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ORIGINAL ARTICLE

Tumor suppressive *microRNA-133a* regulates novel molecular networks in lung squamous cell carcinoma

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Analysis of the microRNA (miRNA) expression signature of lung squamous cell carcinoma (lung-SCC) revealed that the expression levels of *miR-133a* were significantly reduced in cancer tissues compared with normal tissues. In this study, we focused on the functional significance of *miR-133a* in cancer cell lines derived from lung-SCC and the identification of *miR-133a*-regulated novel cancer networks in lung-SCC. Restoration of *miR-133a* expression in PC10 and H157 cell lines resulted in significant inhibition of cell proliferation, suggesting that *miR-133a* functions as a tumor suppressor. We used genome-wide gene expression analysis to identify the molecular targets of *miR-133a* regulation. Gene expression data and web-based searching revealed several candidate genes, including *transgelin 2* (*TAGLN2*), *actin-related protein2/3 complex, subunit 5, 16kDa* (*ARPC5*), *LAG1 homolog, ceramide synthase 2* (*LASS2*) and *glutathione S-transferase pi 1* (*GSTP1*). *ARPC5* and *GSTP1* likely represent bona fide targets as their expression is elevated in lung-SCC clinical specimens. Furthermore, transient transfection of *miR-133a*, repressed *ARPC5* and *GSTP1* mRNA and protein levels. As cell proliferation was significantly inhibited in lung-SCC cells following RNAi knock down of either gene, *ARPC5* and *GSTP1* may function as oncogenes in the development of lung-SCC. The identification of a tumor suppressive miRNA and the novel cancer pathways it regulates could provide new insights into potential molecular mechanisms of lung-SCC carcinogenesis.
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INTRODUCTION

Lung cancer is clearly the primary cause of cancer-related deaths worldwide. In Japan, it accounts for about 60 000 deaths every year, and is the leading cause of cancer-related deaths in men and the third leading cause in women. Approximately 80% of lung cancers are classified histopathologically as non-small cell lung cancers (NSCLC). NSCLC is subdivided into four major histological subtypes with distinct pathological characteristics: adenocarcinoma, squamous cell carcinoma, large cell carcinoma and neuroendocrine cancer.[1,2] Curative outcomes are found only in patients with early stage disease who undergo surgery, with a 5-year overall survival rate of 50–60%,[1,2] whereas patients in advanced stages rarely survive more than 5 years despite aggressive chemotherapy, molecular-targeted therapy or chemoradiotherapy.[3,4]

It is well known that altered expression of cell surface growth factor receptors, including the receptor tyrosine kinase (RTK) family, has a major role in the development of many types of cancer, including NSCLC.[5] Over-expression of epidermal growth factor receptor (also known as Erb1) and other members of the ErbB family of receptor tyrosine kinases (ErbB2 and ErbB3) is often observed in many cancers and contributes to increased cell proliferation.[6] On the basis of this fact, several therapeutic agents have been designed in lung cancers, such as gefitinib and erlotinib for the mutation of epidermal growth factor receptor gene, and crizotinib for the EML4-ALK fusion gene.[7] However, such a molecular-targeted strategy has not been developed for squamous cell carcinoma, but only for adenocarcinoma of the lung. The new genome-wide RNA analysis of lung squamous cell carcinoma (lung-SCC) may provide new avenues for research, and the development of novel diagnostic approaches and therapeutics for lung-SCC.

For the past several decades, many genes (such as *RAS*, *MYC*, *PTEN* and *EGFR*) have been identified and implicated in lung cancer that also have a role in normal cell growth and differentiation. Recent studies have revealed that the normal regulatory mechanisms in these cancer pathways are disrupted by aberrant expression of microRNA (miRNA).[8] MiRNAs are a class of small non-coding RNA molecules consisting of 19–22 nucleotides that regulate gene expression through translational repression and mRNA cleavage,[8] and have an important role in a variety of biological processes, including development, differentiation, apoptosis and cell proliferation. Bioinformatic

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predictions indicate that miRNAs regulate more than 30% of protein coding genes.[9] Currently, 1424 human miRNAs have been registered at miRBase release 17.0 (http://microrna.sanger.ac.uk/).

