Original Research

Identification of lymph node metastasis-related microRNAs in lung adenocarcinoma and analysis of the underlying mechanisms using a bioinformatics approach

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Abstract

This study aimed to screen lymphatic metastasis-related microRNAs (miRNAs) in lung adenocarcinoma and explore their underlying mechanisms using bioinformatics. The miRNA expression in primary lung adenocarcinoma, matched adjacent non-tumorigenic and lymph node metastasis tissues of patients were profiled via microarray. The screened metastasis-related miRNAs were then validated using quantitative real-time PCR in a second cohort of lung adenocarcinoma patients with lymphatic metastasis. Significance was determined using a paired *t*-test. Target genes of the metastasis-related miRNAs were predicted using TargetScan, and transcription factors (TFs) were predicted based on the TRANSFAC and ENCODE databases. Furthermore, the related long non-coding RNAs (lncRNAs) were screened with starBase v2.0. The miRNA-TF-mRNA and lncRNA-miRNA-mRNA networks were constructed to determine the key interactions associated with lung adenocarcinoma metastasis. According to the miRNA microarray results, there were 10 miRNAs that were differentially expressed in metastatic tissues compared with primary tumor and adjacent non-tumorigenic tissues. Among them were increased levels of miR-146a-5p, miR-342-3p, and miR-150-5p, which were validated in the second cohort. Based on the miRNA-TF-mRNA network, vascular endothelial growth factor A and transcription factors (TFs) including TP53, SMAD4, and EP300 were recognized as critical targets of the three miRNAs. Interactions involving SNHG16–miR-146a-5p–SMAD4 and RP6-24A23.7–miR-342-3p/miR-150-5p–EP300 were highlighted according to the lncRNA-miRNA-mRNA network. miR-146a-5p, miR-342-3p, and miR-150-5p are lymphatic metastasis-related miRNAs in lung adenocarcinoma. Bioinformatics analyses demonstrated that SNHG16 might inhibit the interaction between miR-146a-5p and SMAD4, while RP6-24A23.7 might weaken miR-342-3p–EP300 and miR-150-5p–EP300 interactions in metastasis.

Keywords: Lung adenocarcinoma, lymphatic metastasis, long non-coding RNA, microRNAs, underlying mechanisms, bioinformatics approach

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Introduction

Pulmonary adenocarcinoma is an admixture of acinar, papillary, solid, and lepidic patterns. It is the leading histological form of lung cancer and presents as the most common cause of cancer-related death with a five-year survival rate lower than 15%.[1,2] Pulmonary adenocarcinoma is asymptomatic in the early stages and, in most cases, progresses to advanced stages with various metastases when diagnosed.[3] These are the primary contributing factors of its poor clinical outcomes. Hematogenous, lymphatic, and transcoelomic spread are the three major mechanisms that are responsible for metastasis in primary lung cancer.[4] It has been reported that the rate of lymphatic metastasis is 32.2% in Chinese patients with lung adenocarcinoma less than 2 cm in diameter[5] and increases to as much as 60.2% in patients with larger tumor size.[6,7] The high incidence and low survival rate associated with pulmonary adenocarcinoma emphasize the need to elucidate the underlying molecular mechanisms of lymph node metastasis to provide a theoretical basis for new diagnostic strategies and treatments.

Lymphatic spread is considered to be a consequence of the invasion of interstitial lymphatic vessels in the lungs.[4] In addition to epithelial–mesenchymal transition (EMT), metastatic competencies of the primary cancer cells, including increased motile and invasive properties, resistance to

hypoxia, cell survival after detachment, enhanced lymphangiogenesis, and evasion of immune surveillance, are essential.[8,9] As a group of short non-coding RNA, microRNA (miRNA) can post-transcriptionally suppress gene expression and thereby affect the disease progression.[10] An accumulation of evidence suggests that miRNAs have a role in metastasis either by promoting or suppressing these associated malignant processes. For instance, miR-483-5p promotes lung adenocarcinoma by down-regulating activated leukocyte cell adhesion molecule (ALCAM) to improve cell motility and invasiveness, and inhibits RhoGDl1 during the EMT process[11]; miR-200 inhibits cell invasion by targeting Flt1/VEGFR1[12]; and miR-31 functions as a biomarker for lymph node metastasis by increasing lung adenocarcinoma cell migration and invasion through ERK1/2.[13] Despite these encouraging findings, data on the contributions of miRNAs in lung adenocarcinoma lymph node metastasis are seriously limited.

Long non-coding RNAs (lncRNAs) have lengths exceeding 200 bp. lncRNAs are critical for the regulation of diseases,[14] such as lung cancer.[15] Recent studies have shown that lncRNAs act as regulatory molecules impacting multiple progresses in the cancer cell cycle. lncRNAs are key competing endogenous RNAs (ceRNAs) with common miRNA recognition elements that regulate the expression of the miRNA-targeted genes by competitively binding to miRNAs.[16] The involvement of metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in the metastasis of non-small cell lung cancer (NSCLC), in particular adenocarcinoma metastasis, is well accepted[17]; however, the functions of other lncRNAs in lung adenocarcinoma metastasis in the lymph nodes remain largely unknown.

Microarray profiling of miRNA expression is useful for determining the global expression patterns of miRNA in developmental processes or diseases.[18] Bioinformatics analysis plays a key role in large-scale expression profiling and lays the groundwork for biological interpretation.[19] Herein, using microarrays, we conducted miRNA expression profiling to comprehensively screen the lymph node metastasis-related miRNA signatures in Chinese Han patients with lung adenocarcinoma. The expression alterations of the top three most significant miRNAs were validated using quantitative real-time PCR (qRT-PCR). Subsequently, miRNA-TF-mRNA and lncRNA-miRNA-mRNA network analyses were performed to predict the key interactions among miRNAs, their target genes, and related lncRNAs. Based on these findings, we discuss the possible mechanisms of miRNA signatures of lung adenocarcinoma lymph node metastasis.

