Clinicopathological Significance of Up-Regulated Pir-1366 in NSCLC and its Effect on Tumor Migration and Metastasis

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Abstract

**Background:** piRNAs are a kind of noncoding RNAs that involve in tumorigenesis and development, but the role of piRNAs in NSCLC remain unclear. In this study, we explored the role of piR-1366 in NSCLC.

**Methods:** The identification of piRNAs was performed using Human Arraystar piRNA Array in NSCLC tissues and adjacent normal tissues. up-regulated piR-1366 were validated in the NSCLC and selected for further study of migration and metastasis. In addition, the expression of piR-1366 in 87 cases of NSCLC was detected by in situ hybridization and the correlation between piR-1366 expression and pathological parameters of NSCLC patients was analyzed. meanwhile, bioinformatics, qRT-PCR and West blotting were used to examine the signal pathway of piR-1366 in migration.

**Results:** piR-1366 was up-regulated and positive expression in NSCLC, The positive expression of piR-1366 was closely related to lymph node metastasis of NSCLC, but not with age, gender, tumor location, tumor size, tumor grade and TNM stage. piR-1366 is transfected into lung cancer cells to promote its migration and metastasis. Mechanistically, WT1-3'UTR was complementary combination with piR-1366 by bioinformatic prediction and identified as a direct target of piR-1366 through dual-luciferase reporter assay. Over-expression and knockdown of WT1 could respectively rescue and simulate the effects induced by piR-1366. Finally, piR-1366 promoted migration and metastasis by the WT1/CDH1 pathway in lung cancer cells.

**Conclusions:** Thus, our article suggested that piR-1366 was a novel pro-metastasis oncogene and may represent a novel marker of diagnosis and treatment for Metastatic NSCLC.

Introduction

Lung cancer (LC) a common cancer of respiratory diseases in the world and its mortality rate and recurrence rate are the highest among all malignant tumors (1). 60% of the patients will eventually die from lung cancer according to statistics (2). LC can be divided into small cell lung cancer and non-small cell lung cancer (NSCLC) according to pathological classification, the most common type of LC is NSCLC. the incidence of LC increased year by year and the average survival time of NSCLC patients was less than 6 months, and (3–4). Despite the continuous progress of molecular biomedicine research and the improvement of medical treatment technology, the therapeutic effect of lung cancer has not been significantly improved. Therefore, it is very important and urgent to find new diagnostic indicators and treatment methods of LC.

PIWI-associated RNAs (piRNAs) are a kind of non-coding RNA with 21–35 nucleotide sequences (5), and combine with PIWI protein to play important biological functions (6–7). In recent years, the research on the function of piRNAs has become one of the hotspots in tumor research. Studies have shown that piRNAs could regulate sperm epigenetics and maintaining germ stem cells in male germ cells (8). Many piRNAs are abnormally expressed in malignant tumor tissues, and can promote or inhibit the proliferation, apoptosis, invasion and migration of cancer cells (9–11). For example, the results of Qi et al. (12)
suggested that piR-19166 target the CTTN gene and inhibit the invasion and distant metastasis of prostate cancer cells, which is a new marker for early diagnosis and treatment of prostate cancer.

This project aims to study the expression of piRNA-1366 (piR-1366) in NSCLCs and explore its role and mechanism in the development of NSCLCs, so as to provide a strong theoretical basis for clinical diagnosis and treatment of NSCLCs.

Materials And Methods

NSCLC specimens’ collection

87 pairs of NSCSC tissues and adjacent normal lung tissues were obtained from the Affiliated Hospital of Yangzhou University, Yangzhou University (Yangzhou, China) from January 2018 to December 2019. NSCSC was confirmed by two pathologists. The age of NSCLC patients ranged from 39 to 76 years old, including 56 males and 31 females. All fresh specimens were stored in liquid nitrogen before use. The use of specimens and the collection of clinical data were approved by the ethics committee of the Affiliated Hospital of Yangzhou University (Approval No. 2018-08). Written informed consent was also provided for the patients. All the clinicopathological parameters are summarized in Table 1, including age, gender, smoking, tumor size, grade, lymphatic metastasis and TNM stage.