Signaling by the *RAS* oncogene pathway has a central role in lung cancer and approximately 30% of lung cancers have activating mutations in this cascade.[10] A growing body of studies has revealed that aberrant expression of miRNAs is associated with the initiation and development of NSCLC,[11] and alterations of RAS signaling. For example, downregulation of the *let-7* family of miRNAs, which normally reduces *RAS* expression levels by directly binding to the 3′UTR of *RAS* mRNA,[12] was observed in cancer tissues from a NSCLC patient. Restoration of *let-7* expression inhibited the growth of cancer cells, suggesting that *let-7* reduction leads to over-expression of *RAS* and is a primary mediator of NSCLC carcinogenesis.

Recent studies from our group have identified tumor suppressive miRNAs and the molecular pathways they control in various human cancers.[13–18] As there are few studies about the role of miRNAs in lung-SCC, we sought to identify tumor suppressive miRNAs based on our lung-SCC expression signature. We focused on *miR-133a*, which was the most significantly downregulated miRNA in our signature, and we provide evidence that it functions as a tumor suppressor that inhibits cancer cell proliferation. To identify the target genes regulated by *miR-133a*, we also performed genome-wide gene expression analysis and found several candidate genes such as *actin-related protein2/3 complex, subunit 5, 16 kDa* (*ARPC5*) and *glutathione S-transferase pi 1* (*GSTP1*). Insights into the association between tumor suppressive *miR-133a* and their target oncogene networks could enhance our understanding of the molecular mechanism of lung-SCC carcinogenesis.

MATERIALS AND METHODS

Clinical lung-SCC specimens and lung-SCC cell culture

A total of 25 pairs of primary lung-SCC tissue and corresponding normal tissue samples were obtained from patients in Chiba University Hospital (Chiba, Japan) from 2004 to 2007 (clinical features of patients with lung-SCC are shown in Table 1). Normal tissues were obtained far from the center of the cancer in surgical specimens. No cancer cells were detected in neighboring formalin-fixed paraffin-embedded tissues. Written consent was obtained from each patient authorizing tissue donation for research purposes before surgery, following a protocol approved by the Institutional Review Board of Chiba University. The specimens were immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at −20 °C until RNA was extracted. Human lung-SCC cell lines, PC10 and H157, were grown in RPMI1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO2 at 37 °C.

RNA isolation

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentrations were determined using a UV spectrophotometer and molecule integrity was checked by gel electrophoresis. RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

miRNA expression signature of lung-SCC

Tissue specimens used for miRNA screening of a low-density array were obtained from five lung-SCC patients (Table 1; patient number 1–5). miRNA expression patterns were evaluated using the TaqMan LDA Human microRNA Panel v2.0 (Applied Biosystems, Foster City, CA, USA). The assay was composed of two steps: generation of cDNA by reverse transcription and a TaqMan real-time PCR assay. Description of real-time PCR and the list of human miRNAs can be found on the company's website (http://www.