Patients and methods

Clinical samples

Samples of primary tumors, matched adjacent normal tissues and metastatic tissues from 10 lung adenocarcinoma patients (6 females and 4 males; mean age = 56; mean tumor size = 3.22 cm). All patients had lymphatic metastasis (1 patient in T1N2M0 stage, six patients in T2N1M0 stage, two patients in T2N2M0 stage, and one patient in T3N1M0 stage), and all samples were used for miRNA microarray profiling. Metastasis-related miRNAs were validated in tumor tissues isolated from 40 lung adenocarcinoma patients (19 females and 21 males; mean age = 60.70; mean tumor size = 3.51 cm; 12 patients with lymphatic metastasis and 28 patients without lymphatic metastasis; 19 patients in T1N0M0 stage, 1 patient in T1N1M0 stage, 2 patients in T1N2M0 stage, 7 patients in T2N0M0 stage, 2 patients in T2N1M0 stage, 2 patients in T2N2M0 stage, 1 patient in T3N0M0 stage, 1 patient in T3N0M1 stage, 1 patient in T3N1M0 stage, 3 patients in T4N1M0 stage, and 1 patient in T4N2M1 stage) using qRT-PCR. All patients had a histopathologic diagnosis of lung adenocarcinoma with or without lymphatic metastasis according to the 2015 WHO classification.[20] All frozen samples were obtained from the No. 117 Hospital of People's Liberation Army (PLA). This study was approved by the Hospital Ethics Committee and informed consents were obtained from all patients enrolled.

miRNA microarray assay and identification of metastasis related miRNAs

Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quality was assessed using an UV spectrophotometer Q3000 bioanalyzer (Quawell, San Jose, CA). Subsequently, total RNA was quantified using NanoDrop™2000 (Thermo, Waltham, MA).

miRNA microarray profiling was conducted using a service provider (LC Sciences, Houston, TX) employing methods described previously.[21,22] Briefly, 4–8 μg samples of total RNA were fractionated to less than 200 nucleotides and 3′-polyadenylated. Then, an oligonucleotide tag (conjugated with Cy3 dye) was ligated to the poly (A) tail. Hybridization was performed overnight at 34°C on a μParaflo microfluidic chip. A 100 μL 6× SSPE buffer (0.90 mol/L NaCl, 60 mmol/L Na_2HPO_4, 6 mmol/L EDTA, pH 6.8) containing 25% formamide was used.[23] Subsequently, the chip was scanned using a GenePix 4000B laser scanner (Molecular Device, Sunnyvale, CA). The data were then digitized using Media Cybernetics Array-Pro image analysis software (Carlsbad, CA). After background subtraction, the signals were normalized using a Locally weighted Regression (LOWESS) filter.[24]

Metastasis-related miRNAs were defined as differentially expressed miRNAs (DEMs) in metastatic tissues compared with primary tumor and carcinoma adjacent tissues. DEMs were identified using a two-tailed paired *t*-test[25] and defined when P value < 0.05. In the present study, only the top three DEMs were further subjected to further analysis in the following validation experiments.

miRNA verification by qRT-PCR

qRT-PCR was performed using SYBR Premix Ex Taq™ miRNA assays (TAKARA, Tokyo, Japan) and the ABI StepOne Plus Real-time PCR system (ABI PRISM® 7900HT; Applied Biosystems, Foster City, CA). The PCR protocol was as follows: 50°C for 2 min, 95°C for 2 min, 39 cycles of 95°C for 15 s, and 60°C for 30 s. The following

Table 1 Lymph node metastatic lung adenocarcinoma associated miRNAs which were identified by microarray analysis

	Metastatic tissues *vs.* carcinoma adjacent tissues			Metastatic tissues *vs.* primary tumor tissues		
	FC	log_2FC	*P*	FC	log_2FC	*P*
miR-146a-5p	8.47	3.08	1.37E – 06	4.28	2.10	5.29E – 04
miR-342-3p	3.07	1.62	3.79E – 05	2.85	1.51	3.29E – 04
miR-150-5p	6.87	2.78	6.15E – 05	7.95	2.99	9.65E – 05
miR-30a-5p	0.39	−1.35	1.77E – 04	0.48	−1.06	1.76E – 03
miR-125a-5p	0.49	−1.03	7.24E – 04	0.55	−0.86	2.43E – 04
let-7g-5p	1.69	0.76	5.31E – 03	2.74	1.45	2.54E – 03
miR-126-3p	0.57	−0.81	6.45E – 03			
miR-146b-5p	1.99	0.99	7.04E – 03			
miR-29a-3p	1.96	0.97	8.34E – 03	1.71	0.77	4.70E – 03
let-7c-5p	0.71	−0.50	1.02E – 02			
miR-16-5p	1.48	0.57	1.33E – 02	1.41	0.50	1.61E – 02
miR-30b-5p	0.52	−0.94	2.62E – 02	0.45	−1.14	2.72E – 02
miR-23b-3p	0.72	−0.48	2.76E – 02	0.77	−0.38	3.87E – 02
miR-125b-5p	0.49	−1.04	3.71E – 02			
miR-21-5p	3.48	1.80	4.22E – 02			

Differently expressed miRNAs (DEMs) between metastatic and carcinoma adjacent tissue tissues and DEMs between metastatic and primary tumor tissues were screened in 10 lung adenocarcinoma patients with lymph node metastasis by microarray analysis.

primers were used: miR-146a-5p forward primer, 5′-CGGCGGCTGAGAACTGAAT-3′; miR-146a-5p reversed primer, 5′-CAGTGCAGGGTCCGAGGTAT-3′; miR-342-3p forward primer, 5′-GGCGCTCTCACACAGAAATC-3′; miR-342-3p reversed primer, 5′-CAGTGCAGGGTCC GAGGTAT-3′; miR-150-5p forward primer, 5′-GAGCG TCTCCCAACCCTTG-3′; miR-150-5p reversed primer, 5′-CAGTGCAGGGTCCGAGGTAT-3′; RNU6B forward primer, 5′-CAAATTCGTGAAGCGTTCCATA-3′; RNU6B reversed primer, 5′-AGTGCAGGGTCCGAGGTATTC-3′. The miRNA expression levels of each sample were normalized to the internal control (RNU6B), and expressed according to the comparative threshold (Ct) cycle method ($2^{-\Delta Ct}$). Between group differences were assessed using either paired Student's *t*-test or Mann-Whitney test where appropriate.

Bioinformatics analysis

Bioinformatics analyses were performed on the qRT-PCR validated DEMs to predict their underlying mechanisms in metastasis. The analytical strategy was as follows: (1) miRNA target prediction; (2) Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/pathway.html) pathway[26] enrichment analysis of the target genes; (3) TF identification; (4) construction of the miRNA-TF-mRNA network, in which the interactions between miRNA and its target genes, between TF and its target gene, and between encoding genes (based on the protein–protein interaction) were identified; (5) extraction of the miRNA-TF-mRNA sub-network including only the relationships between miRNA-targets and TF-targets; and (6) construction of the lncRNA-miRNA-mRNA network.

