European Review for Medical and Pharmacological Sciences 2017; 21: 115-119

The use of lncRNA analysis for stratification management of prognostic risk in patients with NSCLC

C.-G. ZHANG[1], D.-D. YIN[2], S.-Y. SUN[3], L. HAN[3]

[1]Department of Oncology, Second Affiliated Hospital of Nanjing Medical University, Nanjing, China
[2]Central Laboratory, the Second Affiliated Hospital of Southeast University, Nanjing, China
[3]Department of Oncology, Xuzhou Central Hospital, Affiliated Xuzhou Hospital, College of Medicine, Southeast University, Xuzhou, Jiangsu, China

Chong-Guo Zhang and *Dan-dan Yin* contributed equally to this work and should be regarded as joint first authors

Abstract. – OBJECTIVE: Lung cancer is the most frequent cancer in China and worldwide. Long noncoding RNAs (lncRNAs) have been shown to play important regulatory roles in human cancer biology. The aim of the present study was to investigate the relationship between genomics and prognosis among lung cancer patients.

PATIENTS AND METHODS: We collected specimens from non-small cell lung cancer (NSCLC) patients after surgery. Q-PCR was performed to investigate the expression level of lncRNAs in cancerous and adjacent normal tissue. Patients were divided into different risk groups according to lncRNA expression levels and then follow-up.

RESULTS: The lncRNAs HOTAIR, H19 and MALAT1 were up-regulated, while PANDAR and TUG1 were down-regulated in NSCLC cancer tissues compared with the corresponding adjacent normal tissue. After two years of follow-up time, the disease-free survival time (DFS) curves were significantly different between the high-risk, moderate-risk and low-risk patient groups.

CONCLUSIONS: Our results suggest that lncRNAs are involved in the process of NSCLC and that the use of genetic analysis for stratification management of prognostic risk could help us to implement individualized treatment for patients with NSCLC and ultimately to improve the patient prognosis.

Key Words:
Non-small cell lung cancer (NSCLC), Long noncoding RNAs (lncRNAs), Disease-free survival time (DFS).

Introduction

Lung cancer is the most frequent cause of cancer-related death worldwide[1]. Approximately 80-85% of lung cancer patients are diagnosed with non-small cell lung cancer (NSCLC)[2]. According to NCCN guidelines, adjuvant chemotherapy is not recommended for patients with stage IA NSCLC. However, adjuvant chemotherapy may be suggested for patients with stage IB NSCLC when poor prognostic factors, such as poor differentiation, vascular invasion, pulmonary wedge resection, visceral pleura invasion, tumors larger than 4 cm or insufficient lymph node dissection exist[3]. In clinical practice, despite strict accordance with TNM staging and treatment recommended by the NCCN guidelines, we often encounter some patients with late-stage NSCLC have a relatively long disease-free survival time, while other patients with early stage disease experience recurrence and metastasis quickly. Thus, other potential prognostic factors in addition to TNM stage are worth exploring further. Studies of mechanisms of tumorigenesis have primarily focused on protein-coding genes. With the completion of the human genome project, mounting evidence has shown that lncRNAs are capable of influencing various cellular processes, such as proliferation, cell-cycle progression, cell growth and apoptosis[4-7], and thus play an important role in carcinogenesis and cancer metastasis[8-11].

In this context, we collected clinical specimens from NSCLC patients who underwent surgery at our hospital in 2013. Genetic analysis was carried out to analyze the relationship between the expression levels of lncRNAs and DFS among these patients.

Corresponding Author: Liang Han, MD; e-mail: 6468837@qq.com
San-Yuan Sun, MD; e-mail: sanyuan_sun@sina.com

Patients and Methods

Tissue Collection and Ethics Statement

A total of 50 primary NSCLC patients who had undergone surgery at Xuzhou Central Hospital in 2013 (China) were included in the study. The included patients did not receive chemotherapy or radiotherapy prior to surgery. The Ethics Committee of XuZhou Central Hospital approved this study, and each patient participated after providing informed consent. Tumor stage was evaluated according to the tumor-node-metastasis (TNM) classification system 2002. Patients discharged from the hospital were followed up routinely according to a scheduled program, at least once every six months.

