Original Paper

Quantitative Proteomic Analysis of the Metastasis-Inhibitory Mechanism of miR-193a-3p in Non-Small Cell Lung Cancer

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Key Words

MiR-193a-3p • NSCLC • iTRAQ • Proteome

Abstract

Background: microRNAs can repress the expression of target genes by destabilizing their mRNAs or by inhibiting their translation. Our previous findings suggested that miR-193a-3p inhibited the progression of NSCLC both *in vitro* and *in vivo*. However, the biological processes and molecular pathways through which this miRNA exerts its positive effects are unknown.

Methods: To explore the molecular mechanisms by which miR-193a-3p inhibited NSCLC metastasis, we investigated the changes in the protein profile of SPC-A-1sci (highly metastatic) cells in response to the up-regulation of miR-193a-3p expression using a proteomics approach (iTRAQ combined with NanoLC-MS/MS). Changes in the profiles of the expressed proteins were verified using western blotting and were analyzed using the DAVID and STRING programs. **Results:** In the two replicated experiments, 4962/4946 proteins were identified, and the levels of expression of 4923/4902 proteins were quantified. In total, 112 of these proteins were differentially expressed. Among them, the up-regulated levels of expression of two of the 62 proteins with up-regulated expression (PPP2R2A and GSN) and the down-regulated levels of expression four of the 50 proteins with down-regulated expression (LMNB2, UHRF1, G3BP1, and HNRNPU) were verified using western blotting. The bioinformatics analysis revealed the interactions and signaling networks of these differentially expressed proteins. **Conclusion:** miR-193a-3p inhibited the metastasis of lung cancer cells by deregulating the expression of tumor-related proteins. These findings may improve the understanding of the molecular mechanisms underlying the metastatic-inhibitory effect of miR-193a-3p on lung cancer cells.

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Introduction

Lung cancer is the most prevalent cancer in humans and is the leading cause of cancer-related deaths worldwide [1]. Although diagnostic and treatment methods have recently markedly improved, the 5- and 10-year survival rates remain at <15% and <7%, respectively [2]. Lung cancer has the lowest survival outcomes due to the very high metastatic potential of these cancers. The majority of the mortalities due to lung cancers are attributable to tumor metastases [3]. Because the majority of lung cancer patients present with invasive, metastatic disease, it is vital to understand the molecular mechanisms underlying metastasis [4].

In our previous study, the highly metastatic cell line SPC-A-1sci was isolated from its parental SPC-A-1 cell line after several cycles of *in vivo* selection in NOD/SCID mice [5]. Proteomic approaches, which enable relatively comprehensive global analyses, have been widely used in studying complex biological functions [6, 7]. The isobaric tags for relative and absolute quantitation (iTRAQ) method combined with nano liquid chromatography-mass spectrometry (NanoLC-MS/MS), which was developed for protein quantitation, is a high-throughput quantitative technique [8, 9].

MicroRNAs (miRNA) are endogenous ~23-nucleotide non-coding RNA molecules that regulate the expression protein-coding genes through degradation of the target mRNAs or inhibition of protein translation [10, 11]. Emerging evidence shows that miRNAs are implicated in various biological processes, such as proliferation, apoptosis, cellular migration, and differentiation. It was demonstrated that dysregulation of miRNA expression caused abnormal gene expression in a variety of cancers [12, 13]. In the field of miRNA research, the most important step is identifying the multiple target genes of miRNAs. Computational programs have been applied to predict the targets of miRNAs. However, these computational programs have inherent limitations, such as not considering the evidence for functional targeting within the 5'-untranslated region and protein-coding region [14-16] and not adjusting the predictions according to the co-expression of an miRNA and a target, which has been proposed to be an effective way of improving the accuracy of predictions [17], so that many of the predicted targets may be false and many genuine targets could be missed [18].

In the present study, with the aim of improving the understanding of the underlying molecular mechanisms underlying the metastasis-inhibitory effect of miR-193a-3p on lung cancer, a strategy that combined iTRAQ with NanoLC-MS/MS was utilized to analyze the alterations in the protein profile of the lung adenosquamous carcinoma cell line SPC-A-1sci following miR-193a-3p treatment.

Materials and Methods

Cell lines and cell culture

The highly metastatic human lung-cancer cell line SPC-A-1sci was established in our laboratory during a previous study through *in vivo* selection of clones of the cell line SPC-A-1, which has a low metastatic potential, grown in NOD/SCID mice [5]. The NSCLC cell line SPC-A-1sci was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Biowest, South America Origin), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO), and 100 µg/mL streptomycin (Sigma-Aldrich) in a humid chamber at 37°C in 5% CO₂.

Creation of stably transfected cell lines

The miRNA mimic and the negative control miRNA of hsa-miR-193a-3p and the lentiviral vector were purchased from the GenePharma Company. The sequence of the miRNA mimic was 5'-AACTGGCCTACAAAGTCCCAGT-3', and that of the miR-control was 5'-TTCTCCGAACGTGTCACGT-3'.

Stably transfected cell lines were generated by transfecting SPC-A-1sci cells with the miRNA mimic or the miR-control lentiviral vector.

RNA Extraction and Quantitative Real-time PCR Assays

The total RNA was extracted from cultured cells using a mirVana™ miRNA isolation Kit (Ambion) according to the manufacturer's protocol. The concentration and purity of the RNA were determined using ultraviolet spectrophotometry using the Nanodrop 2000 system (Thermo, Japan). To evaluate the miR-193a-3p expression levels, quantitative real-time PCR was performed using TaqMan miRNA assay kits (Applied Biosystems) according to the manufacturer's instructions. U6 mRNA was used as an endogenous reference, and its levels were used for normalization.