In detail, target genes were predicted using TargetScan (version release 6.2, http://www.targetscan.org)[27] target prediction programs. KEGG pathway enrichment analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.Ncifcrf.gov/)[28] with a significance threshold of $P < 0.05$ for all terms. All target genes were mapped onto the TF (TRANSFAC)[29,30] and Encyclopedia of DNA elements (ENCODE) databases to screen the potential TFs. Interactions between the protein products of the target genes were determined using the Search Tool for the Retrieval of Interacting Genes (STRING) database,[31] where a combined score >0.9 was set as the criterion. Interactions between lncRNA and miRNA, and lncRNA and mRNA were determined using starBase v2.0 (http://starbase.sysu.edu.cn/) where the number of supporting experiments ≥2 was set as threshold.

Results

DEMs linked to metastatic lung adenocarcinoma

Transcriptional expression levels of 1570 miRNAs were analyzed in 10 paired samples of lymph node metastasis in primary lung adenocarcinoma, as well as in the adjacent normal lung tissues using microarray technology. As shown in Table 1, eight up-regulated and seven down-regulated miRNAs were identified in metastatic tissues compared with the non-tumorigenic tissues. Among them, the expressions levels of six and four miRNAs were increased and decreased, respectively, in metastatic tissues compared with primary tumor tissues.

To screen the miRNAs that were most specifically linked to metastasis, the top 3 DEMs (miR-146a-5p, miR-342-3p and miR-150-5p) were selected for validation in an additional 40 lung adenocarcinoma cases by qRT-PCR. Consistent with the microarray analysis results, the

expression levels of miR-146a-5p, miR-342-3p, and miR-150-5p were significantly higher in metastatic tissues than those found in the primary tumor tissues ($P < 0.01$) (Table 2).

Table 2 Metastatic lung adenocarcinoma associated miRNAs which were validated by qRT-PCR

	Primary tumor tissues	Metastatic tissues	*P*
miR-146a-5p	0.54 ± 0.34	1.22 ± 0.59	6.59E − 05
miR-342-3p	6.34 ± 4.95	12.78 ± 5.37	5.14E − 05
miR-150-5p	13.44 ± 11.29	29.60 ± 19.69	2.89E − 03

The top 3 differently expressed miRNAs between metastatic and primary tissue were validated in a new cohort of 40 lung adenocarcinoma cases.

Target genes for the metastasis-related miRNAs and enrichement analyses

Totally, miR-146a-5p, miR-342-3p, and miR-150-5p were predicted to be targeted by 225, 284, and 282 genes, respectively. These target genes were found to be enriched in different sets of KEGG pathways associated with cancer (Figure 1), indicating their distinct functions. miR-146a-5p target genes were found to be involved in seven pathways including the neurotrophin signaling pathway, adherens junction, regulation of autophagy, and notch signaling pathways. Target genes for miR-342-3p were enriched in 13 pathways such as ECM-receptor interaction, focal adhesion, endocytosis, TGF-beta signaling, mechanistic target of rapamycin (mTOR) signaling, and calcium signaling pathways, while miR-150-5p was found to be associated with 23 KEGG

Figure 1 Enriched KEGG pathways of the target genes for miR-146a-5p, miR-150-5p and miR-342-3p. Red represents the significantly enriched pathway of the target genes, while black represents the pathway not enriched by the target genes. (A color version of this figure is available in the online journal.)