RNA Extraction and qPCR Analyses

Total RNA was extracted from the frozen tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentrations were estimated by spectrophotometer absorbance readings of 260 and 280 nm. One μg of total RNA was reverse transcribed to cDNA using a Reverse Transcription Kit (Takara, Dalian, China). An ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used to quantify the expression levels of lncRNAs. Ten microliters of SYBR Premix ExTaq (Takara, Dalian, China) was mixed according to the manufacturer's instructions. The results were normalized to the expression of GAPDH. Primer sequences used in our study are shown in Table I.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Survival curves were estimated using the Kaplan-Meier method. A log-rank test was used to estimate the statistical difference between survival curves. A two-tailed p-value of 0.05 or less was considered statistically significant.

Table I. Primer sequences in this study.

HOTAIR FWD	CAGTGGGGAACTCTGACTCG
HOTAIR REV	GTGCCTGGTGCTCTCTTACC
H19 FWD	CCCACAACATGAAAGAAATGGTGC
H19 REV	CACCTTCGAGAGCCGATTCC
MALAT1 FWD	AGCGGAAGAACGAATGTAAC
MALAT1 REV	GAACAGAAGGAAGAGCCAAG
PANDAR FWD	TGCACACATTTAACCCGAAG
PANDAR REV	CCCCAAAGCTACATCTATGACA
TUG1 FWD	TAGCAGTTCCCCAA0TCCTTG
TUG1 REV	CACAAATTCCCATCATTCCC
GAPDH FWD	AGCCACATCGCTCAGACAC
GAPDH REV	GCCCAATACGACCAAATCC

Results

The pathological features of all 50 patients are shown in Table II.

The Expression Levels of lncRNAs in NSCLC Cancer Tissues

Q-PCR was performed to investigate the expression levels of lncRNAs in cancerous and adjacent normal tissue. Consistent with previous studies, we found that the lncRNAs HOTAIR, H19 and MALAT1 were up-regulated while lncRNAs PANDAR and TUG1 were down-regulated in NSCLC cancer tissues compared with the corresponding adjacent normal tissues. As shown in Figure 1A, the change in the level of HOTAIR, H19 and MALAT1 was 9.83±13.05, 6.68 ± 10.12 and 9.70 ± 18.69-fold, respectively. The change in expression of PANDAR and TUG1 was 0.50 ± 0.52 and 0.83 ± 0.99-fold, respectively, as shown in Figure 1B. There was a significant difference in the expression of lncRNAs in cancer and paracancerous tissues ($p < 0.05$).

lncRNAs Could Be Used to Predict DFS in Patients with NSCLC

To determine the prognostic value of lncRNAs in NSCLC patients, we explored the correlation between their expression and clinical outcomes. Patients were included into different groups according to the median gene expression. According to our previous results, high expression of HOTAIR, H19, MALAT1 and low expression of PANDA and TUG1 were defined as poor prognostic factors. Patients who had only one adverse factor were grouped into the low-risk group, two into the moderate-risk group, and three or more into the high-risk group. As a result, there were 6 patients in the low-risk group, 21 in the moderate-risk group and 23 in the high-risk group. Patients could receive routine chemotherapy, radiotherapy or follow up in accordance with the NCCN guidelines. After a 2-year follow-up period, the DFS curve of the three groups was measured (Figure 2). The median DFS time for the high-risk group was 13.5 to 22.5 months, whereas the moderate-risk and low-risk groups had not yet

Table II. The clinic-pathological factors of NSCLC patients.

Clinical factors		Number of cases	Percent of patients
Sex	Male	29	58
	Female	21	42
Age	≤ 60	22	44
	> 60	28	56
Histological grade	High	8	16
	Middle	20	40
	Middle to low	8	16
	Low	13	26
	Other	1	2
Histological classification	SCC (squamous cell carcinoma)	28	56
	AD (adenocarcinoma)	20	40
	Other	2	4
Tumor stage	I	13	26
	II	21	42
	III	15	30
	IV	1	2
Tumor (T)	T1	15	30
	T2	15	30
	T3	15	30
	T4	5	10
Lymph node metastasis (N)	N0	26	52
	N1	19	38
	N2	4	8
	N3	1	2
History of smoking	Ever	29	58
	Never	21	42

reached their median DFS time at 2 years. There was a statistically significant difference among the three groups ($p = 0.041$).