Cell culture

NSCLC cell lines (A549, H1650 and H460) and normal Lung epithelial cell (BEAC-2B) were purchased from the Chinese Peking Union Medical College Cell Bank (Beijing, China). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) (HyClone, Australia), 120 IU/ml penicillin, and 120 mg/ml streptomycin at 37°C in humidified incubators with 5% CO2.

Plasmid and Transfection

Plasmid of piR-1366 mimics/inhibitor and piRNA negative control (NC) were all purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Plasmids were transfected into cultured cells using Lipofectamine 2000 Reagent (Invitrogen) according to the product protocol, Then tumor cells were incubated for 36 hour at 37°C with 5% CO2 before next experiments.

qRT-PCR

Total RNAs were isolated using TRIzol reagent (Invitrogen). The RNAs were set at an optical density (OD) A260/280 ratio between 1.8 and 2.1 and an OD A260/230 ratio >1.8. RNAs were reverse-transcribed to cDNA using the PrimeScript First Strand cDNA synthesis kit (Takara) according to the instructions(13). qRT-PCR was performed with fluorescent quantitative Kit (2 × SYBR Green qPCR mastermix) on an Applied Biosystems 7500 Real Time PCR system. The related primers were in table S1. The expression of piRNAs and mRNAs was normalized to U6 and GAPDH, respectively.
Arraystar piRNA array

The extracted total RNAs were submitted to AKSOMICS (Shanghai) Biotechnology Co., Ltd. (Shanghai, China) for The Human Arraystar piRNA array, which is designed for profiling 23000 human piRNAs (ArrayStar, Rockville, MD). Data analysis of expression profiling and image acquisition were provided by AKSOMICS.

RNA in situ hybridization (RISH)

Paraffin sections were dewaxed to water. Endogenous enzymes were eliminated by hydrogen peroxide treatment at room temperature. The exposed tissues were digested with pepsin. Tissues were incubated in an incubator with pre-hybridizing solution, and then hybridizing solution was added and incubated 12 hours. Add sealing solution after washing. Biotinylated digoxin was added. The tissues were added with SABC. Biotinylated peroxidase was added. The tissues were stained with DAB, hematoxylin re-dyeing, washing, dehydration, transparency, sealing. The hybridization solution containing the probe was replaced by pre-hybridization solution as blank control.

Nude mice lung metastasis models

For metastasis models, LC cells (5 × 106) suspended by PBS were injected subcutaneously into the tail veins of null mice (BABL/c, nu/nu, 23–27 g, 5–7 weeks of age, 10 mice/group) from Animal Center of Yangzhou University. At the end of the arrays, the mice were scarified and the tumors and their lungs were removed, quantified and frozen for further assay. All nude mouse studies were approved in the animal facility at Ethics Committee on Animal Experimentation of Yangzhou University accordance with institutional guidelines.

Western blot assay

Western blotting has been described in the previous article (14). The simple steps are as follows: Total protein extraction, Protein content determination, SDS-PAGE electrophoresis, Transfer membrane, Immune reaction, Final gel image analysis. The primary antibodies included WT1(#ab180840), CDH1(E-cadherin,#ab231303), MGE3(#ab223162) and GAPDH(#2118), the second antibody was anti-rabbit immunoglobulin G antibody (which had been combined with horseradish peroxidase). CD9 (#ab2215), CD81(#ab109201), WT1 (#ab180840), CDH1(E-cadherin, #ab231303), MGE3(#ab223162) and anti rabbit immunoglobulin G antibody (#ab6721) were purchased from Abcam Biotechnology company, GAPDH antibody (#2118) were purchased from CST Biotechnology company.

Dual-luciferase reporter assay

Bioinformatics method was used to predict the piR-1366 binding sites in 3'UTR of WT1. The length 368 nt of WT1 3'UTR was amplified and inserted into the luciferase reporter gene plasmid pGL3-BS. Wild type (WT) gene plasmid and mutation type(Mut) gene plasmid were co-transfected into A549 cells. The protein was extracted and used for luciferase detection. The activity of luciferase was determined by
adding substrate. The relative fluorescence intensity was calculated, Firefly luciferase signal was used for normalization. The primers of WT1 3'-UTR are shown in table S1.

