Biochemical and Biophysical Research Communications 458 (2015) 714–719

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

miR-1271 promotes non-small-cell lung cancer cell proliferation and invasion via targeting HOXA5

Yongfang Wang, Lianhong Xu, Lixin Jiang*

Department of Clinical Laboratory, Wujin Hospital Affiliated to Jiangsu University, Changzhou 213002, China

ARTICLE INFO

Article history:
Received 25 January 2015
Available online 14 February 2015

Keywords:
miR-1271
Non-small-cell lung cancer
Proliferation

ABSTRACT

MicroRNAs (miRNAs) are short, non-coding RNAs (~22 nt) that play important roles in the pathogenesis of human diseases by negatively regulating numerous target genes at posttranscriptional level. However, the role of microRNAs in lung cancer, particularly non-small-cell lung cancer (NSCLC), has remained elusive. In this study, two microRNAs, miR-1271 and miR-628, and their predicted target genes were identified differentially expressed in NSCLC by analyzing the miRNA and mRNA expression data from NSCLC tissues and their matching normal controls. miR-1271 and its target gene HOXA5 were selected for further investigation. CCK-8 proliferation assay showed that the cell proliferation was promoted by miR-1271 in NSCLC cells, while miR-1271 inhibitor could significantly inhibited the proliferation of NSCLC cells. Interestingly, migration and invasion assay indicated that overexpression of miR-1271 could significantly promoted the migration and invasion of NSCLC cells, whereas miR-1271 inhibitor could inhibited both cell migration and invasion of NSCLC cells. Western blot showed that miR-1271 suppressed the protein level of HOXA5, and luciferase assays confirmed that miR-1271 directly bound to the 3'untranslated region of HOXA5. This study indicated indicate that miR-1271 regulates NSCLC cell proliferation and invasion, via the down-regulation of HOXA5. Thus, miR-1271 may represent a potential therapeutic target for NSCLC intervention.

1. Introduction

Lung cancer is one of the most common cancers and the leading cause of cancer-related deaths worldwide so far [1]. About 80% of lung cancer patients are diagnosed to be non-small-cell lung cancer (NSCLC) at the advanced stages of this disease [2]. The average survival time is usually very short after the diagnosis, mainly due to unsatisfactory outcomes of conventional chemotherapeutic treatment, especially in patients of metastatic lung cancer [3]. Fortunately, during the past several years, the treatment outcome has been improved significantly due to increased understanding on molecular mechanisms of carcinogenesis. Although with improved surveillance and clinical treatment strategies, the reported 5-year survival of NSCLC patients after curative resection is only 30–60% [4]. Therefore, exploring the potential mechanisms that underling the initiation and progression of NSCLC is of great interest and urgency.

MicroRNAs (miRNAs) are small, non-coding RNAs that negatively regulate the expression of target genes via translational repression or mRNA degradation [5]. There are more than one thousand miRNAs in human genome, targeting 30%–60% of all protein-coding genes [6]. They are involved in many important biological processes, including cell growth, differentiation, apoptosis, and organ development [7]. Recent studies have shown relationships between aberrant expression of miRNAs and carcinogenesis [8]. In recent years, miRNAs have received great attention in NSCLC research. Several deregulated miRNAs in NSCLCs such as miR-221 [9], miR-222 [9], miR-449a [10], miR-21 [11,12], miR-205 [13], miR-10b [14], miR-143 [12] and miR-181a [12] have been shown to regulate cell growth, apoptosis, migration and invasion. These findings indicate that deregulation of miRNA expression may be associated with carcinogenesis of NSCLCs.

In this study, we compared the miRNA and mRNA expression data from 6 NSCLC tissues and their matching normal control from adjacent tissues to identify potential NSCLC-related miRNAs and genes. Then we investigated the molecular functions of the identified potential NSCLC-related miRNAs and their target genes in the NSCLC cell lines to identify their potential roles in carcinogenesis of NSCLC.

* Corresponding author.
E-mail address: jianglx66766@163.com (L. Jiang).

http://dx.doi.org/10.1016/j.bbrc.2015.02.033
0006-291X/

2. Materials and methods

2.1. miRNA and mRNA profile data collection

miRNA and mRNA profiles data of NSCLC tissues and their matching normal control from adjacent tissues collected from GEO database (www.ncbi.nlm.nih.gov/gds, GSE29250). After quality control, 19 normal gastroduodenal biopsy samples, 6 NSCLC tissues and their matching normal biopsy samples were used in further miRNA and mRNA differential expression analysis.