Table 1 Clinical features of patients with lung squamous cell carcinoma

Case	*Gender*	*Age*	*Smoking index*	*Differentiation*	*TNM classification*	*Patient outcomes*	*Recurrence-free survival period (months)*
1	Male	68	480	Moderately	T1N0M0	Recurrence −	60
2	Male	71	1225	Moderately	T4N0M0	Recurrence −	60
3	Male	69	900	Well	T2N0M0	Recurrence −	60
4	Female	68	380	Well	T3N1M0	Recurrence +	13
5	Male	60	450	Moderately	T2N1M0	Recurrence +	15
6	Male	77	1140	Moderately	T2N0M0	Recurrence −	60
7	Male	77	2750	Poorly	T2N0M0	Recurrence −	60
8	Male	74	2200	Moderately	T3N2M0	Recurrence −	14
9	Male	70	700	Well	T2N0M0	Recurrence −	60
10	Male	78	1120	Poorly	T2N0M0	Recurrence +	12
11	Male	76	150	Moderately	T2N0M0	Recurrence −	28
12	Male	60	450	Moderately	T2N1M0	Recurrence +	15
13	Female	68	380	Well	T3N1M0	Recurrence +	13
14	Male	69	900	Well	T2N0M0	Recurrence −	60
15	Male	71	1225	Moderately	T4N0M0	Recurrence −	60
16	Male	68	480	Moderately	T1N0M0	Recurrence −	60
17	Male	55	280	Moderately	T1N1M0	Recurrence +	24
18	Male	63	1730	Poorly	T1N0M0	Recurrence −	28
19	Male	71	1760	Poorly	T2N1M0	Recurrence −	27
20	Male	66	1200	Moderately	T2N0M0	Recurrence −	27
21	Male	70	1500	Poorly	T2N0M0	Recurrence −	26
22	Male	72	800	Poorly	T4N1M0	Recurrence −	24
23	Male	73	480	Moderately	T1N0M0	Recurrence −	24
24	Male	83	450	Moderately	T2N1M0	Recurrence +	5
25	Male	72	750	Moderately	T1N2M0	Recurrence −	24

appliedbiosystems.com). Analysis of relative miRNA expression data was performed using GeneSpring GX version 7.3.1 software (Agilent Technologies) according to the manufacturer's instructions. A cutoff P value of <0.05 was used to narrow down the candidates after global normalization of the raw data. After global normalization, additional normalization was done with *RNU48*.

Mature miRNA transfection and small interfering RNA treatment

Mature miRNA molecules, Pre-miR miRNA precursors; (*hsa-miR-133a*; Pre-miR ID: AM10413 and negative control miRNA; P/N: AM17111, Applied Biosystems), small interfering RNA; si-*ARPC5* (P/N: HSS145450 and HSS145450, Invitrogen), si-*GSTP1* (P/N: s194475 and s194476, Applied Biosystems) and negative control siRNA (Stealth RNAi Negative Control Medium GC Duplex; 12935-300, Invitrogen) were incubated with Opti-MEM (Invitrogen) and Lipofectamine RNAiMax reagent (Invitrogen). Transfection efficiency of Pre-miR in cell lines was confirmed on the basis of downregulation of *PTK9* mRNA following transfection with *miR-1* as described previously.[13,14]

Cell proliferation assays

Cells were transfected with 10 nM miRNA or siRNA by reverse transfection and plated into 96-well plates at 3×10^3 cells per well. After 72 and 96 h, cell proliferation was determined by the XTT assay, using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Mannheim, Germany).[14,15] Triplicate wells were measured for cell viability in each treatment group.

Target gene search for *miR-133a*

To identify target genes of *miR-133a*, a genome-wide screen was performed in two lung-SCC cell lines, PC10 and H157, which were transfected with *miR-133a*. Expression profiles were generated using Oligo-microarray Human 44 K (Agilent Technologies) from the transfected cells and compared with a miRNA-negative-control sample. Hybridization and wash steps were performed as previously described.[19] The arrays were scanned using a Packard GSI Lumonics ScanArray 4000 (Perkin Elmer, Boston, MA, USA). The data were analyzed by means of DNASIS array software (Hitachi Software Engineering, Tokyo, Japan), which converted the signal intensity for each spot into text format. The $\log_2$ ratios of the median-subtracted background intensity were analyzed. Data from each microarray study were normalized by a global normalization method.[19]

Predicted target genes and their miRNA binding site seed regions were investigated using TargetScan (release 5.1, http://www.targetscan.org/). The sequences of the predicted mature miRNAs were confirmed using miRBase (release 17.0, http://microrna.sanger.ac.uk/).