**Transwell migration**

In 180 µl culture media of the upper wells, ten thousand of lung cancer cells without serum were seeded on a fibronectin-coated polycarbonate membrane. In the lower wells, culture solutions contain 10% FBS. After 12 hours, the tumor cells migrated to the membrane' bottom surface and were fixed with methanol for 20 minutes, then stained with 0.1% crystal violet for 15 minutes, observed and photographed under a light microscope (Leica, Germany) at 10×20 or 10×400 magnification.

**Statistical analysis**

SPSS V.16 software and GraphPad Prism V5.0 software were used for statistical analysis and all graphs. Students’ T test was used to evaluate the differences between groups. The variables of count data were analyzed by chi square test (χ²-test). P < 0.05 as a significant difference between the two groups.

**Results**

**Human piRNA Array’ data analysis**

A piRNA expression profiling of five sample of NSCLC tissue and five normal lung tissues (NC) was detected by Arraystar Human piRNA Array in AKSOMICS (Shanghai) Biotechnology Co., Ltd. The screen and data analysis were performed by AKSOMICS. The results showed that a box plot (Figure 1A) formed a normalized log2-ratio distribution of intensities between the NSCLC group and NC group. Hierarchical clustering was performed Hundreds piRNAs were aberrant expression that appear to up-regulated and down-regulated in this data by Hierarchical clustering (Figure 1B). a Volcano Plot and a Scatter Plot filtered between the NSCLC and control with a threshold fold change > = 2.0 and p-value < = 0.05 was performed (Figure 1C) to identify differentially expressed piRNAs with statistical significance.

**piRNAs validation in NSCLCs**

Human piRNAs screen showed 5 up-regulated and 5 down-regulated piRNAs with P value less than 0.01 between NSCLC and NC, their expression levels were listed in Table 1. 2 up-regulated and 1 down-regulated piRNAs have been validated by qRT-PCR in 87 pairs of tumor sample and normal tissues. The expression of piR-1366 and piR-26323 were up-regulated while piR-31424 was down-regulated in sample tissues (Figure 2). According to the results, up-regulated piR-1366 was only selected to do future experiment.

**Expression of piR-1366 in NSCLC tissues and cell lines**

To further confirm high expression of piR-1366 in piRNAs screen, RNA in situ hybridization (ISH) and qRT-PCR were used to explore its RNA expression in 87 NSCLC tissues. As shown in Figure.3A, piR-1366
expression was positive (78/87) compared with the adjacent normal tissues (negative, 6/87) via ISH. By qRT-PCR, expression of piR-1366 in NSCLC was obviously higher than that of in the adjacent normal lung tissues (Figure 3B). In the lymph node metastasis (LNM)-positive group (n=46), piR-1366 is a higher expression level than that of in LNM-negative group (n=21, Figure 3C). There is closely relation between expression of piR-1366 and LNM (Pearson correlation coefficient = 0.384, p < 0.05). Similarly, up-regulation of piR-1366 was detected using qRT-PCR in three lung cancer cell lines (A549, H1650 and H460) compared with a normal Lung epithelial cell of BEAC-2B using qRT-PCR (Figure 3D).

Collectively, the above findings suggest that piR-1366 expression may play a role of inhibitor in development and metastasis of NSCLCs.

Clinicopathological significance of up-regulated piRNA-1366 in NSCLC

Our experiments had shown that the expression of piR-1366 is up-regulated in NSCLCs, so we explored the correlation between up-regulated piR-1366 RNA level and clinical parameters of NSCLC patients. As shown in Table 2, there was a significant difference in lymphatic metastasis (Pearson Chi-Square = 12.102, P = 0.001) between patients with low/unchanged versus high expression levels of in NSCLCs. Tumor grade (Pearson Chi-Square = 3.057, P = 0.080) and TNM stage (Pearson Chi-Square = 2.909, P = 0.088) are almost statistically different. But no significant correlations between piR-1366 expression levels and other clinicopathologic variables, including age, gender, smoking, and tumor size (all P > 0.05, Table 2). These results suggested that high expression of piR-1366 is closely related to lymph node metastasis, and it is involved in the process of NSCLC metastasis.