2.2. Comparison of miRNA and mRNA profiles

The comparison of miRNA and mRNA profiles between NSCLC tissues and their matching normal control biopsy samples were performed with Limma package on R platform using raw microarray data. The cutline of significant differentially expressed miRNA and mRNA is identified with fold change and t test methods by the cutline |logFC|>1 (fold change) and P.value<0.05 (t test).

2.3. miRNA target genes prediction

Human miRNA sequences that satisfy established criteria [15] were downloaded from the Rfam website (http://www.sanger.ac.uk/Software/Rfam). 3' UTR sequences for all human genes were retrieved using EnsMart [16]. Repetitive elements were masked in these sequences using RepeatMasker [17] with repeat libraries for primates, rodents, or vertebrates, as appropriate. The target genes of miRNAs were predicted using both mirSVR [18] and PicTar [19] methods. The predicted target genes supported by both the methods were selected for further analysis.

2.4. Integrative network analysis

The integrative analysis of miRNAs and mRNAs networks was performed with Cytoscape software, which creates molecular networks with predicted miRNA-Gene interactions and downloaded human Protein–Protein interactions (BioGrid database). The in-depth analysis of the networks has deciphered the complex interplay of miRNAs and corresponding mRNA targets and suggested their possible roles in the carcinogenesis of NSCLC.

2.5. Cell culture

The human NSCLC cell line A549 was obtained from the American Type Culture Collection (Manassas, Virginia, USA). The A549 cell line was cultured in Ham's F12K media (Invitrogen, Beijing, China) supplemented with 10% (vol/vol) fetal bovine serum (FBS)

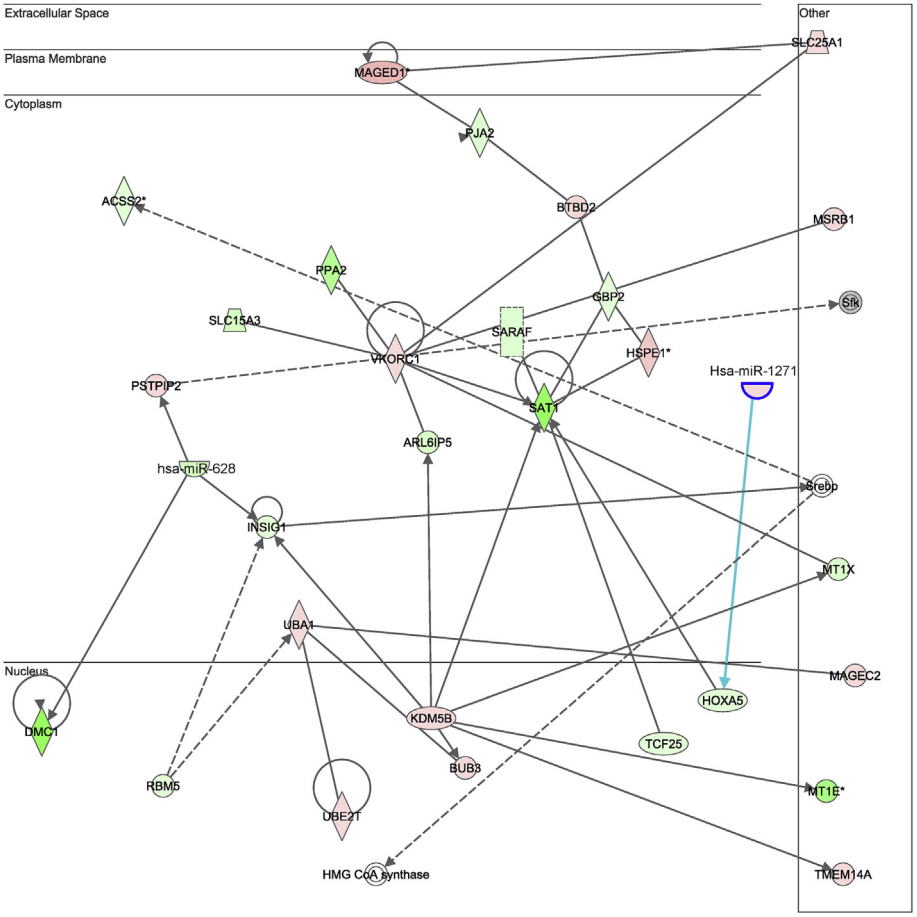

Fig. 1. Regulatory networks of mRNAs and miRNAs involved in NSCLC. Red means this molecule was up-regulated in NSCLC, while green means down-regulated. Blue line indicates the predicted regulatory relationships between miR-1271 and HOXA5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Invitrogen, Beijing, China). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO2.

