expression of long noncoding RNA GAS6-AS1 predicts a poor prognosis in patients with NSCLC. Med Oncol 30:–694
17. Ren S, Peng Z, Mao JH, Yu Y, Yin C, Gao X, Cui Z, Zhang J, Yi K, Xu W, Chen C, Wang F, Guo X, Lu J, Yang J, Wei M, Tian Z, Guan Y, Tang L, Xu C, Wang L, Gao X, Tian W, Wang J, Yang H, Wang J, Sun Y (2012) RNA-seq analysis of prostate cancer in the Chinese population identifies recurrent gene fusions, cancer-associated long noncoding RNAs and aberrant alternative splicings. Cell Res 22:806–821
18. Drobnik W, Liebisch G, Biederer C, Tr mbach B, Rogler G, Muller P, Schmitz G (1999) Growth and cell cycle abnormalities of fibroblasts from Tangier disease patients. Arterioscler Thromb Vasc Biol 19:28–38
19. Wang KC, Chang HY (2011) Molecular mechanisms of long non-coding RNAs. Mol Cell 43:904–914
20. Khachane AN, Harrison PM. Mining mammalian transcript data for functional long non-coding RNAs. PLoS One 2010;5:e10316.
21. Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38:431–440
22. Mattick JS, Gagen MJ (2001) The evolution of controlled multitasked gene networks: the role of introns and other noncoding RNAs in the development of complex organisms. Mol Biol Evol 18:1611–1630
23. Mattick JS (2010) Linc-ing long noncoding RNAs and enhancer function. Dev Cell 19:485–486
24. Orom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang Q, Guigo R, Shiekhattar R (2010) Long noncoding RNAs with enhancer-like function in human cells. Cell 143:46–58