piR-1366 promoted cancer cell migration and lung metastasis

Our above data demonstrated piR-1366 was relation closely with LN metastasis in NSCLC patients, so the role of piR-1366 will be investigated in migration though Transwell assay. Firstly, lentiviral vector overexpressing or silencing of piR-1366 and corresponding negative control (NC) was transfected and confirmed into A549 and H1650 by qRT-PCR analysis (P < 0.01, Figure 4A-B). Then, the cancer cells were used for migration. The results of migration showed overexpression of piR-1366 significantly promoted migration in lung cancer cells, but silencing of piR-1366 dramatically impeded the migration of A549 and H1650 (Figure 4C-D), both groups had significant statistical significance. To further investigate the role of piR-1366 in driving lung cancer metastasis, lung metastasis models of nude mice were also conducted. A549 cells with stably silencing of piR-1366 (si-piR-1366) and control were injected through the tail vein as compared with controls. The nude mice test result showed a significant difference between the si-piR-1366 animals and control groups in macroscopic observation of lung metastasis. Furthermore, Histologic analysis revealed significantly less and smaller metastatic foci in the harvested lung tissues of nude mice injected with A549 cells with silencing of piR-1366 (Figure 4E-F). Collectively, these results suggested that piR-1366 serves as a tumor oncogene in migration of lung cancer and a role of promoter for piR-1366 in the regulation of metastasis in lung cancer.

WT1 is a direct target of piR-1366
First, piR-1366 was predicted the possible target of by bioinformatics. piR-1366 is highly expressed in lung cancer, so we assumed that its target gene might be a tumor suppressor gene. Then we compared the gene sequence of piR-1366 with the 3' UTR of common tumor suppressor genes (Rb, p53, PTEN, WT1 and so on). The results showed that piR-1366 only had four complementary binding sites with the 3' UTR of WT1 (Figure 5A), and had no complementary binding site with other tumor suppressor genes. Then, the relationship between piR-1366 and WT1 were confirmed by Dual-luciferase reporter assay, q-RT PCR and western blotting. The binding sequences of piR-1366, WT1 of wild type (WT) and WT1 of mutation type (Mut) were showed in Figure 5B. Co-expression with WT1 -3'UTR/pGL3-BS and piR-1366 in lung cancer cells caused significant decrease in the luciferase activity compared with the negative control, but this repressive effect disappeared by mutation WT1 (p < 0.05, Figure. 5C). This result indicated that piR-1366 exerts inhibitory effects on WT1 expression via binding with the 3'UTR of WT1. Meanwhile, overexpression of piR-1366 suppressed WT1 expression, and silencing of piR-1366 promoted WT1 expression in levels of RNA and protein (Figure. 5D-E). These data suggested that piR-1366 may inhibit WT1 expression by blocking WT1 post transcriptional translation, which is consistent with other non coding RNAs.

PiR-1366 exerts to regulate WT1 expression

Rescue assays were conducted to determine whether WT1 was regulated though piR-1366-induced in lung cancer cells. Firstly, Over-expression WT1 rescued the down-regulation of WT1 via high levels of piR-1366. Further functional studies confirmed that Over-expression of WT1 could abrogate piR-1366 mediated promotion of migration, whereas silencing WT1 could provoke anti-piR-1366 abilities to induce migration (Fig. 6A-D). Collectively, these results indicated that piR-1366 inhibited WT1 expression and then hindered migration in lung cancer cells.

PiR-1366 promotes metastasis though WT1/CDH1 pathway in lung cancer

Previous evidence has suggested that CDH1 (also called E-cadherin) is a direct target of WT1, the WT1/CDH1 pathways are involved in tumor metastasis (15-16). So whether the WT1/CDH1 pathways could be activated by piR-1366, qRT-PCR and western blot assay were used to test this effect after piR-1366 overexpression or knockdown. These results showed that WT1 was obviously decreased and CDH1 were increased in high piR-1366 groups rather than those with low piR-1366 groups in mRNA (Figure.7A-B) and protein (Figure.7C) level. Therefore, this study was concluded that the piR-1366 suppressed expression of WT1, and then promoted CDH1 expression, the WT1/CDH1 signaling pathways might work in lung cancer cells. Therefore, these data also demonstrated that the up-regulated piR-1366 effectively regulates CTTN/CDH1 signaling pathways in vivo to promote metastasis of lung cancer.