2.6. Cell transfection

A549 cells were seeded at 3 × 105 cells/wells in 6-well plates and incubated overnight. Transfection of the miR-1271 miRNA mimic, the anti-miR-1271, inactive control cel-mir-67(Thermo Scientific, Shanghai, China), or pMIR-Report vectors was performed with Lipofectamine 2000 transfection reagent (Invitrogen, Beijing, China) using 300 nmol of miRNA or 1 μg/ml DNA plasmid, respectively. Total RNAs were extracted at 24 h after transfection. Total proteins were isolated at 48 h after transfection.

2.7. Western blot

The Western blot followed the protocol as previously described [20]. Proteins were separated on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad, Shanghai, China). The membrane was blocked with 5% non-fat milk and then incubated with anti-HOXA5 antibody (Sigma, Shanghai, China) or anti-β-actin antibody (Sigma, Shanghai, China). After being extensively washed, secondary antibody (Sigma, Shanghai, China) was added to the system. Immunoreactive protein bands were detected using the Enhanced Chemiluminescence (ECL) system.

2.8. Cell proliferation

Cell proliferation measure was performed using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). A549 NSCLC cells were plated in 24-well plates at 2 × 10^5 cells per well. Then cells were incubated in 10% CCK-8 that was diluted in normal culture medium at 37 °C until the occurrence of visual color conversion. Proliferation rates were measured at 0, 24, 48 and 72 h after transfection.

2.9. Migration and invasion assays

Cell invasion and migration assay were performed using a transwell chamber (Millipore, USA) with and without Matrigel (BD, Franklin Lakes, USA). For the invasion assay, a transwell chamber was placed into a 24-well plate, coated with 30 μl Matrigel and incubated for 40 min at 37 °C. In both transwell assay with and without Matrigel, A549 cells that 48 h after transfection were trypsinized and then seeded in chambers at the density of 8 × 10^4 cells/well. They were cultured in medium with RPMI 1640 medium with 2% serum. There was 600 μl of 10% FBS-1640 added to the lower chamber. 24 h later, migrated A549 cells were fixed in 100% methanol for 30 min. These non-migrated A549 cells were removed by cotton swabs. After that cells on bottom surface of the membrane were stained by the 0.1% crystal violet for 20 min. Images of cell were taken under a phase-contrast microscope.

2.10. Luciferase assay

A549 cells were seeded in 24-well plates (1 × 10^5 cells/well) and incubated for 24 h before transfection. For the reporter gene assay, the cells were co-transfected with 0.5 μg of pGL3-HOXA5-3′UTR or pGL3-HOXA5-3′UTR Mut plasmid, 0.05 ng of the phRL-SV40 control vector (Promega, Beijing, China), and 100 nM miR-1271 or control RNA using Lipofectamine 2000 (Invitrogen, Beijing, China). The firefly and renilla luciferase activities were determined consecutively through a dual luciferase assay (Promega, Beijing, China) 24 h after transfection.

2.11. Statistical analysis

The fold changes of gene expression were calculated by the equation $2^{-\Delta\Delta CT}$. Values were expressed as means ± S.D. Differences between groups were estimated with T-test. All analysis were