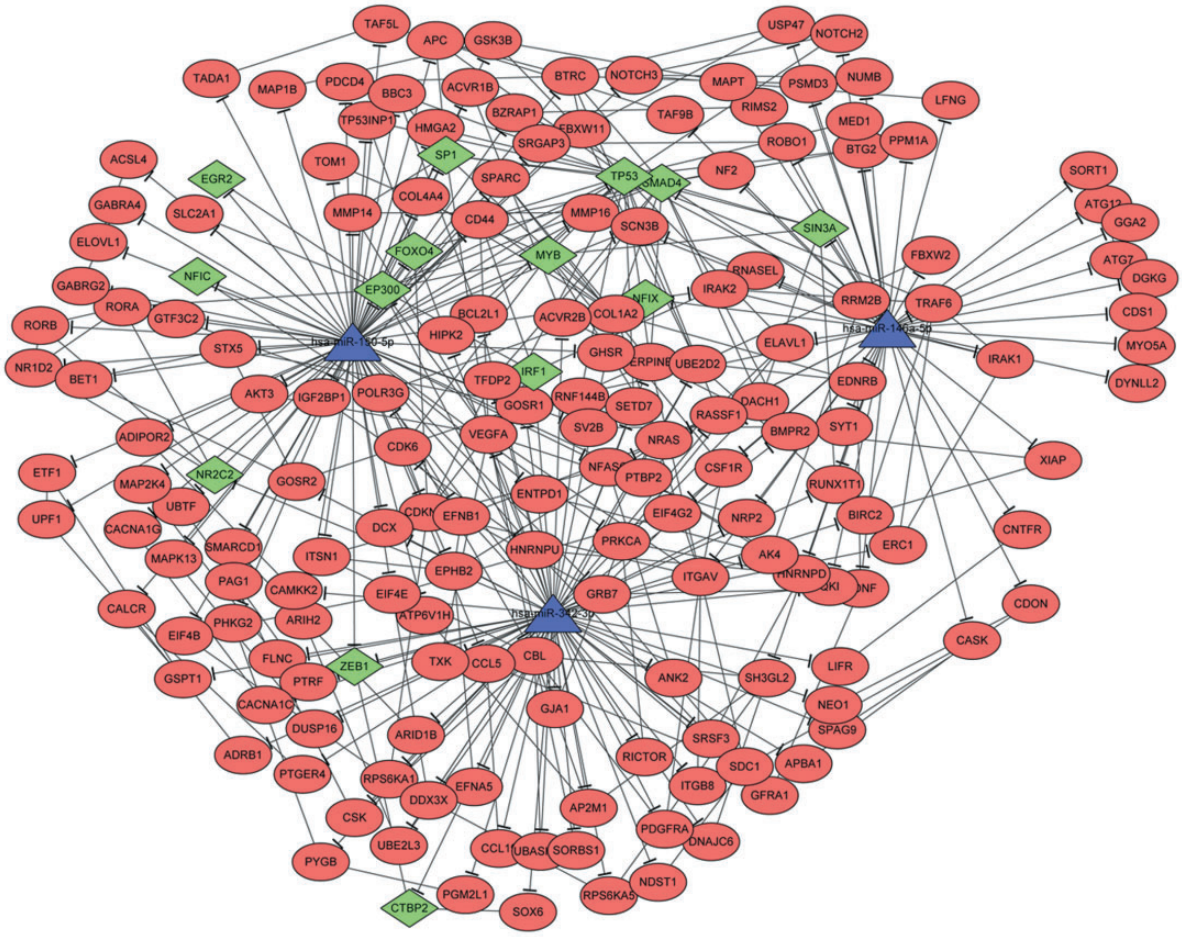

Figure 2 miRNA-TF-target network associated with lung adenocarcinoma metastasis. The interaction network where in miRNAs are represented as purple triangle nodes, transcription factors (TFs) are represented as green diamond nodes and targets of miRNAs or TFs represented as red circle nodes. (A color version of this figure is available in the online journal.)

pathways including the Wnt signaling pathway, cell adhesion molecules, and Toll-like receptor signaling pathway.

Interaction network of miRNAs, TFs, and mRNAs

The miRNA-TF-mRNA network involving three miRNAs, 166 mRNAs, and 14 TFs was constructed (Figure 2). A total of 214 pairs of interactions were identified within this network. Among the genes in the network, TP53 (tumor protein p53, degree = 23), SMAD4 (SMAD family member 4, degree = 14), VEGFA (vascular endothelial growth factor A, degree = 12), CBL (Cbl proto-oncogene, E3 ubiquitin protein ligase, degree = 11), and EP300 (E1A Binding Protein P300, degree = 10) were the top five genes with the highest degrees of interaction in this network, which may have significant roles in tumor metastasis. To further screen the genes most correlated with the three miRNAs and 14 TFs, we extracted a sub-network, in which only interactions of miRNA–mRNA and TF–mRNA were included (Figure 3). Similarly, VEGFA mRNA and TFs including TP53, SMAD4, and EP300 were highly correlated within this sub-network. Additionally, correlations between miR-146a-5p and SMAD4, miRNA-342-3p and EP300, miRNA-342-3p and VEGFA, and miRNA-150-5p and EP300 were found in this sub-network.

Interaction network of lncRNA, miRNA, and mRNA

The lncRNA, miRNA, and mRNA interaction network consisted of 11 pairs of lncRNA–miRNA interactions, nine pairs of miRNA–mRNA interactions, and 65 pairs of lncRNA–mRNA interactions (Figure 4). In addition to the well-recognized MALAT1, 49 lncRNAs were shown within the network. Additionally, five genes (CBL, MYB, SMAD4, EP300 and BTRC) were found to interact with both lncRNAs and miRNAs. Combined with the miRNA-TF-mRNA network results, several interactions of lncRNA, miRNA, and mRNA were highlighted, including SNHG16–miR-146a-5p–SMAD4, RP6-24A23.7–miR-342-3p–EP300, and RP6-24A23.7–miR-150-5p–EP300.

Discussion

In the current report, we identified and validated three miRNAs (miR-146a-5p, miR-342-3p and miR-150-5p) as lymph node metastasis-related miRNAs in pulmonary adenocarcinoma. Subsequently, the mechanisms of miRNA

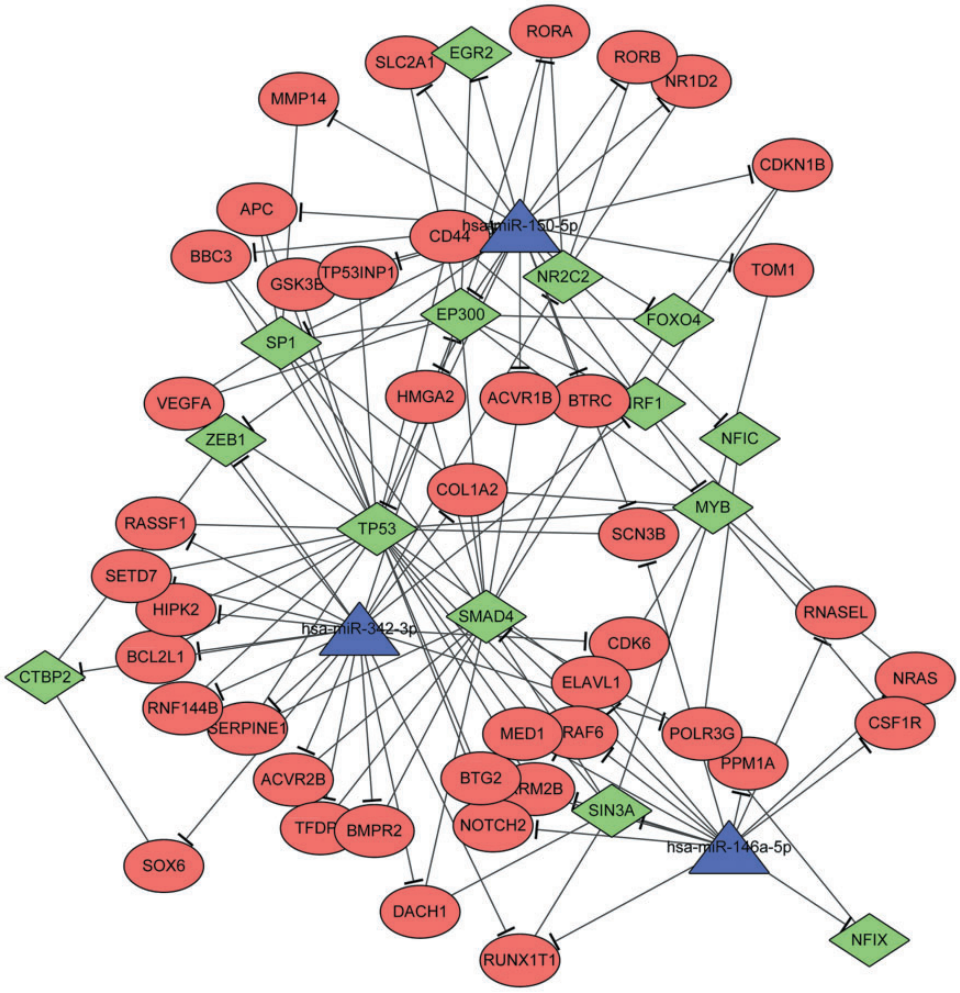

Figure 3 miRNA-TF-target sub-network. The sub-network only included interactions between miRNAs and transcription factors (TFs) and their associated target genes. Purple triangle nodes represent miRNAs, green diamond nodes represent TFs and red circle nodes represent their target genes. (A color version of this figure is available in the online journal.)