Discussion

According to the global cancer database in 2018, lung cancer is the most common type of cancer in all the population (11.6% of the total cases), and it is also the main cause of cancer death (18.4% of the total cancer deaths)(17). In China, lung cancer now ranks first in the incidence of malignant tumors with an
annual incidence of about 781,000, and its death rate of six tumor is the first, the number of death is about 626,000 (18). With the continuous development of treatment methods in recent years, more and more targeted drugs and the comprehensive application of new biological immunotherapy, the survival period and prognosis of patients with lung cancer have been improved (19).

piRNAs are a kind of non-coding small RNA and piRNA-1366 is one of piRNAs with the length of 24nt. Gene alteration of tumor cells are regulated by a series of small molecules, including piRNAs. Existing studies have shown that piRNAs played the role of oncogene or tumor suppressor gene in tumor formation, development and progression of cancer (20). For example, piR-651, piR-4987, piR-20365, piR-20485 and piR-20582 were highly expressed in breast cancer (21). The expression of piR-823 decreased in gastric cancer, and the level of piR-823 was positively correlated with the stage of gastric cancer (22). Law et al. (23) reported that the level of piR-hep1 was positively correlated with the invasion and metastasis of hepatoma cells, indicating that piRNA might also participate in the invasion and metastasis of tumor cells and other pathological processes. The abnormal high expression of piR-594040 was found in bladder cancer (24). PiR-19166 inhibited the invasion and metastasis targeting CTTN gene in prostate cancer cells. However, there are few studies on the mechanism of piR-1366 regulating development and progression of NSCLC.

In the current study, piR-1366 was obviously up-regulated in NSCLC tissues and cell lines compared to control group via ISH and qRT-PCR, and the higher the expression level of piR-1366 is, the more likely lung cancer is to have lymph node metastasis. These data demonstrated piR-1366 expression may play a role of tumor oncogene and pro-metastasis in development of NSCLC. Similarly, in vitro suggested that piR-1366 could block cell migration of lung cancer cells and decrease the number of metastatic tumors in lung model of nude mice. The observations explored piR-1366 was served as a biomarker of metastatic NSCLC.

WT1 has been confirmed as a tumor suppressor and negative regulator of metastasis by previous study (25-29). By bioinformatics predict that WT1 was a target gene of piR-1366 and there is four complementary binding sites between piR-1366 and WT1 3'UTR region. Our arrays showed piR-1366 could inhibit directly protein translation of CNNT via 3'UTR region. These data confirmed that piR-1366 is one of direct inhibitor of WT1, piR-1366/ WT1 axis involved in metastasis of NSCLC. Previous considerable research has suggested that a number of WT1 signal pathways (WT1/ PEI, WT1/ alpha 4 integrin, WT1/ VEGF and WT1/CDH1) are involved in the formation and development of tumors (15,16,25,30-31). Our results only confirmed WT1/CDH1 signal pathway was valid in NSCLC, that the piR-1366 overexpression significantly suppressed the expression of WT1 and promoted CDH1 expression, while silencing of piR-1366 expression showed the opposite results. These data showed that the piR-1366 activated WT1/ CDH1 signaling pathways and promoted migration and metastasis of NSCLC cells. These are important markers of personalized therapeutics for early-stage NSCLC patients with LN metastasis. Meanwhile, our experiments suggested that inhibition of WT1 expression could promote the metastasis of lung cancer cells, which is contrary to the results reported [80-81]. Therefore, we speculated
that the expression level of WT1 in lung cancer was different from that in other cancers, and it was also related to whether WT1 itself has gene mutation.

In conclusion, up-regulated piR-1366 was related with metastatic NSCLC, piR-1366 promoted migration and metastasis by activating WT1/ CDH1 signaling pathway, piR-1366 is an important oncogene associated with metastatasis in NSCLC. These newly identified results could provide a novel diagnostic and therapeutic target for NSCLC patients with lymph node metastasis.

**Declarations**

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**Contributions**

Chenghai Wang and Hongchan Shi conceived and designed the whole experiments and manuscript. Jun Zhou, Hong Guo, Wen Lu and Lei Wang performed the major work of the assay. Lei Wang and Chenghai Wang analyzed and did data statistics. All authors read and approved the final manuscript.

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**Ethics declarations**

Ethics approval and consent to participate
Animal experiment was approved by the Ethics committee of The Affiliated Hospital of Yangzhou University, Yangzhou University in accordance with guidelines and protocols for animal care and protection.

Consent for publication

All authors approved this manuscript to publication in *Cancer Cell International*.

Competing interests

The authors have no conflicts of interest.