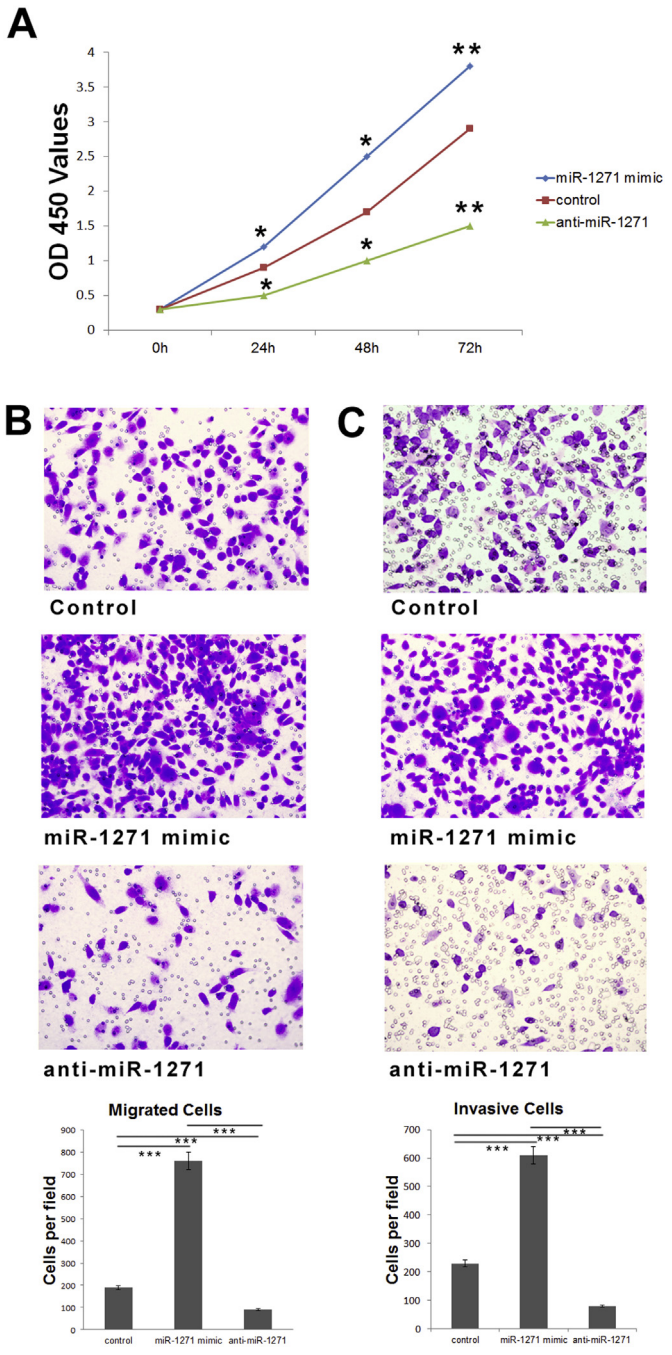

Fig. 2. Overexpression of miR-1271 promoted NSCLC cell proliferation, migration and invasion. (A) Growth of A549 cells was shown after transfection with miR-1271 mimics or inhibitor or inactive control. The growth index as assessed at 0, 24, 48 and 72 h. (B) Transwell analysis of A549 cells migration after treatment with miR-1271 mimics, inhibitors or inactive control; (C) Transwell analysis of A549 cells invasion after treatment with miR-1271 mimics, inhibitors or inactive control; *p < 0.05, **p < 0.01, and ***p < 0.001.

considered to be significant when P. value<0.05. Statistical analysis was performed on R.

3. Results

3.1. miR-1271 and miR-628, and their predicted target genes were differentially expressed in NSCLC

The comparison of the miRNA and mRNA expression data from 6 NSCLC tissues and their matching normal control from adjacent tissues identified 35 miRNAs and 128 genes were differentially expressed in NSCLC. However, further miRNA targets prediction and integrative network analysis indicated that only miR-1271 and miR-628, as well as their predicted target genes, respectively, were both differentially expressed in NSCLC (Fig. 1). In this study miR-1271 and miR-628 were found to be up-regulated in the 6 NSCLC tissues. Till now, miR-1271 has been found to participate the carcinogenesis of some types of cancers, including gastric cancer [21] and hepatocellular carcinoma [22]. On the other side, although miR-628 has been found to be deregulated in Wilms' tumor [23] and neuroblastoma [24], there is no other evidence to support the relationship between miR-628 and carcinogenesis. These evidences imply that miR-1271 might have more possibility to take part in the carcinogenesis of NSCLC. Therefore, we selected miR-1271 for further functional investigation in the human NSCLC cell line A549.