dysfunction regulated by miRNA-TF-mRNA and lncRNA-miRNA-mRNA were revealed. The miRNA-TF-mRNA network demonstrated correlations between miR-146a-5p and SMAD4, miRNA-342-3p and EP300, miRNA-342-3p and VEGFA, and miRNA-150-5p and EP300, while the lncRNA-miRNA-mRNA network revealed the interactions of SNHG16–miR-146a-5p–SMAD4 and RP6-24A23.7–miR-342-3p/miR-150-5p–EP300, indicating their significance in lymph node metastasis.

Several studies have investigated the functions of miR-146a/miR-146a-5p, miR-342/miR-342-3p, and miR-150/miR-150-5p in tumor invasion and metastasis; however, their roles vary in different tumor types. It has been reported that the expression of miR-146a in breast and gastric cancer cell lines inhibits cell invasion *in vitro* and results in suppressed distant metastasis in mice,[32,33] implying an inhibitory effect of miR-146a/miR-146a-5p on tumor metastasis. On the other hand, miR-146a-5p was up-regulated in osteosarcoma cell lines with excellent invasive ability, demonstrating that it may also have a role in the process of metastasis.[34] It has been found that the overexpression of miR-342/miR-342-3p in cervical, colorectal and NSCLC cancer cell lines[35–37] and miR-150/miR-150-5p in colorectal cancer, pancreatic cancer, and cutaneous T-cell lymphoma cell lines,[38–40] could attenuate cell invasion *in vitro*. Nevertheless, when the levels of miRNAs in paired primary tumors and metastatic lymph nodes were compared, increases of miR-146a and miR-342-3p expression levels in metastatic bladder cancer, as well as increased miR-150 expression in metastatic bladder and breast cancers were detected.[41] Similarly, our findings revealed higher levels of miR-146a-5p, miR-342-3p, and miR-150-5p in metastatic tissues *vs.* non-metastatic tumor tissues, indicating that these miRNAs might also promote lymph node metastasis in lung adenocarcinoma.

SMAD4, a gene that encodes a member of the SMAD protein family, was predicted to be a target gene of miR-146a-5p. It has been found that low levels of SMAD4 can reflect lymph node metastasis in several cancers, such as esophagus and lung squamous cell carcinomas, pancreatic, and colorectal cancers.[42–45] Using immunohistochemical methods, Bian *et al*. found that TP53 mutant expression correlated with pathological stage and tumor size, and decreased SMAD4 expression correlated with tumor size,

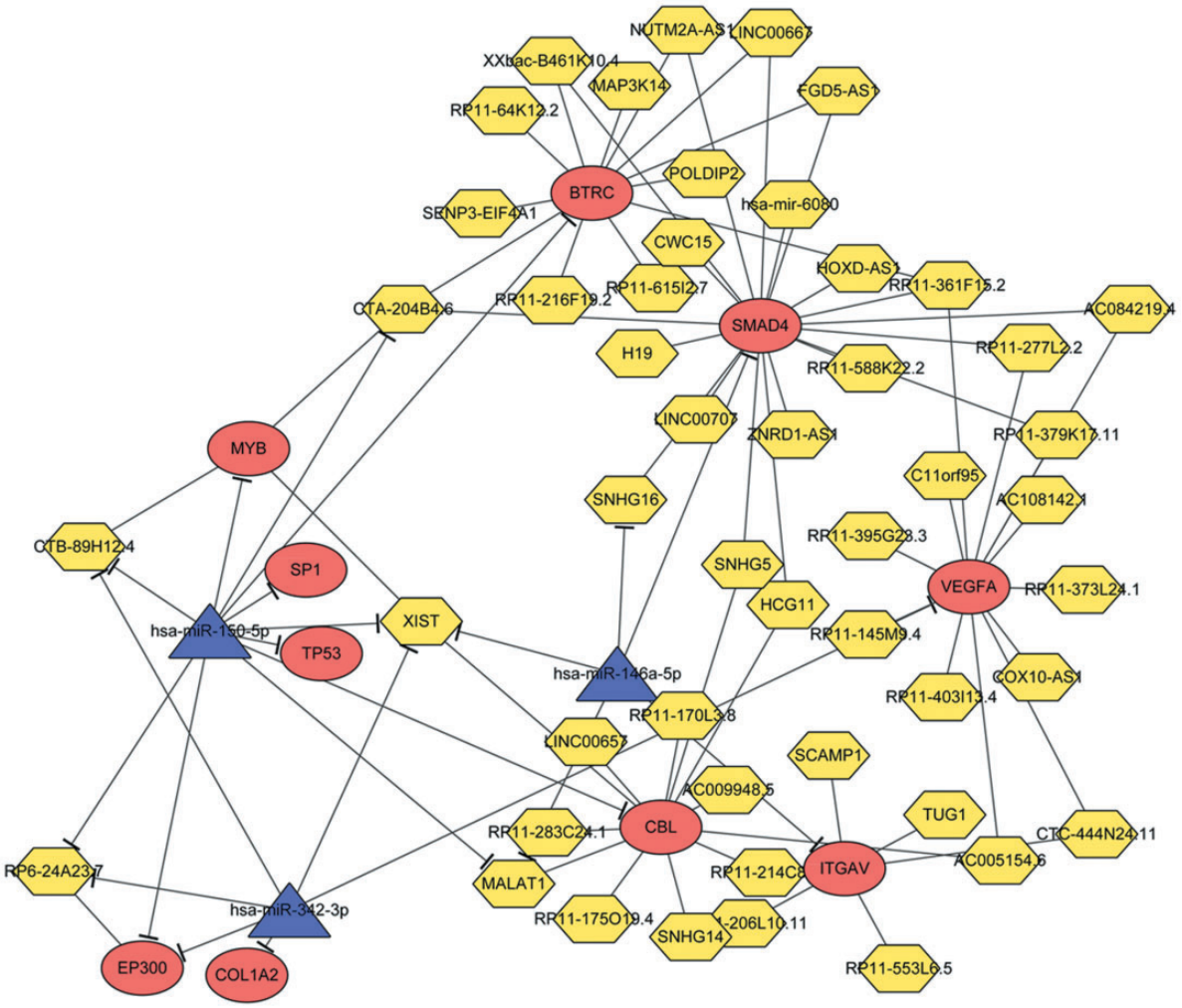

Figure 4 Interaction network of lncRNA, miRNA and mRNA. The interaction network wherein miRNAs are represented as purple triangle nodes, lncRNAs are represented as yellow hexagon nodes and mRNAs represented as red circle nodes. (A color version of this figure is available in the online journal.)