Discussion

According to the latest statistical data, lung cancer has the highest incidence and mortality among malignant tumors in China. There are approximately 705,000 new cases and 569,000 deaths each year, with an incidence rate of 36.28/10 million and a mortality rate of 28.81/10 million. In recent years, in addition to traditional chemotherapy, many new treatments, such as monoclonal antibodies, including bevacizumab[12,13] and cetuximab[14,15], and small-molecule TKI inhibitors, such as gefi-

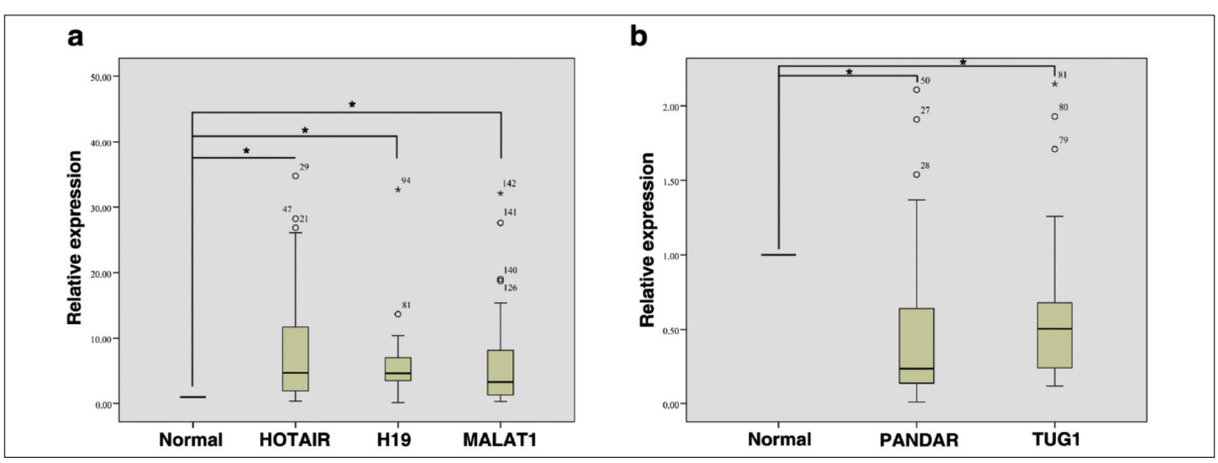

Figure 1. ***A,*** and ***B,*** Relative expression of lncRNAs in NSCLC tissues compared with corresponding non-tumor tissues (n=50). LncRNAs expression was examined by qPCR and normalized to GAPDH expression.

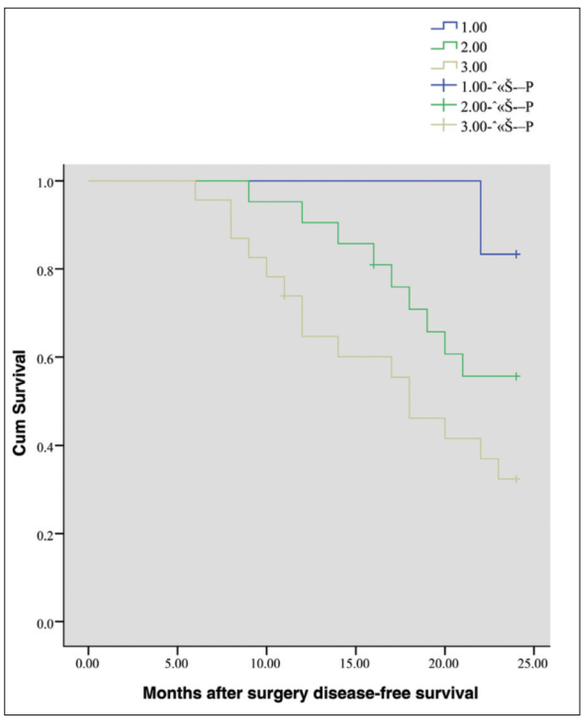

Figure 2. The DFS curve of three groups of patients with different prognostic risk. 1: low-risk group, 2: moderate-risk group, 3: high-risk groups. Log-rank test was performed between three groups, $p = 0.041$.

tinib and erlotinib[16,17], have prolonged the survival time of patients in advanced stages of NSCLC. However, the overall prognosis of advanced NSCLC patients remains very poor. The main reasons for this poor prognosis are that molecular mechanisms of the development and metastasis of NSCLC have not been fully characterized, and most patients are diagnosed at an advanced stage[2]. Gene therapy or immunotherapy have good potential but are still being researched in the laboratory or are in pre-clinical stages, and we are currently lacking an effective molecular target. Therefore, further exploration and research of new gene functions in NSCLC ARE needed to reveal the precise molecular mechanism of lung cancer, improve early diagnosis of the disease, practice reasonable clinical treatment, and finally improve the diagnosis and prognosis of patients with NSCLC.