3.2. Overexpression of miR-1271 promoted NSCLC cell proliferation, migration and invasion

We explored the potential impact of miR-1271 in NSCLC cell proliferation, migration and invasion. Cells were transfected with inactive control cel-mir-67 or miR-1271 mimics and inhibitor. CCK-8 proliferation assay showed that the cell proliferation was promoted in miR-1271-mimics-transfected NSCLC cells compared with inactive control cel-mir-67-transfected cells (Fig. 2A). Conversely,

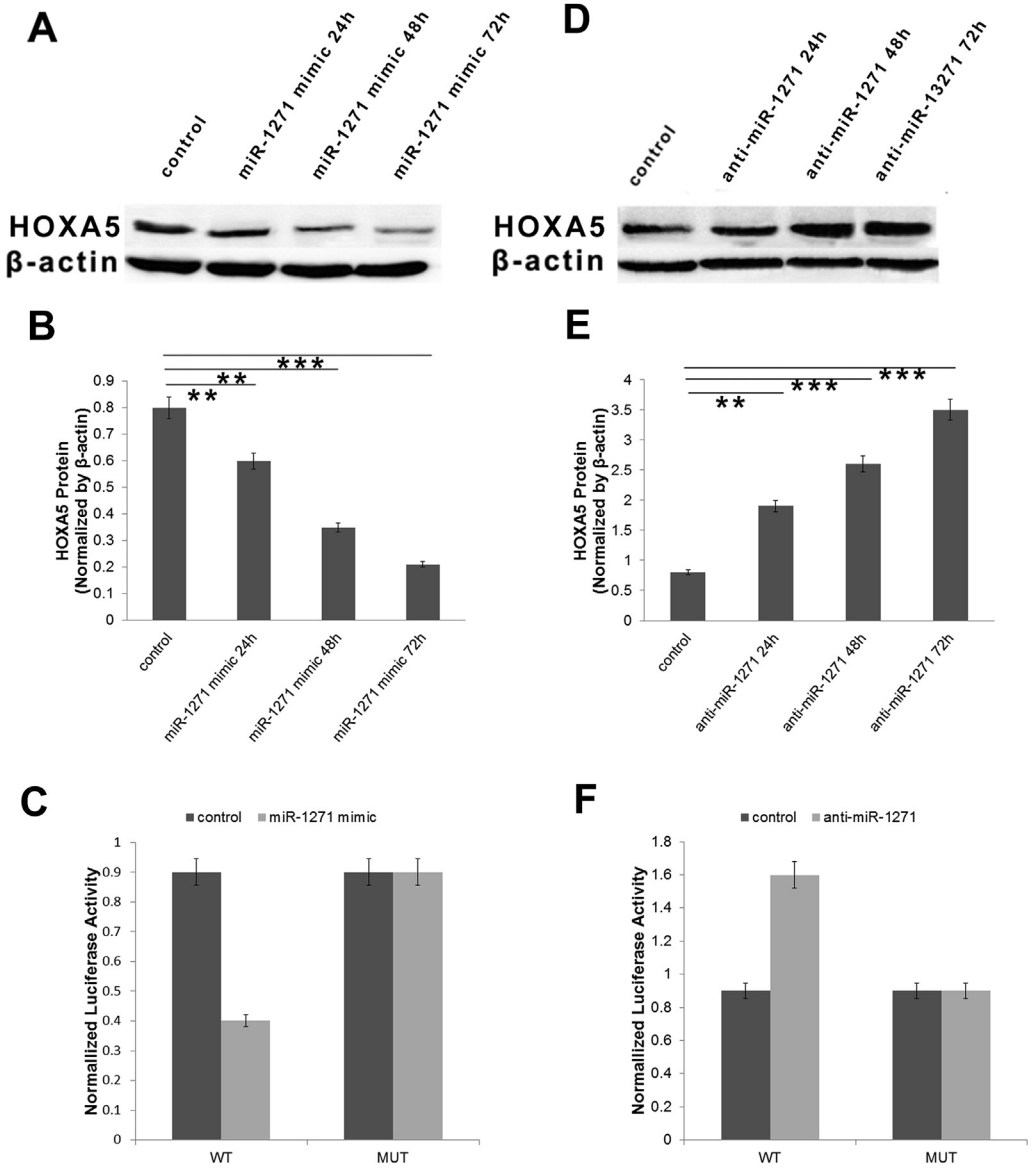

Fig. 3. miR-1271 targets HOXA5 in NSCLC cells. (A,B) Protein of HOXA5 decreased with time after transfection with miR-1271 mimics; (C) The analysis of the relative luciferase activities of HOXA5-WT, HOXA5-MUT in A549 cells after transfection with miR-1271 mimics; (D,E) Protein of HOXA5 increased with time after transfection with anti-miR-1271; (F) The analysis of the relative luciferase activities of HOXA5-WT, HOXA5-MUT in A549 cells after transfection with anti-miR-1271; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