pathological stage, lymph node metastasis, and tumor differentiation in patients with lung adenocarcinoma.[46] SMAD family proteins are signal transducers that mediate TGF-β signaling.[47] Enhanced TGF-β signaling is involved in the induction of the EMT process, which could lead to tumor invasion and metastasis.[48] A recent study reported that via the SMAD pathway, TGF-β may also induce the expression of DOCK4, promoting the lung adenocarcinoma cell extravasation and metastasis without affecting EMT.[49] Combined, these results suggest that miR-146a-5p participates in lymph node metastasis by affecting the TGF-β/Smad signaling pathway, and involving both EMT dependent and independent processes. Furthermore, interactions between SNHG16 (Ensemble cytogenetic band: 17q25.1), miR-146a-5p, and SMAD4 were predicted by the network analysis. Zhu *et al*. demonstrated that SNHG16 was positively correlated with bladder cancer invasion[50]; however, there have been no reports elucidating the underlying molecular mechanisms involved. Based on our findings, we speculate that, in patients with lung adenocarcinoma, SNHG16 might also function as a metastatic suppressor by competitively binding to miR-146a-5p and thereby impair its regulatory effect on SMAD4. To determine the exact mechanisms of miR-146a-5p would require further investigation both *in vitro* and *in vivo*.

The bioinformatic analyses reported in this study revealed that EP300 targeting miRNAs (including miR-342-3p and miR-150-5p) were found to be up-regulated potentially contributing to the occurrence of lymph node metastasis. EP300 acetylase, which regulates the transcription of its target genes via chromatin remodeling, is well recognized as a modulator of cell growth and division in tumors.[51] Using qRT-PCR and Western blot analyses, Mees *et al*. detected decreases in EP300 mRNA and protein expression in highly metastatic cell lines of ductal adenocarcinomas of the pancreas, and demonstrate that EP300 may act as a distant liver and lymph node metastasis suppressor.[52] However, its role in lung adenocarcinoma is uncertain. The miRNA-TF-mRNA network analysis demonstrated that EP300 interacts with VEGFA and SP1. VEGFA is a member of the VEGF family, which is responsible for the spread of cancer cells via lymphatic vessels.

Niki *et al*. reported that VEGFA was essential for the growth of metastatic tumors via vasculogenesis and angiogenesis and was not involved in the metastasis of pulmonary adenocarcinoma cells into lymph vasculature.[53] Another study showed that exogenous treatment with VEGFA potentiated the migration and invasion abilities of lung adenocarcinoma cell line via FLJ10540.[54] Whether EP300–VEGFA is involved in miR-342-3p and miR-150-5p induced lymphatic metastasis requires further investigation. SP1 is also an invasion and metastasis-related gene found in many cancers such as breast and gastric cancers[55,56]; however, its involvement in the lymphatic metastasis of lung adenocarcinoma and its interactions with miR-342-3p/miR-150-5p and EP300 are unknown. RP6-24A23.7 was predicted to be a ceRNA of miR-342-3p and miR-150-5p in the regulation of EP300. Functional analysis of RP6-24A23.7 in tumor metastasis, however, has not yet been conducted.

Based on miRNA microarray profiling and bioinformatic analysis, various hypotheses regarding the mechanisms of lymphatic metastasis-related miRNAs have been proposed. The limitation of the present study was the lack of experimental evidence to either prove or disprove these hypotheses. Detection of expression alterations in larger sample sizes of clinical tissues, invasion and motility assays *in vitro*, luciferase assays to investigate the interactions, tumor growth and metastasis evaluation and determination of expression levels of the possible involved genes *in vivo* by up- or down-regulating miRNAs/lncRNAs, should be performed in future studies.