Real-time quantitative RT-PCR

First-strand cDNA was synthesized from 1 μg total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene-specific PCR products were assayed continuously using a 7900-HT Real-Time PCR System according to the manufacturer's protocol. The initial PCR step consisted of a 10-min hold at 95 °C, followed by 40 cycles of a 15-sec denaturation at 95 °C and a 1-min annealing/extension at 63 °C. TaqMan probes and primers for *ARPC5* (P/N: Hs00271722_m1), *GSTP1* (P/N: Hs00168310_m1) and *GUSB* (P/N: Hs99999908_m1) as an internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products). The expression levels of *miR-133a* (Assay ID: 002246) were analyzed by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalized to *RNU48* (Assay ID: 001006). All reactions were performed in triplicate and included negative control reactions that lacked cDNA.

Western blot

Cells were harvested 72 h after transfection and lysates were prepared. Using SDS-PAGE, 50 μg protein from each lysate was separated on Mini-PROTEAN TGX (Tris-Glycine eXtended) precast gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was performed with diluted (1:500) monoclonal ARPC5 antibody (ab51243; Abcam, Cambridge, UK), monoclonal GSTP1 antibody (#3369; Cell Signaling, Danvers, MA, USA) or GAPDH antibody (ab8245, Abcam) used as an internal control. The membrane was washed and incubated with goat anti-rabbit or anti-mouse IgG (H+L)–HRP conjugate (Bio-Rad). Specific complexes were visualized by echochemiluminescence (Immun-Star WesternC Chemiluminescence Kit #170-5070, Bio-Rad) and the expression level of each protein was evaluated by ImageJ software (ver.1.44; http://rsbweb.nih.gov/ij/index.html).

Statistical analysis

The relationships between the two groups and the numerical values obtained by real-time RT-PCR were analyzed using the nonparametric Mann–Whitney U test or the paired t-test. The relationship among three variables and numerical values was analyzed using the Bonferroni-adjusted Mann–Whitney U test; a non-adjusted statistical level of significance of $P<0.05$ corresponded to a Bonferroni-adjusted level of $P<0.0167$. Spearman's rank test was used to evaluate the relationships among the relative expression levels of *miR-133a*, *ARPC5* and *GSTP1* mRNA. All analyses were performed using Expert StatView (version 4, SAS Institute Inc., Cary, NC, USA).

RESULTS

Identification of downregulated miRNAs in lung-SCC by miRNA expression signatures

We evaluated mature miRNA expression levels of five pairs of normal tissues and lung-SCC by miRNA expression signatures. A total of 24 significantly downregulated miRNAs were selected after the normalization using *RNU48*, and then 21 miRNAs were selected as their fold change were less than 0.5 (Table 2). Among them, *miR-133a* was the most downregulated and was selected for further study.

Expression of *miR-133a* in lung-SCC clinical specimens and effect of *miR-133a* transfectants on cell proliferation in PC10 and H157

Quantitative stem-loop RT-PCR demonstrated that the expression level of *miR-133a* was significantly reduced in 20 lung-SCC specimens (Table 1; patient number 6–25) in comparison with normal tissues ($P<0.0001$, Figure 1a).