miR-1271 inhibitor could significantly inhibited the proliferation of the A549 cells (Fig. 2A). Interestingly, migration and invasion assay indicated that overexpression of miR-1271 could significantly promoted the migration and invasion of A549 cells compared with the cel-mir-67 control whereas anti-miR-1271 could inhibited both cell migration and invasion of the A549 cells (Fig. 2B and C).

3.3. MiR-1271 targets HOXA5 in NSCLC cells

As predicted by mirSVR [18] and PicTar [19], there was complementarity between has-miR-1271 and the HOXA5 3′ UTR. Overexpression of miR-1271 reduced the protein levels of HOXA5 in NSCLC cells (Fig. 3A and B). Conversely, miR-1271 inhibitor significantly increased the protein levels of HOXA5 in NSCLC cells (Fig. 3D and E). The effect of miR-1271 on the translation of HOXA5 mRNA into protein was then assessed by using a luciferase reporter assay (Fig. 3C and F). Enforced expression of miR-1271 remarkably reduced the luciferase activity of the reporter gene with the wild type construct but not with the mutant HOXA5 3′UTR construct (Fig. 3C), while the miR-1271 inhibitor remarkably increased the luciferase activity of the reporter gene with the wild type construct but not with the mutant HOXA5 3′UTR construct (Fig. 3F). All these evidences indicate that miR-1271 directly targeted the HOXA5 3′UTR. Since the down-regulation of HOXA5 will promote NSCLC cell proliferation, migration and invasion [25], the targeting of HOXA5 might be responsible for the miR-1271 involved regulation of NSCLC cell proliferation and invasion.

4. Discussion

During the last decades, microRNAs have be found to be major regulators involved in diverse biological processes such as transcriptional regulation, cell differentiation and carcinogenesis [26]. Globally microRNA expression profiles of tumors have provided valuable insights of the molecular mechanisms of carcinogenesis [27]. Thus we compared miRNA and mRNA expression data from 6 NSCLC tissues and their matching normal control from adjacent tissues, and made further miRNA targets prediction and integrative network analysis to identify potential miRNAs and genes involved in carcinogenesis of NSCLC. The results indicated that miR-1271 and miR-628, as well as their predicted target genes, respectively, were both differentially expressed in NSCLC.

miR-1271 has been found to participate the carcinogenesis of some types of cancers, including gastric cancer [21] and hepatocellular carcinoma [22]. However, the exact role of miR-1271 in NSCLC is still unclear. Thus, our current study intended to clarify the biological function of miR-1271 in NSCLC. Enforced expression of miR-1271 enhanced proliferation, migration and invasion of A549 cells, which suggest that miR-1271 plays a critical role in the invasive and metastatic potential of NSCLC and may be potential diagnostic and predictive biomarkers. Next, we addressed the molecular mechanism of miR-1271 in promoting proliferation, migration and invasion in NSCLC cells. In this study, Western blots and luciferase assays showed that HOXA5 is a target of miR-1271. HOXA5 is the homeobox protein that has been shown to participate in the developmental regulation of the lung [28]. Since the inhibition of HOXA5 expression in A549 cells significantly promotes cell proliferation, migration and invasion [25], consistent with the results of ectopic miR-1271 expression in the same cells, we believe that miR-1271 plays a role in the promotion of proliferation and invasion in NSCLC by down-regulating the protein expression of HOXA5.

miR-628 is another miRNA found in the first step analysis. miR-628 has been found to be deregulated in Wilms' tumor [23] and neuroblastoma [24]. However, there is no evidence to support the relationship between miR-628 and lung cancer till now. Meanwhile, the study of its' predicted target gene PSTPIP2 is also at the very beginning stage. Therefore, lots of works needed to be done to explorer the roles of miR-628 and it's predicted target gene PSTPIP2 in NSCLC in the future.