Taken together, we performed a global miRNA expression profile to screen lymph node metastases-related miRNAs in lung adenocarcinoma. Using qRT-PCR, we found that three miRNAs (miR-146a-5p, miR-342-3p and miR-150-5p) were positively correlated with metastasis. Furthermore, miR-146a-5p may be involved in lymph node metastasis by affecting the TGF-β/Smad signaling pathway. SNHG16 might function as a metastatic suppressor by competitively binding to miR-146a-5p. In addition, miR-342-3p and miR-150-5p may have a common mechanism mediated by EP300; RP6-24A23.7 may act as the ceRNA. However, these hypotheses need to be tested both *in vitro* and *in vivo*. Identification of metastatic miRNAs provides new insights into the molecular mechanisms of lung adenocarcinoma metastasis. The miRNAs may serve as the ideal diagnostic and therapeutic biomarkers.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; LY, DJ, and QC conducted the experiments, HH, JW, XT, and JC analyses of the data, LY, DJ, and QC wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Kamiya K, Hayashi Y, Douguchi J, Hashiguchi A, Yamada T, Izumi Y, Watanabe M, Kawamura M, Horinouchi H, Shimada N, Kobayashi K, Sakamoto M. Histopathological features and prognostic significance of the micropapillary pattern in lung adenocarcinoma. *Mod Pathol* 2008;**21**:992–1001
2. Youlden DR, Cramb SM, Baade PD. The international epidemiology of lung cancer: geographical distribution and secular trends. *J Thorac Oncol* 2008;**3**:819–31
3. Xie L, Yang Z, Li G, Shen L, Xiang X, Liu X, Xu D, Xu L, Chen Y, Tian Z, Chen X. Genome-wide identification of bone metastasis-related microRNAs in lung adenocarcinoma by high-throughput sequencing. *PloS One* 2013;**8**:e61212
4. Gaikwad A, Souza CA, Inacio JR, Gupta A, Sekhon HS, Seely JM, Dennie C, Gomes MM. Aerogenous metastases: a potential game changer in the diagnosis and management of primary lung adenocarcinoma. *AJR Am J Roentgenol* 2014;**203**:W570–82
5. Wei L, Zhao S, Chen M, Chen Z, Chen X. [Clinical research on surgical treatment for non-small cell lung cancer of diameter less than 2 cm]. *Zhonghua yi xue za zhi* 2013;**93**:2502–4
6. Liu S, Li Y, Qi W, Zhao Y, Huang A, Sheng W, Lei B, Lin P, Zhu H, Li W, Shen H. Expression of Tiam1 predicts lymph node metastasis and poor survival of lung adenocarcinoma patients. *Diagn Pathol* 2014;**9**:69
7. Chao L, Yi-Sheng H, Yu C, Li-Xu Y, Xin-Lan L, Dong-Lan L, Jie C, Yi-Lon W, Hui LY. Relevance of EGFR mutation with micropapillary pattern according to the novel IASLC/ATS/ERS lung adenocarcinoma classification and correlation with prognosis in Chinese patients. *Lung Cancer* 2014;**86**:164–9
8. Bernards R, Weinberg RA. A progression puzzle. *Nature* 2002;**418**:823
9. Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* 2009;**9**:274–84
10. Wong KY, Huang X, Chim CS. DNA methylation of microRNA genes in multiple myeloma. *Carcinogenesis* 2012;**33**:1629–38
11. Song Q, Xu Y, Yang C, Chen Z, Jia C, Chen J, Zhang Y, Lai P, Fan X, Zhou X, Lin J, Li M, Ma W, Luo S, Bai X. miR-483-5p promotes invasion and metastasis of lung adenocarcinoma by targeting RhoGDI1 and ALCAM. *Cancer Res* 2014;**74**:3031–42
12. Roybal JD, Zang Y, Ahn YH, Yang Y, Gibbons DL, Baird BN, Alvarez C, Thilaganathan N, Liu DD, Saintigny P, Heymach JV, Creighton CJ, Kurie JM. miR-200 Inhibits lung adenocarcinoma cell invasion and metastasis by targeting Flt1/VEGFR1. *Mol Cancer Res* 2011;**9**:25–35
13. Meng W, Ye Z, Cui R, Perry J, Dedousi-Huebner V, Huebner A, Wang Y, Li B, Volinia S, Nakanishi H, Kim T, Suh SS, Ayers LW, Ross P, Croce CM, Chakravarti A, Jin VX, Lautenschlaeger T. MicroRNA-31 predicts the presence of lymph node metastases and survival in patients with lung adenocarcinoma. *Clin Cancer Res* 2013;**19**:5423–33
14. Wapinski O, Chang HY. Long noncoding RNAs and human disease. *Trends Cell Biol* 2011;**21**:354–61
15. Thai P, Statt S, Chen CH, Liang E, Campbell C, Wu R. 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* 2013;**49**:204–11
16. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell* 2011;**146**:353–8
17. Ji P, Diederichs S, Wang W, Böing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E. MALAT-1, a novel noncoding RNA, and thymosin β4 predict metastasis and survival in early-stage non-small cell lung cancer. *Oncogene* 2003;**22**:8031–41
18. Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA* 2005;**11**:241–7