Table 2 Downregulated miRNAs in lung squamous cell carcinoma

				ΔΔ Ct		
No.	*microRNA*	*Accession number*	*P value*	*Tumor*	*Normal*	*Fold change*
1	miR-133a	MIMAT0000427	0.01649	0.00224	0.03655	0.06133
2	miR-1247	MIMAT0005899	0.00619	0.00048	0.00695	0.06952
3	miR-206	MIMAT0000462	0.04275	0.00012	0.00116	0.10556
4	miR-99b*	MIMAT0004678	0.0353	0.00043	0.00351	0.12171
5	miR-139-5p	MIMAT0000250	0.00264	0.01138	0.0921	0.1236
6	miR-30a-3p	MIMAT0000088	0.01166	0.05335	0.30994	0.17214
7	miR-138	MIMAT0000430	0.02002	0.01959	0.09074	0.21595
8	miR-126	MIMAT0000445	0.04921	2.4132	11.1672	0.2161
9	miR-30e-3p	MIMAT0000693	0.01517	0.05111	0.20773	0.24604
10	miR-26a-1*	MIMAT0004499	0.00952	0.00033	0.00131	0.25273
11	miR-140-3p	MIMAT0004597	0.00729	0.01209	0.04569	0.26464
12	miR-34b	MIMAT0004676	0.02123	0.00863	0.03143	0.27452
13	miR-574-3p	MIMAT0003239	0.01686	0.12074	0.42691	0.28281
14	miR-628-5p	MIMAT0004809	0.00317	0.00366	0.01294	0.28308
15	miR-186	MIMAT0000456	0.0089	0.11448	0.39553	0.28943
16	miR-628-3p	MIMAT0003297	0.00736	0.00013	0.00045	0.29053
17	miR-146b-5p	MIMAT0002809	0.02777	0.69287	1.84479	0.37558
18	miR-16	MIMAT0000069	0.03184	1.31416	3.14414	0.41797
19	miR-125a-5p	MIMAT0000443	0.02874	0.02617	0.06222	0.42058
20	miR-320	MIMAT0000510	0.01465	0.12608	0.2791	0.45173
21	miR-191	MIMAT0000440	0.00526	0.607	1.31828	0.46044

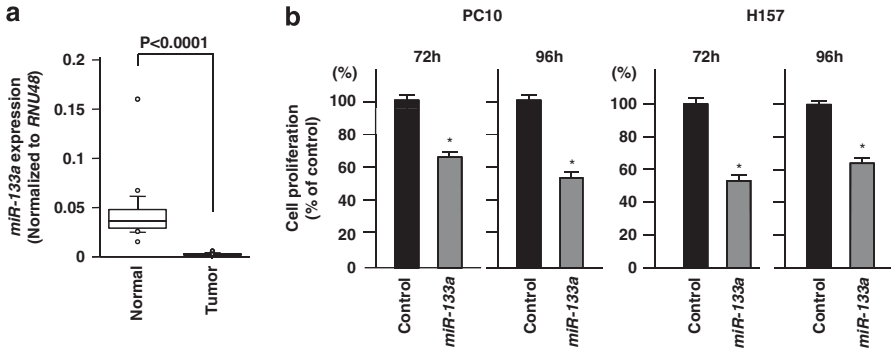

Figure 1 *miR-133a* functions as a tumor suppressor in lung-SCC. (**a**) The expression level of *miR-133a* was significantly downregulated in tumor tissues compared with non-tumor tissues. *RNU48* was used as an internal control. (**b**) Cancer cell proliferation was determined by XTT assay in PC10 and H157 cell lines. The XTT assay was performed 72 and 96 h after transfection of 10 nM *miR-133a* or miR-control and showed significant inhibition of cell proliferation in *miR-133a* transfectants in comparison with controls. $*P<0.05$.

Table 3 Top 30 downregulated genes 2 ratio) with *miR-133a* transfectants in PC10 and H157