In conclusion, our results have shown that enforced expression of miR-1271 promoted NSCLC cell proliferation, migration and invasion through directly targeting HOXA5. This novel miR-1271/HOXA5 axis may provide new insights into the mechanisms underlying tumor metastasis, and inhibition of miR-1271 may be a potential therapeutic strategy for the treatment of NSCLC in the future.

Conflict of interest

Authors have declared that no competing interest exists.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.033.

References

[1] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, D. Forman, Global cancer statistics, CA Cancer J. Clin. 61 (2011) 69–90.
[2] W. Smith, F.R. Khuri, The care of the lung cancer patient in the 21st century: a new age, Semin. Oncol. 31 (2004) 11–15.
[3] J.H. Schiller, D. Harrington, C.P. Belani, C. Langer, A. Sandler, J. Krook, J. Zhu, D.H. Johnson, G. Eastern Cooperative Oncology, Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer, N. Engl. J. Med. 346 (2002) 92–98.
[4] J. Ferlay, H.R. Shin, F. Bray, D. Forman, C. Mathers, D.M. Parkin, Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008, Int. J. Cancer 127 (2010) 2893–2917.
[5] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2009) 215–233.
[6] J.M. Noto, R.M. Peek, The role of microRNAs in Helicobacter pylori pathogenesis and gastric carcinogenesis, Front. Cell. Infect. Microbiol. 1 (2011) 21.
[7] W.P. Kloosterman, R.H. Plasterk, The diverse functions of microRNAs in animal development and disease, Dev. Cell. 11 (2006) 441–450.
[8] J. Lu, G. Getz, E.A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B.L. Ebert, R.H. Mak, A.A. Ferrando, J.R. Downing, T. Jacks, H.R. Horvitz, T.R. Golub, MicroRNA expression profiles classify human cancers, Nature 435 (2005) 834–838.
[9] M. Acunzo, R. Visone, G. Romano, A. Veronese, F. Lovat, D. Palmieri, A. Bottoni, M. Garofalo, P. Gasparini, G. Condorelli, M. Chiariello, C.M. Croce, miR-130a targets MET and induces TRAIL-sensitivity in NSCLC by downregulating miR-221 and 222, Oncogene 31 (2012) 634–642.
[10] H.S. Jeon, S.Y. Lee, E.J. Lee, S.C. Yun, E.J. Cha, E. Choi, M.J. Na, J.Y. Park, J. Kang, J.W. Son, Combining microRNA-449a/b with a HDAC inhibitor has a synergistic effect on growth arrest in lung cancer, Lung Cancer 76 (2012) 171–176.
[11] J.G. Zhang, J.J. Wang, F. Zhao, Q. Liu, K. Jiang, G.H. Yang, MicroRNA-21 (miR-21) represses tumor suppressor PTEN and promotes growth and invasion in non-small cell lung cancer (NSCLC), Clin. Chim. Acta 411 (2010) 846–852.
[12] W. Gao, Y. Yu, H. Cao, H. Shen, X. Li, S. Pan, Y. Shu, Deregulated expression of miR-21, miR-143 and miR-181a in non small cell lung cancer is related to clinicopathologic characteristics or patient prognosis, Biomed. Pharmacother. 64 (2010) 399–408.
[13] D. Lebanony, H. Benjamin, S. Gilad, M. Ezagouri, A. Dov, K. Ashkenazi, N. Gefen, S. Izraeli, G. Rechavi, H. Pass, D. Nonaka, J. Li, Y. Spector, N. Rosenfeld, A. Chajut, D. Cohen, R. Aharonov, M. Mansukhani, Diagnostic assay based on hsa-miR-205 expression distinguishes squamous from nonsquamous non-small-cell lung carcinoma, J. Clin. Oncol. 27 (2009) 2030–2037.
[14] X. Liu, L.F. Sempere, Y. Guo, M. Korc, S. Kauppinen, S.J. Freemantle, E. Dmitrovsky, Involvement of microRNAs in lung cancer biology and therapy, Transl. Res. 157 (2011) 200–208.
[15] V. Ambros, B. Bartel, D.P. Bartel, C.B. Burge, J.C. Carrington, X. Chen, G. Dreyfuss, S.R. Eddy, S. Griffiths-Jones, M. Marshall, M. Matzke, G. Ruvkun, T. Tuschl, A uniform system for microRNA annotation, RNA 9 (2003) 277–279.
[16] A. Kasprzyk, D. Keefe, D. Smedley, D. London, W. Spooner, C. Melsopp, M. Hammond, P. Rocca-Serra, T. Cox, E. Birney, EnsMart: a generic system for fast and flexible access to biological data, Genome Res. 14 (2004) 160–169.
[17] M. Tarailo-Graovac, N. Chen, Using RepeatMasker to Identify Repetitive Elements in Genomic Sequences, Curr Protoc Bioinformatics Chapter 4, 2009. Unit 4 10.