Protein Preparation

Harvested cells were washed three times using ice-cold PBS and were disrupted using a cell lysis buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitors for 45 min on ice, followed by centrifugation at 12000 ×g for 15 min at 4°C. Next, the protein concentrations was determined using a bicinchoninic acid (BCA) protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as the standard.

iTRAQ Labeling and High-pH RPLC Fractionation

The iTRAQ Reagent 4-Plex kit (Applied Biosystems, Foster City, CA) was used according to the manufacturer's protocol to label peptides. Equal amounts of protein (100 µg per sample) obtained from the stably transfected cell lines (SPC-A-1sci NC and SPC-A-1sci miRNA mimic lines) were labeled using iTRAQ labeling reagents 116 and 117, respectively. Briefly, the proteins in each sample were reduced using tris-(2-carboxyethyl) phosphine (TCEP) and were alkylated using methyl methane-thiosulfonate (MMTS). Afterward, the samples were digested overnight at 37 °C using trypsin (mass spectrometry grade, Promega, Madison, WI) at a trypsin:protein ratio of 1:20 (W/W). Then, the tryptic peptides were labeled using iTRAQ reagents. The iTRAQ-labeled samples were combined in a fresh tube, desalted using Oasis HLB cartridges (Waters, Milford, MA, USA) and dried using a vacuum centrifuge (Concentrator Plus, Eppendorf, Germany). The iTRAQ-labeled peptides were fractionated using an Agilent 1290 system using a Durashell C18 column (4.6×250 mm, 5 µm, 100 Å, Agela technologies, China) for high-pH RP chromatography. A total of 20 RP fractions were collected and were then desalted using C18 cartridges (UltraMicroSpin, The Nest Group, Southborough, MA), after which they were dried and were reconstituted using 20 µL of 0.1% FA for NanoLC-MS/MS analysis.

NanoLC-MS/MS Analysis

The RP fractions were analyzed in duplicate using NanoLC-MS/MS using a NanoLC system (NanoLC-2D Ultra, Eksigent, Dublin, CA) connected to a Triple TOF 5600 mass spectrometer (AB SCIEX, USA). Briefly, the peptides were enriched on an RP Trap column (ProteoPepC18 column, 5 µm, 300 Å, 0.15×25 mm, IntegraFrit, New Objective, Woburn, MA) and were then eluted onto an analytical column (ProteoPepC18 column, 5 µm, 300 Å, 0.075 ×150 mm, IntegraFrit, Woburn, MA) and were separated using a 120-min gradient of 5 to 35% Buffer B (98% ACN, 2% H₂O, 0.1% FA) at a flow rate of 300 nL/min. The MS analysis was performed using a nano ion spray voltage that was typically maintained at 2.3 kV and a scan range of 350 to 1500 (m/z) in the positive-ion mode. Full-scan MS spectra were acquired from 40 precursors that had been selected for MS/MS from the m/z 100–1500 range using a dynamic exclusion setting of 30 s. The IDA CE parameter script, which selected up to 40 precursors with charge states of 2+ to 4+, automatically controlled the collision energy (CE). The mass spectrometer was calibrated using the tryptic peptides of a beta-galactosidase (BG).

Protein Identification and Quantitation

Analysis of the proteins was performed using ProteinPilot4.1 software (AB SCIEX, USA). The search parameters were as follows: (1) sample type, iTRAQ 4-plex (Peptide Labeled); (2) cysteine alkylation, MMTS; (3) digestion, trypsin; (4) instrument, Triple TOF5600; (5) special factors, none; (6) species, Homo sapiens; (7) ID Focus, biological modifications; (8) database, UniProtKB/Swiss-Prot FASTA (which was released in November, 2013 and consists of 176592 human sequences); and (9) search effort, Thorough ID. In the iTRAQ quantitation, the Pro Group algorithm had been automatically selected to calculate the reporter-peak areas. To estimate the FDR for peptide identification, a decoy database search strategy was adopted. For this study, a strict unused confidence score of >1.3 were used as the qualification criteria, which corresponded to

a peptide confidence level of 95%. If the iTRAQ ratios were >1.5 or <0.67 in the samples obtained from the highly metastatic SPC-A-1sci cells containing miR-193a-3p relative to those of the miR-control, the proteins were considered to be differentially expressed.

Western Blotting (WB)

Protein samples of the stably transfected cell lines SPC-A-1sci miR-control and SPC-A-1sci miRNA mimic were harvested. Twenty micrograms of protein/well was loaded on 8% gels for separation using sodium dodecyl sulfate–polyacrylamide gels electrophoresis (SDS–PAGE) and subsequent electroblotting onto NC membranes (Millipore, Billerica, MA, USA). The membranes were then blocked for one hour using 5 % nonfat dry milk in PBS-T (0.05 % Tween-20 in PBS). After blocking, the membranes were rinsed and were incubated with rabbit anti-PPP2R2A (1:100), rabbit anti-G3BP1 (1:500) and rabbit anti-UHRF1 (1:250) antibodies purchased from Proteintech™; rabbit anti-GSN (1:500) antibodies purchased from Cell Signaling Technology and rabbit anti-HUNRNPU (1:50) antibodies purchased from Abcam; mouse anti-LMNB2 (1:20) antibodies purchased from Santa Cruz Biotechnology. HRP-conjugated anti-goat IgG (1:20000) purchased from Sigma-Aldrich was used as a secondary antibody for an incubation period of 1 h. The labeled bands were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Japan), and the β -actin levels were used as the loading controls.