19. Zhang Y, Szustakowski J, Schinke M. Bioinformatics analysis of microarray data. *Cardiovasc Genom* 2009;**573**:259–84
20. Travis WD, Brambilla E, Nicholson AG, Yatabe Y, Austin JH, Beasley MB, Chirieac LR, Dacic S, Duhig E, Flieder DB. The 2015 World Health Organization classification of lung tumors: impact of genetic, clinical and radiologic advances since the 2004 classification. *J Thorac Oncol* 2015;**10**:1243–60
21. Lin F, Li R, Pan ZX, Zhou B, Yu de B, Wang XG, Ma XS, Han J, Shen M, Liu HL. miR-26b promotes granulosa cell apoptosis by targeting ATM during follicular atresia in porcine ovary. *PloS One* 2012;**7**:e38640
22. Ma Y, Zhang P, Wang F, Zhang H, Yang J, Peng J, Liu W, Qin H. miR-150 as a potential biomarker associated with prognosis and therapeutic outcome in colorectal cancer. *Gut* 2012;**61**:1447–53
23. Gao X, Gulari E, Zhou X. In situ synthesis of oligonucleotide microarrays. *Biopolymers* 2004;**73**:579–96
24. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003;**19**:185–93
25. Markou A, Sourvinou I, Vorkas P, Yousef G, Lianidou E. Clinical evaluation of microRNA expression profiling in non small cell lung cancer. *Lung Cancer* 2013;**81**:388–96
26. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 2000;**28**:27–30
27. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;**120**:15–20
28. Huang da W, Sherman BT, Tan Q, Collins JR, Alvord WG, Roayaei J, Stephens R, Baseler MW, Lane HC, Lempicki RA. The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. *Genome Biol* 2007;**8**:R183
29. Jia AY, Castillo-Martin M, Bonal DM, Sanchez-Carbayo M, Silva JM, Cordon-Cardo C. MicroRNA-126 inhibits invasion in bladder cancer via regulation of ADAM9. *Br J Cancer* 2014;**110**:2945–54
30. Matys V, Fricke E, Geffers R, Gossling E, Haubrock M, Hehl R, Hornischer K, Karas D, Kel AE, Kel-Margoulis OV, Kloos DU, Land S, Lewicki-Potapov B, Michael H, Munch R, Reuter I, Rotert S, Saxel H, Scheer M, Thiele S, Wingender E. TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res* 2003;**31**:374–8
31. Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, Lin J, Minguez P, Bork P, von Mering C, Jensen LJ. STRING v9.1: protein–protein interaction networks, with increased coverage and integration. *Nucleic Acids Res* 2012;**41**:D808–D15
32. Hurst DR, Edmonds MD, Scott GK, Benz CC, Vaidya KS, Welch DR. Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis. *Cancer Res* 2009;**69**:1279–83
33. Zhou L, Zhao X, Han Y, Lu Y, Shang Y, Liu C, Li T, Jin Z, Fan D, Wu K. Regulation of UHRF1 by miR-146a/b modulates gastric cancer invasion and metastasis. *FASEB J* 2013;**27**:4929–39
34. Lauvrak S, Munthe E, Kresse S, Stratford E, Namløs H, Meza-Zepeda L, Myklebost O. Functional characterisation of osteosarcoma cell lines and identification of mRNAs and miRNAs associated with aggressive cancer phenotypes. *Br J Cancer* 2013;**109**:2228–36
35. Wang H, Wu J, Meng X, Ying X, Zuo Y, Liu R, Pan Z, Kang T, Huang W. MicroRNA-342 inhibits colorectal cancer cell proliferation and invasion by directly targeting DNA methyltransferase 1. *Carcinogenesis* 2011;**32**:1033–42
36. Li X-r, Chu H-j, Lv T, Wang L, Kong S-f, Dai S-z. miR-342-3p suppresses proliferation, migration and invasion by targeting FOXM1 in human cervical cancer. *FEBS Lett* 2014;**588**:3298–307
37. Xie X, Liu H, Wang M, Ding F, Xiao H, Hu F, Hu R, Mei J. miR-342-3p targets RAP2B to suppress proliferation and invasion of non-small cell lung cancer cells. *Tumour Biol* 2015;**36**:5031–8
38. Ito M, Teshima K, Ikeda S, Kitadate A, Watanabe A, Nara M, Yamashita J, Ohshima K, Sawada K, Tagawa H. MicroRNA-150 inhibits tumor invasion and metastasis by targeting the chemokine receptor CCR6, in advanced cutaneous T-cell lymphoma. *Blood* 2014;**123**:1499–511
39. Wang WH, Chen J, Zhao F, Zhang BR, Yu HS, Jin HY, Dai JH. MiR-150-5p suppresses colorectal cancer cell migration and invasion through targeting MUC4. *Asian Pac J Cancer Prev* 2014;**15**:6269–73
40. Srivastava SK, Bhardwaj A, Singh S, Arora S, Wang B, Grizzle WE, Singh AP. MicroRNA-150 directly targets MUC4 and suppresses growth and malignant behavior of pancreatic cancer cells. *Carcinogenesis* 2011;**32**:1832–9
41. Baffa R, Fassan M, Volinia S, O'Hara B, Liu CG, Palazzo JP, Gardiman M, Rugge M, Gomella LG, Croce CM. MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. *J Pathol* 2009;**219**:214–21
42. Jiang H, He C, Geng S, Sheng H, Shen X, Zhang X, Li H, Zhu S, Chen X, Yang C. RhoT1 and Smad4 are correlated with lymph node metastasis and overall survival in pancreatic cancer. *PloS One* 2012;**7**:e42234
43. Natsugoe S, Xiangming C, Matsumoto M, Okumura H, Nakashima S, Sakita H, Ishigami S, Baba M, Takao S, Aikou T. Smad4 and transforming growth factor beta1 expression in patients with squamous cell carcinoma of the esophagus. *Clin Cancer Res* 2002;**8**:1838–42
44. Tanaka T, Watanabe T, Kazama Y, Tanaka J, Kanazawa T, Kazama S, Nagawa H. Loss of Smad4 protein expression and 18qLOH as molecular markers indicating lymph node metastasis in colorectal cancer—a study matched for tumor depth and pathology. *J Surg Oncol* 2008;**97**:69–73
45. Liu J, Cho S-N, Akkanti B, Jin N, Mao J, Long W, Chen T, Zhang Y, Tang X, Wistub II. ErbB2 pathway activation upon Smad4 loss promotes lung tumor growth and metastasis. *Cell Rep* 2015;**10**:1599–613
46. Bian C, Li Z, Xu Y, Wang J, Xu L, Shen H. Clinical outcome and expression of mutant P53, P16, and Smad4 in lung adenocarcinoma: a prospective study. *World J Surg Oncol* 2015;**13**:1
47. Xu P, Liu J, Derynck R. Post-translational regulation of TGF-β receptor and Smad signaling. *FEBS Lett* 2012;**586**:1871–84
48. Katsuno Y, Lamouille S, Derynck R. TGF-β signaling and epithelial–mesenchymal transition in cancer progression. *Curr Opin Oncol* 2013;**25**:76–84
49. Yu J-R, Tai Y, Jin Y, Hammell MC, Wilkinson JE, Roe J-S, Vakoc CR, Van Aelst L. TGF-β/Smad signaling through DOCK4 facilitates lung adenocarcinoma metastasis. *Genes Dev* 2015;**29**:250–61
50. Zhu Y, Yu M, Li Z, Kong C, Bi J, Li J, Gao Z, Li Z. ncRAN, a newly identified long noncoding RNA, enhances human bladder tumor growth, invasion, and survival. *Urology* 2011;**77**:510:e1–e5
51. Gayther SA, Batley SJ, Linger L, Bannister A, Thorpe K, Chin SF, Daigo Y, Russell P, Wilson A, Sowter HM, Delhanty JD, Ponder BA, Kouzarides T, Caldas C. Mutations truncating the EP300 acetylase in human cancers. *Nat Genet* 2000;**24**:300–3
52. Mees ST, Mardin WA, Wendel C, Baeumer N, Willscher E, Senninger N, Schleicher C, Colombo-Benkmann M, Haier J. EP300ndel C, regulated metastasis suppressor gene in ductal adenocarcinomas of the pancreas. *Int J Cancer* 2010;**126**:114–24
53. Niki T, Iba S, Tokunou M, Yamada T, Matsuno Y, Hirohashi S. Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. *Clin Cancer Res* 2000;**6**:2431–9
54. Chen C-H, Lai J-M, Chou T-Y, Chen C-Y, Su L-J, Lee Y-C, Cheng T-S, Hong Y-R, Chou C-K, Whang-Peng J. VEGFA upregulates FLJ10540 and modulates migration and invasion of lung cancer via PI3K/AKT pathway. *PLoS One* 2009;**4**:e5052
55. Qiu T, Zhou X, Wang J, Du Y, Xu J, Huang Z, Zhu W, Shu Y, Liu P. MiR-145, miR-133a and miR-133b inhibit proliferation, migration, invasion and cell cycle progression via targeting transcription factor Sp1 in gastric cancer. *FEBS Lett* 2014;**588**:1168–77
56. Kong L-M, Liao C-G, Zhang Y, Xu J, Li Y, Huang W, Zhang Y, Bian H, Chen Z-N. A regulatory loop involving miR-22, Sp1, and c-Myc modulates CD147 expression in breast cancer invasion and metastasis. *Cancer Res* 2014;**74**:3764–78

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