LncRNAs are generally defined as transcribed RNA molecules with a length greater than 200 nt that are lacking an open reading frame. They are expressed in tissue- and cell-specific patterns, and they display weaker evolutionary constraints and usually have lower expression levels than protein-coding genes[18]. In recent years, many studies have focused on the relationship between lncRNAs and cancer. In our previous work[10], we found that lncRNA TUG1 is a direct target of the p53 gene and could epigenetically regulate HOXB7 expression, thus affecting NSCLC cell proliferation. The lncRNA PANDAR could affect apoptosis by regulating Bcl-2 and predicts a poor prognosis in patients with NSCLC[9]. LncRNA H19 is induced by proto-oncogene c-Myc and modulates NSCLC cell proliferation[19]. The lncRNAs HOTAIR and MALAT1 have also been confirmed to participate in the development and progression of NSCLC by affecting cellular biological functions[20,21].

In the present paper, we collected specimens from NSCLC patients after surgery, investigated the expression levels of the above-mentioned lncRNAs, and divided patients into three different risk groups. We found that the DFS time was significantly different between the three groups, which suggests that, in addition to the traditional TNM stage and poor prognostic factors (vascular invasion, tumor size, etc.), genomics is also involved in the process of disease and eventually leads to different prognoses. In our clinical practice, in addition to evidence-based medical treatments, such as NCCN guidelines, we may need more powerful chemotherapy and more intensive follow-up time for patients with a high-risk of recurrence to ultimately improve the patients' prognosis.

Conclusions

Altogether, our findings indicate that testing for histological lncRNA expression to predict the risk of progression as well as the prognosis of NSCLC can aid in the individualized treatment of NSCLC patients and has important clinical implications, which warrant further investigation.