Data Analysis

STRING (<http://string.embl.de>) is a database of predicted signaling networks and protein interactions. Ontological analysis of the dysregulated genes was performed using the search tool DAVID (<http://david.abcc.ncifcrf.gov/>).

Statistical Analysis

The levels of miR-193a-3p expression, determined using real-time PCR, and the differences in the protein profiles of the groups of cells were compared using the t-test, using SPSS 19.0 software. A P-value of less than 0.05 was considered significant.

Results

Comparative Proteomics of SPC-A-1sci miRNA mimic Cells versus SPC-A-1sci miR-control Cells

We created stably transfected cell lines by transfecting SPC-A-1sci cells with the miRNA mimic/miR-control lentiviral vectors. The extracted RNA was used to detect the levels of miR-193a-3p expression (Fig. 1A). The proteins were extracted from the two cell lines. The samples were digested using trypsin and labeled using the 116 and 117 iTRAQ tags, respectively. The labeled digests were utilized for MS analysis (Fig. 1B). Using ProteinPilot 4.1, we identified a total of 6127/6118 proteins in these two cell lines (global FDR of $<1\%$). Using the filters of an unused protein score of >1.3 and a number of peptides of ≥ 2 , 4962/4946 proteins were identified and the amounts of 4923/ 4902 proteins were quantified. The labelling rate was 99.2% (4923/4962) and 99.1% (4902/4946) in the two runs (Table 1). In total, we identified 62 up-regulated (with 117:116 iTRAQ ratios of >1.5 in the two runs) and 50 down-regulated proteins (with 117:116 iTRAQ ratios of <0.67 in the two runs) in SPC-A-1sci miRNA mimic cells compared with the SPC-A-1sci miR-control cells (Fig. 1C and Tables 2 and 3).

Table 1. Protein numbers of identified and quantitative by iTRAQ

Experiment NO.	Lobal FDR $<1\%$	Unused protein score(>1.3) and peptides(≥ 2)	Labeled protein	lable ratio
1	6127	4962	4923	99.20%
2	6118	4946	4902	99.10%

Fig. 1. Experimental workflow for the quantitative proteomic analysis and the results. (A) SPC-A-1sci cells were transfected with a miR-control lentiviral vector or a miR-193a-3p-mimic lentiviral vector. The miRNA was extracted from cultured cells using the mirVana miRNA Isolation Kit (Ambion, Carlsbad, CA, USA) and was quantified using probes in TaqMan miRNA assays (Applied Biosystems, Carlsbad, CA, USA) performed according to the manufacturer's instructions. (B) The proteins of the cell lines that were stably transfected with the miR-control and the miR-mimic were digested with trypsin and were labeled using 116/117 ITRAQ tags, respectively. The labeled digests were separated using offline SCX LC and were analyzed using Nano LC-MS/MS. The differentially expressed proteins were evaluated using western blotting and were analyzed through database searches. (C) In total, 112 proteins were identified, including 62 up-regulated proteins and 50 down-regulated proteins, in two physical replicates.

Cluster Analysis of the miR-193a-3p-regulated Proteins

The heatmap contains the data obtained for the 112 dysregulated proteins. The two horizontal clusters represent the technical replicates. The clustering of the globally dysregulated proteins in the miR-193a-3p-overexpressing cells may provide direct evidence that the genes encoding these differentially expressed proteins are regulated by miR-193a-3p (Fig. 2).

Evaluation of iTRAQ Results using Western Blotting

Based on the results of the MS analysis, the levels of expression of six of the dysregulated proteins was validated using western blotting analysis of proteins of the SPC-A-1sci miR-193a-3p mimic cells and the SPC-A-1sci miRNA negative control cells. The expression levels of four proteins (HNRNPU, G3BP1, UHRF1, and LMNB2) were down-regulated (Fig. 3A) and the expression levels of two proteins (PPP2R2A and GSN) were up-regulated (Fig. 3B), which was consistent with the results of the MS analysis. Therefore, the expression levels of 112 proteins were considered to be regulated by miR-193a-3p.

Functional Enrichment of the miR-193a-3p-regulated Proteins

Some of the proteins among the globally dysregulated group have been reported to be involved in lung cancer; for example, UHRF1, G3BP1 and KRT7 were associated with lung cancer metastasis and PSAT1, PRKDC and PPP2R2A were associated with lung-cancer cell proliferation. To further elucidate the biological processes affected by miR-193a-3p expression, these differentially expressed proteins were applied to the DAVID database to achieve an enrichment analysis regarding involvement in a biological process, a cellular component or a molecular function (Fig. 4).

Analysis of the signaling network

We uploaded the globally dysregulated proteins after stably transfecting the miR-193a-3p and their names into the STRING database. Ninety-four proteins were identified by the

Table 2. List of up-regulated proteins

database, whereas 24 proteins did not show any link at the chosen confidence level (STRING score=0.4). A merged network is shown in Fig. 5. The most significant functions of the proteins in the network were cytoskeletal organization, RNA transport, RNA processing and protein localization. KHSRP, RANBP2, XPO1, LRPPRC and TPR are important proteins in RNA transport. DDX23, DHX9, KHSRP, SSB, HNRNPR and HNRNPU are important proteins in the RNA metabolic process. ACTN4, GSN, KRT19, KRT8 and PAFAH1B1 are associated with cytoskeletal organization. The program predicted associations for a particular group of proteins.