References

1. Burns TF, Stabile LP. Targeting the estrogen pathway for the treatment and prevention of lung cancer. Lung Cancer Manag 2014, 3(1), 43-52.

2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2016, 66(2): 115-32.

3. Ruzycka M, Cimpan MR, Rios-Mondragón I, Grudzinski IP. Microfluidics for studying metastatic patterns of lung cancer. J Nanobiotechnology 2019, 17(1): 71.

4. Langer CJ, Obasaju C, Bunn P, Bonomi P, Gandara D, Hirsch FR, et al. Incremental Innovation and Progress in Advanced Squamous Cell Lung Cancer: Current Status and Future Impact of Treatment. J Thorac Oncol 2016, 11(12): 2066-81.

5. Han YN, Li Y, Xia SQ, Zhang YY, Zheng JH, Li W. PIWI proteins and PIWI-interacting RNA: emerging roles in cancer. Cell Physiol Biochem 2017, 4(1): 1-20.

6. Liu Y, Dou M, Song X, Dong Y, Liu S, Liu H, et al. The Emerging Role of the piRNA/piwi Complex in Cancer. Mol Cancer 2019, 18(1): 123.

7. Wenhao Weng, Hanhua Li, Ajay Goel. Piwi-interacting RNAs (piRNAs) and Cancer: Emerging Biological Concepts and Potential Clinical Implications. Biochim Biophys Acta Rev Cancer 2019, 1871(1): 160-9.

8. Yu Y, Xiao J, Hann SS. The emerging roles of PIWI-interacting RNA in human cancers. Cancer Manag Res 2019, 11: 5895-909.

9. Liu P, Dong Y, Gu J, Puthiyakunnel S, Wu Y, Chen XG. Developmental piRNA profiles of the invasive vector mosquito Aedes albopictus. Parasit Vectors 2016, 9(1): 524.

10. Ross RJ, Weiner MM, Lin H. PIWI proteins and PIWI-interacting RNAs in the soma. Nature 2014, 505(7483): 353-9.

11. Siddiqi S, Matushansky I. Piwis and piwi-interacting RNAs in the epigenetics of cancer. J Cell Biochem 2012, 113(2): 373-80.

12. Qi T, Cao H, Sun H, Feng H, Li N, Wang C, et al. piR-19166 inhibits migration and metastasis through CTTN/MMPs pathway in prostate carcinoma. Aging (Albany NY) 2020, 12(18): 18209-220.
13. Wang C, Wang Z, Zhou J, Liu S, Wu C, Huang C, et al. TUC.338 promotes invasion and metastasis in colorectal cancer. Int J Cancer 2017, 140:1457-64.

14. Zhou J, Wang C, Gong W, Wu Y, Xue H, Jiang Z, et al. uc.454 Inhibited Growth by Targeting Heat Shock Protein Family A Member 12B in Non-Small-Cell Lung Cancer. Mol Ther Nucleic Acids 2018, 12:174-83.

15. Brett A, Pandey S, Fraizer G. The Wilms’ tumor gene (WT1) regulates E-cadherin expression and migration of prostate cancer cells. Mol Cancer 2013, 12:3.

16. Chen J, Gao S, Wang C, Wang Z, Zhang H, Huang K, et al. Pathologically decreased expression of miR-193a contributes to metastasis by targeting WT1-E-cadherin axis in non-small cell lung cancers. J Exp Clin Cancer Res 2016, 35(1):173.

17. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018 Nov, 68(6):394-424.

18. Cao M, Chen W. Epidemiology of lung cancer in China. Thorac Cancer 2019, 10(1):3-7.

19. Zeng H, Zheng R, Guo Y, Zhang S, Zou X, Wang N, et al. Cancer survival in China, 2003-2005: a population-based study. Int J Cancer. 2015 Apr 15, 136(8):1921-30.

20. Han YN, Li Y, Xia SQ, Zhang YY, Zheng JH, Li W. PIWI Proteins and PIWI-Interacting RNA: Emerging Roles in Cancer. Cell Physiol Biochem 2017, 44(1):1-20.

21. Huang G, Hu H, Xue X, Shen S, Gao E, Guo G, et al. Altered expression of piRNA and their relation with clinicopathologic features of breast cancer. Clin Transl Oncol 2013, 15(7):563-8.