[18] D. Betel, A. Koppal, P. Agius, C. Sander, C. Leslie, Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites, Genome Biol. 11 (2010) R90.
[19] A. Krek, D. Grun, M.N. Poy, R. Wolf, L. Rosenberg, E.J. Epstein, P. MacMenamin, I. da Piedade, K.C. Gunsalus, M. Stoffel, N. Rajewsky, Combinatorial microRNA target predictions, Nat. Genet. 37 (2005) 495–500.
[20] J. Li, X. Kong, J. Zhang, Q. Luo, X. Li, L. Fang, MiRNA-26b inhibits proliferation by targeting PTGS2 in breast cancer, Cancer Cell. Int. 13 (2013) 7.
[21] M. Yang, X. Shan, X. Zhou, T. Qiu, W. Zhu, Y. Ding, Y. Shu, P. Liu, miR-1271 regulates cisplatin resistance of human gastric cancer cell lines by targeting IGF1R, IRS1, mTOR, and BCL2, Anticancer Agents Med. Chem. 14 (2014) 884–891.
[22] M. Maurel, S. Jalvy, Y. Ladeiro, C. Combe, L. Vachet, F. Sagliocco, P. Bioulac-Sage, V. Pitard, H. Jacquemin-Sablon, J. Zucman-Rossi, B. Laloo, C.F. Grosset, A functional screening identifies five microRNAs controlling glypican-3: role of miR-1271 down-regulation in hepatocellular carcinoma, Hepatology 57 (2013) 195–204.
[23] J.A. Watson, K. Bryan, R. Williams, S. Popov, G. Vujanic, A. Coulomb, L. Boccon-Gibod, N. Graf, K. Pritchard-Jones, M. O'Sullivan, miRNA profiles as a predictor of chemoresponsiveness in Wilms' tumor blastema, PLoS One 8 (2013) e53417.
[24] J.H. Schulte, T. Marschall, M. Martin, P. Rosenstiel, P. Mestdagh, S. Schlierf, T. Thor, J. Vandesompele, A. Eggert, S. Schreiber, S. Rahmann, A. Schramm, Deep sequencing reveals differential expression of microRNAs in favorable versus unfavorable neuroblastoma, Nucleic Acids Res. 38 (2010) 5919–5928.
[25] X.H. Liu, K.H. Lu, K.M. Wang, M. Sun, E.B. Zhang, J.S. Yang, D.D. Yin, Z.L. Liu, J. Zhou, Z.J. Liu, W. De, Z.X. Wang, MicroRNA-196a promotes non-small cell lung cancer cell proliferation and invasion through targeting HOXA5, BMC Cancer 12 (2012) 348.
[26] W.K. Wu, C.W. Lee, C.H. Cho, D. Fan, K. Wu, J. Yu, J.J. Sung, MicroRNA dysregulation in gastric cancer: a new player enters the game, Oncogene 29 (2010) 5761–5771.
[27] J. Miao, S. Wu, Z. Peng, M. Tania, C. Zhang, MicroRNAs in osteosarcoma: diagnostic and therapeutic aspects, Tumour Biol. 34 (2013) 2093–2098.
[28] I. Mandeville, J. Aubin, M. LeBlanc, M. Lalancette-Hebert, M.F. Janelle, G.M. Tremblay, L. Jeannotte, Impact of the loss of Hoxa5 function on lung alveogenesis, Am. J. Pathol. 169 (2006) 1312–1327.