Discussion

In a previous study, we demonstrated that miR-193a-3p was involved in metastasis of NSCLC cells. Overexpression of miR-193a-3p suppressed the migration and invasion of NSCLC cells *in vitro* and NSCLC metastasis *in vivo* through down-regulating the expression of ERBB4 and S6K2 [19]. However, the complex cancer inhibitory mechanisms

Accession NO.	Protein name	Unused score (mean±SD)	%Cov(95) (mean±SD)	Peptides(95%) (mean±SD)	iTRAQ Rate (mean±SD)	Pval (mean±SD)
A0MZ66	KIAA1598	26.11±2.47	39.3±1.84	18±3	3.12±0.22	0.01±0
O00159-2	MYO1C	40.08±3.67	39.25±2.19	25±4	2.94±0.15	0±0
O00299	CLIC1	37.97±4.67	83±0	46±4	2.79±1.05	0±0
O14929	HAT1	15.57±0.96	35.8±5.8	15±1	1.9±0.11	0.05±0
O15031	PLXNB2	13.4±1.18	20.4±4.24	11±0	2.21±0.6	0.01±0
O43707	ACTN4	112.36±0.24	80.75±2.76	142±6	2.88±0.04	0±0
O43795-2	MYO1B	17.49±1.9	24.8±2.55	11±2	1.88±0.29	0.02±0.01
O75489	NDUFS3	10.81±1.07	38.45±2.9	7±1	2.93±0.49	0.02±0.01
O75521	ECI2	15.04±1.36	41.8±1.98	11±3	2±0.65	0.04±0
P02786	TFRC	38.81±5.29	47.3±5.09	25±3	2.86±0.52	0±0
P04264	KRT1	15.67±1.92	43±1.27	16±1	2.97±1.08	0.01±0.01
P05023-3	ATP1A1	42.28±4.08	39.65±1.48	39±1	4.17±1.74	0±0
P05455	SSB	35.82±1.5	51.5±8.34	24±3	2.43±0.35	0.03±0.03
P06396	GSN	32.01±3.8	49.05±5.02	27±2	2.08±0.39	0±0
P06576	ATP5B	41.44±0.9	59.85±4.45	45±10	2.85±0.61	0±0
P06756-3	ITGAV	30.43±6.36	34.55±3.18	20±4	2.08±0.15	0.01±0
P07339	CTSD	36.13±2.38	61.3±0.14	37±4	2.68±0.81	0±0
P08758	ANXA5	67.26±5.41	88.75±1.34	73±4	2.54±0.44	0.01±0.01
P09960	LTA4H	36.83±3.68	48±0	39±2	1.59±0.04	0.03±0.02
P10515	DLAT	18.97±3	31±1.84	14±1	6.33±4.68	0±0
P11177	PDHB	22.13±4.54	43.3±4.53	22±1	2.89±0.11	0.01±0
P14625	HSP90B1	93.88±4.55	68.7±1.13	97±1	1.61±0.05	0±0
P22314	UBA1	94.08±13.89	69.75±5.3	119±4	2.98±0.14	0.01±0.01
P25705	ATP5A1	48.12±5.03	54.35±3.46	40±2	2.86±0.19	0±0
P26038	MSN	64.67±4.27	71.05±7.85	90±7	1.71±0.03	0.02±0.01
P30041	PRDX6	33.43±0.87	87.75±0.35	40±1	5.07±1.05	0±0
P30101	PDIA3	53.19±4.97	71.4±2.69	63±1	4.54±1.14	0±0
P31327	CPS1	90.32±5.06	49.3±3.39	78±6	3.08±0.32	0.01±0
P33992	MCM5	47.41±0.08	53.1±0.57	36±2	2.11±0.03	0.01±0
P35221	CTNNA1	50.52±2.64	48.95±2.47	35±2	2.63±0.36	0±0
P38117	ETFB	13.57±0.51	45.9±1.13	8±1	2.33±0.03	0.03±0.01
P40925-3	MDH1	27.13±0.02	61.1±2.4	21±0	1.72±0.13	0.01±0
P40939	HADHA	44.79±1.7	50.6±2.26	31±3	2.18±0.56	0±0
P42704	LRPPRC	81.88±0.11	53.15±1.2	77±1	5.02±0.81	0±0
P43034	PAFAH1B1	17.51±1.35	42.2±0.71	15±2	2.79±0.36	0.03±0.02
P46939	UTRN	38.57±3.13	22.85±4.74	26±4	3.32±1.14	0±0
P46940	IQGAP1	149.37±4.07	62.3±0.28	154±0	2.56±0.23	0±0
P49736	MCM2	47.28±0.59	45.25±1.06	39±5	2.29±0.09	0.01±0
P54709	ATP1B3	14.07±0.64	37.25±6.58	9±1	4.69±0.46	0.01±0.01
P60842	EIF4A1	57.16±2.45	82.75±1.34	76±1	3.83±0.87	0.03±0
P63151	PPP2R2A	30.65±2.21	61.85±4.6	24±2	2.07±0.49	0.03±0
P80303	NUCB2	14.87±1.23	42±5.23	11±1	2.81±0.46	0±0
Q02218	OGDH	34.94±1.26	32.4±0.57	24±1	2.52±0.36	0±0
Q02952-3	AKAP12	30.86±3.98	34.55±7	18±4	6.68±2.1	0±0
Q14697	GANAB	52.88±0.88	49.45±1.63	39±1	4.86±2.64	0±0
Q15147	PLCB4	15.25±1.73	22.05±3.75	9±1	1.95±0.06	0.03±0.01
Q6PID6	TTC33	8.01±0.01	29.4±1.13	6±1	2.94±0.79	0.03±0.01
Q86VS8	HOKK3	18.26±0.07	33.05±5.73	12±0	2.45±0.43	0.01±0.01
Q96199	SUCLG2	19.76±1.82	45.25±0.49	15±1	2.41±0.34	0.02±0.02
Q99436	PSMB7	23.82±1.9	56.7±13.29	17±2	2.51±0.93	0.03±0.01

continued

underlying the effects of this small non-coding RNA and the molecular targets through which miR-193a-3p acts have not yet been fully determined.