No.	Entrez gene ID	Symbol	Gene name	log2 ratio PC10	log2 ratio H157	log2 ratio Average	miR-133a target site
1	51776	*ZAK*	Sterile alpha motif and leucine zipper containing kinase AZK	−2.77	−3.06	−2.91	—
2	8407	*TAGLN2*	Transgelin 2	−2.82	−3.00	−2.91	3
3	23204	*ARL6IP1*	ADP-ribosylation factor-like 6 interacting protein 1	−2.13	−3.13	−2.63	—
4	79026	*AHNAK*	AHNAK nucleoprotein	−2.00	−2.92	−2.46	—
5	1500	*CTNND1*	Catenin (cadherin-associated protein), delta 1	−2.49	−2.37	−2.43	—
6	10092	*ARPC5*	Actin-related protein 2/3 complex, subunit 5, 16kDa	−2.33	−2.51	−2.42	1
7	29956	*LASS2*	LAG1 homolog, ceramide synthase 2	−2.20	−2.63	−2.42	2
8	27166	*PRELID1*	PRELI domain containing 1	−2.46	−2.33	−2.39	—
9	2950	*GSTP1*	Glutathione S-transferase pi 1	−2.21	−2.56	−2.39	1
10	124152	*IQCK*	IQ motif containing K	−2.02	−2.74	−2.38	—
11	255758	*TCTEX1D2*	Tctex1 domain containing 2	−1.86	−2.87	−2.36	—
12	7168	*TPM1*	Tropomyosin 1 (alpha)	−1.95	−2.70	−2.33	—
13	2512	*FTL*	Ferritin, light polypeptide	−2.11	−2.46	−2.29	1
14	65977	*PLEKHA3*	Pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 3	−2.09	−2.46	−2.28	1
15	2760	*GM2A*	GM2 ganglioside activator	−1.68	−2.83	−2.26	—
16	200232	*C20orf106*	Chromosome 20 open reading frame 106	−2.15	−2.17	−2.16	—
17	55435	*AP1AR*	Adaptor-related protein complex 1 associated regulatory protein	−1.78	−2.50	−2.14	1
18	80127	*C14orf45*	Chromosome 14 open reading frame 45	−1.91	−2.31	−2.11	—
19	10944	*C11orf58*	Chromosome 11 open reading frame 58	−1.71	−2.48	−2.10	2
20	10952	*SEC61B*	Sec61 beta subunit	−2.38	−1.81	−2.09	1
21	9208	*LRRFIP1*	Leucine rich repeat (in FLII) interacting protein 1	−2.13	−2.02	−2.08	2
22	359821	*MRPL42P5*	Mitochondrial ribosomal protein L42 pseudogene 5	−1.92	−2.23	−2.07	—
23	58472	*SQRDL*	Sulfide quinone reductase-like (yeast)	−2.51	−1.60	−2.06	1
24	7171	*TPM4*	Tropomyosin 4	−2.14	−1.98	−2.06	1
25	100133263	*LOC100133263*	Hypothetical LOC100133263	−1.91	−2.19	−2.05	—
26	56005	*C19orf10*	Chromosome 19 open reading frame 10	−2.20	−1.85	−2.03	—
27	145694	*LOC145694*	Hypothetical protein LOC145694	−1.78	−2.26	−2.02	—
28	1389	*CREBL2*	cAMP responsive element binding protein-like 2	−1.87	−2.15	−2.01	—
29	10186	*LHFP*	Lipoma HMGIC fusion partner	−2.28	−1.71	−1.99	2
30	54842	*MFSD6*	Major facilitator superfamily domain containing 6	−2.11	−1.87	−1.99	2

To investigate the functional role of *miR-133a*, we performed gain-of-function studies in miRNA transfectants. The XTT assay showed significant inhibition of cell proliferation in *miR-133a* transfectants in comparison with the miR-control transfectants (% of cell viability, 72 h; PC10; 67.1 ± 2.3 and 100.0 ± 2.7, H157; 54.2 ± 3.6 and 100.0 ± 3.9, respectively, 96 h; PC10; 54.9 ± 2.3 and 100.0 ± 2.7, H157; 62.2 ± 3.6 and 100.0 ± 3.1, respectively, $P<0.05$, Figure 1b).