Acknowledgements

This work was supported by National Natural Science Foundation of China (81401873) and the Foundation of Xuzhou Central Hospital (XZB201301).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Bray F, Ren JS, Masuyer E, Ferlay J. Global estimates of cancer prevalence for 27 sites in the adult population in 2008. Int J Cancer 2013; 132: 1133-1145.
2) Goldstraw P, Crowley J, Chansky K, Giroux DJ, Groome PA, Rami-Porta R, Postmus PE, Rusch V, Sobin L. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. J Thorac Oncol 2007; 2: 706-714.
3) Ettinger DS, Wood DE, Akerley W, Bazhenova LA, Borghaei H, Camidge DR, Cheney RT, Chirieac LR, D'Amico TA, Demmy TL, Dilling TJ, Dobelbower MC, Govindan R, Grannis FW Jr., Horn L, Jahan TM, Komaki R, Krug LM, Lackner RP, Lanuti M, Lilenbaum R, Lin J, Loo BW Jr., Martins R, Otterson GA, Patel JD, Pisters KM, Reckamp K, Riely GJ, Rohren E, Schild SE, Shapiro TA, Swanson SJ, Tauer K, Yang SC, Gregory K, Hughes M. Non-Small Cell Lung Cancer, Version 6.2015. J Natl Compr Canc Netw 2015; 13: 515-524.
4) Gutschner T, Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. RNA Biol 2012; 9: 703-719.
5) Clark MB, Mattick JS. Long noncoding RNAs in cell biology. Semin Cell Dev Biol 2011; 22: 366-376.
6) Ginger MR, Shore AN, Contreras A, Rijnkels M, Miller J, Gonzalez-Rimbau MF, Rosen JM. A noncoding RNA is a potential marker of cell fate during mammary gland development. Proc Natl Acad Sci U S A 2006; 103: 5781-5786.
7) Mourtada-Maarabouni M, Hedge VL, Kirkham L, Farzaneh F, Williams GT. Growth arrest in human T-cells is controlled by the non-coding RNA growth-arrest-specific transcript 5 (GAS5). J Cell Sci 2008; 121: 939-946.
8) Kong R, Zhang EB, Yin DD, You LH, Xu TP, Chen WM, Xia R, Wan L, Sun M, Wang ZX, De W, Zhang ZH. Long noncoding RNA PVT1 indicates a poor prognosis of gastric cancer and promotes cell proliferation through epigenetically regulating p15 and p16. Mol Cancer 2015; 14: 82.
9) Han L, Zhang EB, Yin DD, Kong R, Xu TP, Chen WM, Xia R, Shu YQ, De W. Low expression of long noncoding RNA PANDAR predicts a poor prognosis of non-small cell lung cancer and affects cell apoptosis by regulating Bcl-2. Cell Death Dis 2015; 6: e1665.
10) Zhang EB, Yin DD, Sun M, Kong R, Liu XH, You LH, Han L, Xia R, Wang KM, Yang JS, De W, Shu YQ, Wang ZX. P53-regulated long non-coding RNA TUG1 affects cell proliferation in human non-small cell lung cancer, partly through epigenetically regulating HOXB7 expression. Cell Death Dis 2014; 5: e1243.
11) Zhang EB, Kong R, Yin DD, You LH, Sun M, Han L, Xu TP, Xia R, Yang JS, De W, 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) Le Moulec S, Hadoux J, Gontier E, Chargari C, Helissey C, Lamand V, Tanz R, Farace F, Vedrine L, Bonardel G, Soria JC, Besse B. Combination of paclitaxel and bevacizumab in heavily pre-treated non-small-cell lung cancer (NSCLC) patients: a case series study on 15 patients. Bull Cancer 2013; 100: 30-37.
13) Zarza V, Couraud S, Bosc C, Toffart AC, Moro-Sibilot D, Souquet PJ. [Paclitaxel-bevacizumab is a possible alternative as salvage chemotherapy in advanced non-small cell bronchial carcinoma]. Rev Mal Respir 2014; 31: 601-607.
14) Lin CH, Lin MT, Kuo YW, Ho CC. Afatinib combined with cetuximab for lung adenocarcinoma with leptomeningeal carcinomatosis. Lung Cancer 2014; 85: 479-480.
15) Sgambato A, Casaluce F, Maione P, Rossi A, Ciardiello F, Gridelli C. Cetuximab in advanced non-small cell lung cancer (NSCLC): the showdown? J Thorac Dis 2014; 6: 578-580.
16) Ellis PM, Coakley N, Feld R, Kuruvilla S, Ung YC. Use of the epidermal growth factor receptor inhibitors gefitinib, erlotinib, afatinib, dacomitinib, and icotinib in the treatment of non-small-cell lung cancer: a systematic review. Curr Oncol 2015; 22: e183-215.
17) Riely GJ, Yu HA. EGFR: the paradigm of an oncogene-driven lung cancer. Clin Cancer Res 2015; 21: 2221-2226.
18) Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, Guernec G, Martin D, Merkel A, Knowles DG, Lagarde J, Veeravalli L, Ruan X, Ruan Y, Lassmann T, Carninci P, Brown JB, Lipovich L, Gonzalez JM, Thomas M, Davis CA, Shiekhattar R, Gingeras TR, Hubbard TJ, Notredame C, Harrow J, Guigo R. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res 2012; 22: 1775-1789.
19) Zhang E, Li W, Yin D, De W, Sun S, Han L. c-Myc-regulated long non-coding RNA H19 indicates a poor prognosis and affects cell proliferation in non-small-cell lung cancer. Tumour Biol 2015.
20) Zhou C, Ye L, Jiang C, Bai J, Chi Y, Zhang H. Long noncoding RNA HOTAIR, a hypoxia-inducible factor-1alpha activated driver of malignancy, enhances hypoxic cancer cell proliferation, migration, and invasion in non-small cell lung cancer. Tumour Biol 2015; 36: 9179-9188.
21) Schmidt LH, Spieker T, Koschmieder S, Schaffers S, Humberg J, Jungen D, Bulk E, Hascher A, Wittmer D, Marra A, Hillejan L, Wiebe K, Berdel WE, Wiewrodt R, Muller-Tidow C. The long noncoding MALAT-1 RNA indicates a poor prognosis in non-small cell lung cancer and induces migration and tumor growth. J Thorac Oncol 2011; 6: 1984-1992.