In this study, the lung adenocarcinoma cancer cell line SPC-A-1sci, which was established by our laboratory and which has a highly metastatic potential, was used as a model, and the isobaric tags for relative and absolute quantitation (iTRAQ) approach combined with nano liquid chromatography-tandem mass spectrometry (NanoLC-MS/MS) analysis were used to identify the proteins species with altered levels of expression in the SPC-A-1sci cells that overexpressed miR-193a-3p. The proteomic analysis revealed a total of 112 dysregulated protein species, the functions of which were involved in diverse biological processes, such as protein transport, RNA processing, cytoskeletal organization and translation. Among these proteins, 62 proteins exhibited up-regulated expression and 50 proteins exhibited down-regulated expression. The oncoproteins with miR-193a-3p-dysregulated expression are thought to be associated with the inhibition of NSCLC progression.

GSN and PPP2R2A were up-regulated by miR-193a-3p. Gelsolin (GSN) is a cytoskele-

Accession NO.	Protein name	Unused score (mean±SD)	%Cov(95) (mean±SD)	Peptides(95%) (mean±SD)	iTRAQ Rate (mean±SD)	Pval (mean±SD)
Q9BVG4	PBDC1	12.15±0.18	51.3±7.64	9±1	1.96±0.24	0.04±0
Q9BXJ9	NAA15	31.07±1.41	42.7±0	21±5	2.09±0.16	0.02±0.02
Q9H074	PAIP1	12.38±3.02	34.45±5.02	10±2	2.54±0.2	0.03±0.01
Q9NQR4	NIT2	23.42±0.83	58.7±0.57	14±0	2.59±0.24	0±0
Q9NT62	ATG3	15.1±0.74	45.05±7.85	12±0	2.06±0.24	0.04±0
Q9UBT2	UBA2	28.41±4.61	47.5±0.85	22±1	1.77±0.05	0.01±0.01
Q9UKY7	CDV3	14.97±1.24	73.1±0.28	11±2	5.15±2.51	0.01±0.01
Q9ULZ3	PYCARD	5.37±0.9	23.3±2.55	4±0	10.5±4.32	0.03±0.01
B4DWW4	MCM3	56.44±3.22	56.1±1.13	46±6	3.53±1.02	0±0
C9JIZ6	PSAP	30.06±4.87	46.9±2.55	27±1	7.04±3.55	0.03±0.01
E9PRY8	EEF1D	36.65±0.62	48.15±2.33	40±2	3.58±1.58	0.02±0.01
K7ELL7	PRKCSH	32.53±0.54	57.6±1.84	32±4	3±1.44	0.01±0.01

Fig. 2. The expression levels of the 112 dysregulated proteins are shown in a Heatmap. The red-colored clusters represent up-regulated proteins and the green-colored clusters represent down-regulated proteins.