22. Cheng J, Deng H, Xiao B, Zhou H, Zhou F, Shen Z, et al. piR-823, a novel noncoding small RNA, demonstrates in vitro and in vivo tumor suppressive activity in human gastric cancer cells. Cancer Lett 2012, 315(1):12-7.

23. Law PT, Qin H, Ching AK, Lai KP, Co NN, He M, et al. Deep sequencing of small RNA transcriptome reveals novel noncoding RNAs in hepatocellular carcinoma. J Hepatol 2013, 58(6):1165-73.

24. Chu H, Hui G, Yuan L, Shi D, Wang Y, Du M, et al. Identification of novel piRNAs in bladder cancer. Cancer Lett 2015, 356(2):561-7.

25. Kirschner KM, Wagner N, Wagner KD, Wellmann S, Scholz H. The Wilms tumor suppressor Wt1 promotes cell adhesion through transcriptional activation of the alpha4integrin gene. J Biol Chem. 2006 281(42):31930-9.

26. Xie F, Hosany S, Zhong S, Jiang Y, Zhang F, Lin L, et al. MicroRNA-193a inhibits breast cancer proliferation and metastasis by downregulating WT1. PLoS One 2017 10, 12(10):e0185565.

27. Wagner KD, Cherfils-Vicini J, Hosen N, Hohenstein P, Gilson E, Hastie ND, et al. The Wilms’ tumour suppressor Wt1 is a major regulator of tumour angiogenesis and progression. Nat Commun 2014 16, 5:5852.

28. Qi XW, Zhang F, Wu H, Liu JL, Zong BG, Xu C, et al. Wilms’ tumor 1 (WT1) expression and prognosis in solid cancer patients: a systematic review and meta-analysis. Sci Rep 2015, 5:8924.
29. Miyoshi Y, Ando A, Egawa C, Taguchi T, Tamaki Y, Tamaki H, et al. High expression of Wilms’ tumor suppressor gene predicts poor prognosis in breast cancer patients. Clin Cancer Res 2002, 8(5):1167-71.

30. Zamora-Avila DE, Zapata-Benavides P, Franco-Molina MA, Saavedra-Alonso S, Trejo-Avila LM, Reséndez-Pérez D, et al. WT1 gene silencing by aerosol delivery of PEI-RNAi complexes inhibits B16-F10 lung metastases growth. Cancer Gene Ther 2009, 16(12):892-9.

31. Graham K, Li W, Williams BR, Fraizer G. Vascular endothelial growth factor (VEGF) is suppressed in WT1-transfected LNCaP cells. Gene Expr 2006, 13(1):1-14.

### Tables

Table 1: Identified piRNA in NSCLC using piRNA array screen

| PiRNA     | Fold Change | PiRNA     | Fold Change |
|-----------|-------------|-----------|-------------|
| piR-1366  | 17.1        | piR-15151 | -28.3       |
| piR-29595 | 13.2        | piR-7283  | -21.5       |
| piR-26323 | 12.6        | piR-31424 | -16.6       |
| piR-26754 | 9.3         | piR-26522 | -12.1       |
| piR-26593 | 8.3         | piR-6092  | -9.0        |

Table 2 Correlation between piR-1366 expression and clinicopathological parameters
| Clinicopathological Features | High piR-1366 n=67 | low / unchanged piR-1366 n=20 | Pearson Chi-Square | P value |
|------------------------------|---------------------|-------------------------------|-------------------|--------|
| 1. Age (years)               |                     |                               | 0.281             | 0.596  |
| <50                          | 38                  | 10                            |                   |        |
| ≥50                          | 29                  | 10                            |                   |        |
| 2. Gender                    |                     |                               | 0.047             | 0.828  |
| male                         | 42                  | 12                            |                   |        |
| female                       | 25                  | 8                             |                   |        |
| 3. Smoking                   |                     |                               | 0.203             | 0.652  |
| Yes                          | 34                  | 9                             |                   |        |
| No                           | 33                  | 11                            |                   |        |
| 4. Tumor size                |                     |                               | 0.143             | 0.705  |
| ≥3                           | 40                  | 8                             |                   |        |
| <3                           | 37                  | 12                            |                   |        |
| 5. Tumor grade               |                     |                               | 3.057             | 0.080  |
| []                           | 32                  | 14                            |                   |        |
| [1]                          | 35                  | 6                             |                   |        |
| 6. Lymphatic metastasis      |                     |                               | 12.102            | 0.001  |
| Yes                          | 46                  | 5                             |                   |        |
| No                           | 21                  | 15                            |                   |        |
| 7. TNM stage                 |                     |                               | 2.909             | 0.088  |
| []                           | 29                  | 13                            |                   |        |
| [1]                          | 38                  | 7                             |                   |        |