Table 3. List of down-regulated proteins

Accession NO.	Protein name	Unused score (mean±SD)	%Cov(95) (mean±SD)	Peptides(95%) (mean±SD)	iTRAQ Rate (mean±SD)	Pval (mean±SD)
O14980	XP01	58.64±3.42	46.4±0	57±1	0.54±0.06	0±0
O15230	LAMA5	31.42±2.06	15.25±0.64	19±1	0.39±0.04	0±0
O43390	HNRNPR	12.93±0.48	33.2±3.82	16±1	0.36±0.21	0.01±0.02
O75533	SF3B1	47.4±0.75	41.15±2.33	32±1	0.4±0.09	0±0
P02545	LMNA	49.59±2.02	53.95±4.88	40±4	0.37±0.12	0±0
P05783	KRT18	61.98±0.13	81.4±3.96	75±8	0.4±0.03	0±0
P05787	KRT8	77.17±3.97	78.2±0.14	93±12	0.21±0.03	0±0
P06737	PYGL	35.53±8.95	47.35±4.17	31±2	0.57±0.03	0.02±0.02
P08727	KRT19	31.49±1.28	72.3±2.83	25±0	0.58±0.01	0.01±0.01
P08729	KRT7	41.59±1.55	65.15±6.15	51±1	0.25±0.11	0±0
P12270	TPR	67.47±1.46	35.15±1.06	44±4	0.6±0.04	0±0
P15924	DSP	91.83±7.67	41±0.57	59±4	0.58±0.06	0±0
P23246	SFPQ	14.35±0.45	29.35±2.76	12±2	0.51±0.16	0.02±0.01
P23381	WARS	31.38±0.58	60.2±6.79	28±1	0.45±0.08	0±0
P23396	RPS3	31.17±2.14	71.85±3.18	28±4	0.43±0.05	0±0
P26196	DDX6	27.78±1.64	45.55±3.18	24±2	0.64±0.01	0.02±0.01
P28289	TMOD1	7.16±1.19	33.85±1.34	5±1	0.37±0.3	0.02±0.01
P49411	TUFM	40.15±0.54	61.3±1.56	39±6	0.55±0.05	0.03±0
P49588	AARS	71.83±6.77	58.55±1.77	63±5	0.43±0.09	0.01±0.01
P49792	RANBP2	59.85±10.34	29.25±0.35	38±5	0.6±0.05	0.01±0.01
P50454	SERPINH1	40.25±1.66	60.05±1.06	38±6	0.55±0.04	0.01±0.01
P52948-6	NUP98	30.96±2.03	20.45±0.21	21±1	0.57±0.05	0±0
P54136	RARS	53.15±2.3	57.8±0.57	40±1	0.55±0.1	0.01±0.01
P62249	RPS16	13.46±0.86	59.9±2.4	8±0	0.6±0.1	0.04±0.01
P62805	HIST1H4A	8.01±0.01	39.8±1.41	9±3	0.27±0.02	0.02±0
P78527	PRKDC	128.53±8.53	33.15±0.49	103±6	0.5±0.04	0±0
Q00839	HNRNPU	39.34±3.51	42.75±5.73	36±2	0.58±0.05	0.04±0.01
Q08211	DHX9	63.55±2.37	44.85±3.32	51±7	0.52±0.04	0.01±0
Q13085	ACACA	73±2.45	34.8±1.56	43±1	0.52±0.15	0±0
Q13283	G3BP1	21.02±0.37	41.95±2.33	21±1	0.58±0.02	0±0
Q14694-2	USP10	19.38±5.14	24.1±3.82	12±2	0.44±0.01	0.03±0.01
Q14764	MVP	41.48±1.61	47.7±3.68	29±2	0.49±0.04	0±0
Q14847	LASP1	16.86±2.24	76.05±4.03	23±2	0.4±0.03	0.02±0.02
Q16555	DPYSL2	37.09±3.37	59.45±5.44	41±6	0.22±0.05	0±0
Q16822	PCK2	21.86±0.25	32.95±1.77	13±1	0.44±0.06	0.01±0.01
Q8IVF2-3	AHNAK2	135.87±9.67	47.45±0.64	97±3	0.33±0.09	0±0
Q92616	GCN1L1	86.69±1.72	33.9±1.7	60±4	0.29±0.12	0±0
Q92945	KHSRP	26.77±3.58	45.9±2.26	22±2	0.38±0.05	0.02±0.01
Q96T88	UHRF1	16.55±3.56	26.65±4.45	11±1	0.5±0.04	0.02±0
Q9BUQ8	DDX23	26.14±0.06	31±1.98	14±1	0.56±0.04	0.02±0.01
Q9H0A0	NAT10	25.41±1.66	31.8±0.28	17±1	0.53±0.01	0.01±0.01
Q9Y2T3-3	GDA	37.68±1.19	66.45±3.32	28±1	0.33±0.02	0.04±0.01
Q9Y617	PSAT1	32.7±0.34	70.55±0.35	30±5	0.46±0.07	0±0
C9J8U1	SPECC1L	13.39±0.28	33.75±0.92	9±1	0.51±0.04	0.03±0.01
F5GWX5	CHD4	44.48±0.42	28.3±0.85	29±2	0.36±0.03	0±0
F5H0L8	SEC23IP	23.75±1.86	38.9±1.84	14±2	0.26±0.04	0.03±0
F5H2U2	PRPF4B	12.99±0.78	22.15±6.58	7±1	0.41±0.03	0.01±0
I3L4C2	BAIAP2	29.04±0.88	48.3±2.26	19±1	0.45±0.29	0±0.01
J3KTA4	DDX5	38.65±0.95	50.95±8.27	32±0	0.55±0.05	0.03±0.01
J9JID7	LMNB2	14.78±1.04	33.25±2.47	9±1	0.53±0.16	0±0

ton-associated protein that is involved in dynamic changes in the actin cytoskeleton during cell motility [20]. It has been reported that GSN is aberrantly expressed in many types of tumor. Under various pathological conditions and in different types of cancer cells, GSN performs different functions. For example, high GSN expression in oral cancers is related to a poor clinical outcome for patients with metastasis [21]. In contrast, GSN expression is frequently down-regulated in ovarian, stomach and colon cancers [22-24]. In our study, the up-regulation of GSN expression may play a role in inhibiting the metastasis of SPC-A-1sci cells transfected with miR-193a-3p. PPP2R2A is a candidate tumor-suppressor gene that is implicated in the negative control of cell growth and division because it is a member of the protein phosphatase 2 (PP2A) regulatory subunit B family [25]. Many studies have reported that PPP2R2A down-regulation was observed in acute

Fig. 3. The expression levels of the dysregulated proteins were verified using WB analysis. (A) Representative blots show the protein expression level in the miR-193a-3p-overexpressing SPC-A-1sc1 cells. HNRNPU, G3BP1, UHRF1, LMNB2 were down-regulated proteins, and PPP2R2A and GSN were up-regulated proteins (B). β-actin was used as the loading control.

Fig. 4. The 112 dysregulated proteins were analyzed according to GO terms. (A) All of the dysregulated proteins were analyzed by searching the DAVID database, $P < 0.05$. The histograms demonstrate the top 10 counts for a biological process, a cellular component (B) and a molecular function (C).

myelogenous leukemia and prostate adenocarcinoma, as well as in lung cancer [26-28].

The proteins with down-regulated expression mediated by the overexpression of miR-193a-3p are UHRF1, G3BP1, LMNB2 and HNRNPU.

UHRF1 belongs to the UHRF family and is a nucleoprotein involved in cell growth and proliferation [29]. It was found that UHRF1 could promote the G1/S transition as a direct target of E2F1, which is a transcriptional factor essential for S-phase entry [30]. In examining many human cancers, including lung cancer, breast cancer and colorectal cancer [31-33], we have found that UHRF1 could promote their proliferation and metastasis.

G3BP1 is one member of the G3BP family that participates in several signaling pathways involved in carcinogenesis. G3BP1, which was reported to be highly overexpressed in various cancers, including breast cancer, esophageal squamous carcinoma and lung cancer [34-36], was found to be closely associated with the invasion and metastasis of tumor cells.

LMNB2 is a component of the nuclear lamina, which is the intermediate-filament

Fig. 5. The differentially expressed proteins were used to search the STRING database to predict their protein-protein interactions in the miR-193a-3p overexpressing SPC-A-1sci cells. In the network shown, the nodes are the proteins and the lines represent the predicted functional associations. The number of lines indicates the strength of the predicted functional interactions of the proteins.

meshwork lying beneath the inner nuclear membrane [37]. HNRNPU, which plays an important role in the processing and transport of mRNA, is the largest member of the hnRNP protein family [38]. LMNB2 and HNRNPU had not been reported in lung cancer progression. They might be new targets of miR-193a-3p-mediated inhibition of SPC-A-1sci migration.

In summary, we used the iTRAQ quantitative approach combined with nano liquid chromatography-tandem mass spectrometry analysis to identify the proteins species with altered levels of expression in SPC-A-1sci cells overexpressing miR-193a-3p. Our findings may improve the understanding of the molecular mechanisms underlying the metastasis-inhibitory effects of miR-193a-3p on lung cancer cells.

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References

- 1 Siegel R, Naishadham D, Jemal A: Cancer statistics for Hispanics/Latinos, 2012. *CA Cancer J Clin* 2012;5:283-298.
- 2 Crino L, Weder W, van Meerbeeck J, Felip E, Group EGW: Early stage and locally advanced (non-metastatic) non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2010;21:v103-115.
- 3 Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* 2000;1:57-70.
- 4 Gdowicz-Klosok A, Giglok M, Drosik A, Suwinski R, Butkiewicz D: The SIPA1 -313A>G polymorphism is associated with prognosis in inoperable non-small cell lung cancer. *Tumor Biol* DOI 10.1007/s13277-014-2753-8.
- 5 Jia D, Yan M, Wang X, Hao X, Liang L, Liu L, Kong H, He X, Li J, Yao M: Development of a highly metastatic model that reveals a crucial role of fibronectin in lung cancer cell migration and invasion. *BMC Cancer* 2010;10:364.
- 6 Liang S, Xu Z, Xu X, Zhao X, Huang C, Wei Y: Quantitative proteomics for cancer biomarker discovery. *Comb Chem High T Scr* 2012;3:221-231.
- 7 Nikolov M, Schmidt C, Urlaub H: Quantitative mass spectrometry-based proteomics: an overview. *Methods Mol Biol* 2012;893:85-100.
- 8 Masui O, White NM, DeSouza LV, Krakovska O, Matta A, Metias S, Khalil B, Romaschin AD, Honey RJ, Stewart R, Pace K, Bjarnason GA, Siu KW, Yousef GM: Quantitative proteomic analysis in metastatic renal cell carcinoma reveals a unique set of proteins with potential prognostic significance. *Mol Cell Proteomics* 2013;1:132-144.
- 9 Jeppesen DK, Nawrocki A, Jensen SG, Thorsen K, Whitehead B, Howard KA, Dyrskjot L, Orntoft TF, Larsen MR, Ostensfeld MS: Quantitative proteomics of fractionated membrane and lumen exosome proteins from isogenic metastatic and nonmetastatic bladder cancer cells reveal differential expression of EMT factors. *Proteomics* 2014;6:699-712.
- 10 Bartel DP: MicroRNAs: target recognition and regulatory functions. *Cell* 2009;2:215-233.
- 11 Selbach M, Schwanhauser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N: Widespread changes in protein synthesis induced by microRNAs. *Nature* 2008;7209:58-63.
- 12 Calin GA, Croce CM: MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res* 2006;15:7390-7394.
- 13 Ambros V: The functions of animal microRNAs. *Nature* 2004;7006:350-355.
- 14 Shkumatava A, Stark A, Sive H, Bartel DP: Coherent but overlapping expression of microRNAs and their targets during vertebrate development. *Gene Dev* 2009;4:466-481.
- 15 Grimson A, Farh KK, Johnston WK, Garrett-Engle P, Lim LP, Bartel DP: MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* 2007;1:91-105.
- 16 Chi SW, Zang JB, Mele A, Darnell RB: Argonaute HTS-CLIP decodes microRNA-mRNA interaction maps. *Nature* 2009;7254:479-486.
- 17 Ritchie W, Flamant S, Rasko JE: Predicting microRNA targets and functions: traps for the unwary. *Nat Methods* 2009;6:397-398.
- 18 Thomson DW, Bracken CP, Goodall GJ: Experimental strategies for microRNA target identification. *Nucleic Acids Res* 2011;16:6845-6853.
- 19 Yu T, Li J, Yan M, Liu L, Lin H, Zhao F, Sun L, Zhang Y, Cui Y, Zhang F, Li J, He X, Yao M: MicroRNA-193a-3p and -5p suppress the metastasis of human non-small-cell lung cancer by downregulating the ERBB4/PIK3R3/mTOR/S6K2 signaling pathway. *Oncogene* DOI: 10.1038/onc.2013.574.
- 20 Baig RM, Mahjabeen I, Sabir M, Masood N, Ali K, Malik FA, Kayani MA: Mutational spectrum of Gelsolin and its down regulation is associated with breast cancer. *Dis Markers* 2013;2:71-80.
- 21 Shieh DB, Chen IW, Wei TY, Shao CY, Chang HJ, Chung CH, Wong TY, Jin YT: Tissue expression of gelsolin in oral carcinogenesis progression and its clinicopathological implications. *Oral Oncol* 2006;6:599-606.
- 22 Noske A, Denkert C, Schober H, Sers C, Zhumabayeva B, Weichert W, Dietel M, Wiechen K: Loss of Gelsolin expression in human ovarian carcinomas. *Eur J Cancer* 2005;3:461-469.
- 23 Kim JH, Choi YK, Kwon HJ, Yang HK, Choi JH, Kim DY: Downregulation of gelsolin and retinoic acid receptor beta expression in gastric cancer tissues through histone deacetylase 1. *J Gastroen Hepatol* 2004;2:218-224.