**Figures**
Figure 1

piRNAs expression profiling of NSCLC in Arraystar piRNA Microarray. (A) Box plots revealed the distribution of piRNAs in the five samples after normalization. (B) Hierarchical clustering was performed to reveal the differentially expressed piRNAs between NSCLC and NC. (C) Scatter plot and Volcano plots revealed the differentially expressed piRNAs. Green and red dots represent significantly down and
upregulated piRNAs in NSCLC, respectively (fold change ≥ 5.0, P<0.01). Among them, 1247 and 1453 piRNAs were upregulated and downregulated, respectively.

![Graph showing qRT-PCR analysis of select piRNAs.](image)

**Figure 2**

qRT-PCR analysis of select piRNAs. Validation of selected piRNA by qRT-PCR in 87 NSCLC tissues compared to normal lung tissues. The expression of hsa_piR_001366 (piR-19004) and hsa_piR_026323 (piR-26323) were up-regulated while hsa_piR_031424 (piR-31424) was down-regulated in NSCLC tissues, *P < 0.05.
Expression of piR-1366 in NSCLS tissues and cell lines. (A) ISH showed expression of piR-1366 was positive (78/87) compared with the adjacent normal tissues (negative, 6/87). (B) qRT-PCR showed expression of piR-1366 is higher than the adjacent normal tissues. (C) Compared with the negative group of lymph-node metastasis (LM-), a significantly high level of piR-1366 was detected in the LM-positive group (LM+) by qRT-PCR. (D) up-regulation of piR-1366 was detected using qRT-PCR in A549, H1650 and H460 compared with normal lung epithelial cell BEAC-2B.
Figure 4

PiR-1366 promoted migration and metastasis in LC. (A-B) Overexpressing or silencing of piR-1366 was confirmed into A549 and H1650 cells by qRT-PCR. *P<0.05. (C-D) The cell migration of A549 and H1650 was assessed by Transwell assay. *P<0.05. (E-F) The cell metastasis was assessed by assay of lung metastasis models of nude mice after silencing of piR-1366 in A549. *P<0.05.
Figure 5

piR-19166 targeted directly EPHA2 in PC3 cells. (A) DNA binding site sequence between piR-1366 and the 3'-UTR of WT1. (B) the sequence of wild type (WT) or mutant type (MT) of WT1 binding site and piR-1366 were showed. (C) The effect of piR-NC and piR-1366 on the activity of the luciferase reporter containing either WT or Mut was detected by dual-luciferase reporter assay. (D-E) RNA and protein levels of WT1 were, respectively, tested by qRT-PCR and western blot in A549 cells transfected with piR-1366 or anti-piR-1366 compared with those treated with negative control. *P <0.05.
Figure 6

Rescue assays detected the effects of WT1 regulated by piR-1366 in metastasis. (A-B) RNA and protein levels of WT1 were detected respectively by qRT-PCR and western blot in A549 cells with the presence of WT1 overexpression or vector control and si-WT1 or siRNA control. (C-D) Overexpression of WT1 rescued the effects via piR-1366-induced, whereas knockdown of WT1 simulated the effects associated with piR-1366 through cell migration in A549. Error bars represent the mean±SD of three independent experiments. *P <0.05.
piR-19166 activated WT1 /CDH1 pathways. (A) mRNA levels of WT1 were detected in A549 of piR-1366 overexpressing or silencing compared with the negative control (NC) by qRT-PCR. (B) mRNA levels of CDH1 were detected in A549 of piR-1366 overexpressing or silencing compared with the negative control (NC) by qRT-PCR. (C) Protein levels of WT1 and CDH1 were detected in A549 of piR-1366-overexpressing or piR-1366-silencing compared with the negative control (NC) by western blot. Error bars represent the mean±SD of three independent experiments. *P <0.05.

Supplementary Files

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- TableS1.docx