- 24 Gay F, Estornes Y, Saurin JC, Joly-Pharaboz MO, Friederich E, Scoazec JY, Abello J: In colon carcinogenesis, the cytoskeletal protein gelsolin is down-regulated during the transition from adenoma to carcinoma. *Hum Pathol* 2008;10:1420-1430.
- 25 Cheng Y, Liu W, Kim ST, Sun J, Lu L, Sun J, Zheng SL, Isaacs WB, Xu J: Evaluation of PPP2R2A as a prostate cancer susceptibility gene: a comprehensive germline and somatic study. *Cancer Genet-NY* 2011;7:375-381.
- 26 Ruvalo PP, Qui YH, Coombes KR, Zhang N, Ruvalo VR, Borthakur G, Konopleva M, Andreeff M, Kornblau SM: Low expression of PP2A regulatory subunit B55alpha is associated with T308 phosphorylation of AKT and shorter complete remission duration in acute myeloid leukemia patients. *Leukemia* 2011;11:1711-1717.
- 27 Liu W, Xie CC, Zhu Y, Li T, Sun J, Cheng Y, Ewing CM, Dalrymple S, Turner AR, Sun J, Isaacs JT, Chang BL, Zheng SL, Isaacs WB, Xu J: Homozygous deletions and recurrent amplifications implicate new genes involved in prostate cancer. *Neoplasia* 2008;8:897-907.
- 28 Shen S, Yue H, Li Y, Qin J, Li K, Liu Y, Wang J: Upregulation of miR-136 in human non-small cell lung cancer cells promotes Erk1/2 activation by targeting PPP2R2A. *Tumor Biol* 2014;1:631-640.
- 29 Jenkins Y, Markovtsov V, Lang W, Sharma P, Pearsall D, Warner J, Franci C, Huang B, Huang J, Yam GC, Vistan JP, Pali E, Vialard J, Janicot M, Lorens JB, Payan DG, Hitoshi Y: Critical role of the ubiquitin ligase activity of UHRF1, a nuclear RING finger protein, in tumor cell growth. *Mol Biol Cell* 2005;12:5621-5629.
- 30 Unoki M, Nishidate T, Nakamura Y: ICBP90, an E2F-1 target, recruits HDAC1 and binds to methyl-CpG through its SRA domain. *Oncogene* 2004;46:7601-7610.
- 31 Daskalos A, Oleksiewicz U, Filia A, Nikolaidis G, Xinarianos G, Gosney JR, Malliri A, Field JK, Liloglou T: UHRF1-mediated tumor suppressor gene inactivation in nonsmall cell lung cancer. *Cancer* 2011;5:1027-1037.
- 32 Li X-L: Exogenous expression of UHRF1 promotes proliferation and metastasis of breast cancer cells. *Oncol Rep* 2012;28:375-83.
- 33 Wang F, Yang YZ, Shi CZ, Zhang P, Moyer MP, Zhang HZ, Zou Y, Qin HL: UHRF1 promotes cell growth and metastasis through repression of p16(ink(4)a) in colorectal cancer. *Ann Surg Oncol* 2012;8:2753-2762.
- 34 French J, Stirling R, Walsh M, Kennedy HD: The expression of Ras-GTPase activating protein SH3 domain-binding proteins, G3BPs, in human breast cancers. *Histochemical J* 2002;5:223-231.
- 35 Zhang HZ, Liu JG, Wei YP, Wu C, Cao YK, Wang M: Expression of G3BP and RhoC in esophageal squamous carcinoma and their effect on prognosis. *World J Gastroentero* 2007;30:4126-4130.
- 36 Zhang H, Zhang SH, He HW, Zhang CX, Yu DK, Shao RG: Downregulation of G3BPs inhibits the growth, migration and invasion of human lung carcinoma H1299 cells by suppressing the Src/FAK-associated signaling pathway. *Cancer Gene Ther* 2013;11:622-629.
- 37 Yang SH, Chang SY, Yin L, Tu Y, Hu Y, Yoshinaga Y, de Jong PJ, Fong LG, Young SG: An absence of both lamin B1 and lamin B2 in keratinocytes has no effect on cell proliferation or the development of skin and hair. *Hum Mol Genet* 2011;18:3537-3544.
- 38 Spraggon L, Dudnakova T, Slight J, Lustig-Yariv O, Cotterell J, Hastie N, Miles C: hnRNP-U directly interacts with WT1 and modulates WT1 transcriptional activation. *Oncogene* 2007;10:1484-1491.