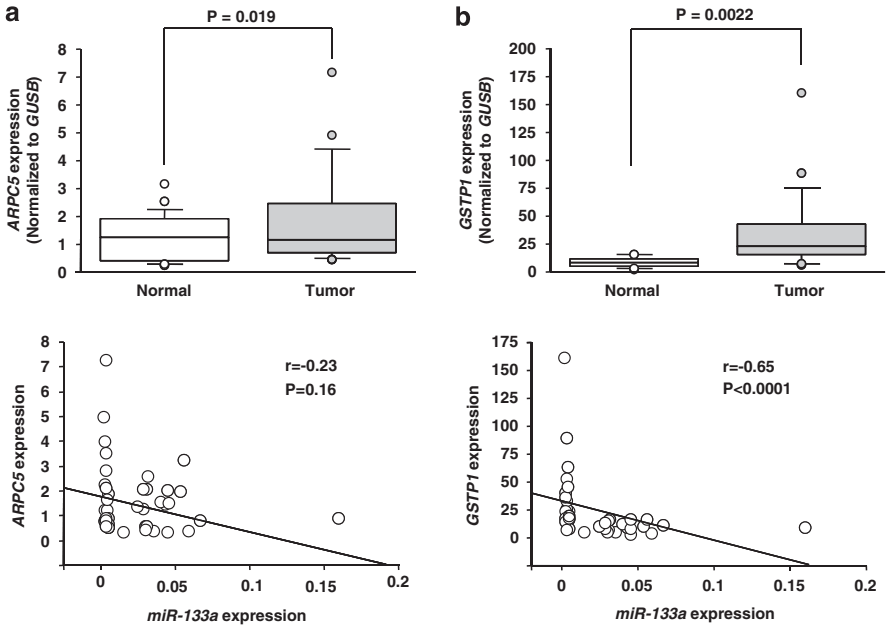

Figure 2 Increased expression levels of *ARPC5* and *GSTP1* mRNA in lung-SCC clinical specimens. (**a**) The mRNA expression levels of *ARPC5* in tumor tissues and non-tumor tissues in 20 clinical specimens (upper). Expression of *ARPC5* mRNA was significantly elevated in tumor tissues. The correlation between *ARPC5* and *miR-133a* expression was investigated in clinical specimens (lower). The trend of inverse correlation was recognized between *ARPC5* and *miR-133a* expression. (**b**) The mRNA expression levels of *GSTP1* in tumor tissues and non-tumor tissues in 20 clinical specimens (upper). Expression of *GSTP1* mRNA was significantly elevated in tumor tissues. The correlation between *GSTP1* and *miR-133a* expression was investigated in clinical specimens (lower). A significant inverse correlation was recognized between *GSTP1* and *miR-133a* expression.

Expression profiling identifies downregulated genes in *miR-133a* transfectants

To gain further insight into which genes are regulated by *miR-133a*, we performed gene expression analysis following *miR-133a* transfection compared with negative controls in PC10 and H157 cells. Signal values less than 1000 from the raw data in miR-control transfectants were not further considered. A total of 116 genes were downregulated with $\log_2$ ratio scores less than -1.0 in *miR-133a* transfectants compared with the controls (top 30 genes are shown in Table 3). The TargetScan program showed that 14 genes had putative target sites of *miR-133a* in their 3′UTR among the top 30 downregulated genes. Entries from the microarray data were approved by the Gene Expression Omnibus and were assigned Gene Expression Omnibus accession number, GSE26032.

Expression levels of candidate target genes of *miR-133a* in lung-SCC clinical specimens

We measured the expression levels of mRNAs of the top four candidate genes in 20 lung-SCC clinical specimens by quantitative real-time RT–PCR. Two genes, *actin-related protein2/3 complex, subunit 5, 16kDa (ARPC5)* and *glutathione S-transferase pi 1 (GSTP1)* were significantly upregulated in the tumor region (P=0.019 and P=0.0022, respectively, Figure 2a and b, upper). The other two genes (*TAGLN2* and *LASS2*) were not upregulated in the tumor region of lung-SCC (data not shown). There was a trend but no significant inverse correlation between *ARPC5—miR-133a* ($r=-0.23$ and P=0.16, Figure 2a, lower) and a significant inverse correlation between *GSTP1—miR-133a* ($r=-0.65$ and $P<0.0001$, Figure 2b, lower).

Silencing of *ARPC5* and *GSTP1* by *miR-133a* transfection

We performed gain-of-function studies using *miR-133a* transfectants, and the ARPC5 and GSTP1 mRNA and protein expression levels were markedly downregulated in comparison with the controls (Figures 3 and 4).

Effect of silencing of *ARPC5* and *GSTP1* on cell proliferation in PC10

To examine the functional role of *ARPC5* and *GSTP1*, we performed loss-of-function studies in PC10 cells using two different si-RNAs for each gene. The ARPC and GSTP1 levels of mRNA and protein expression were markedly repressed by each si-RNA treatment (Figures 5a, b, 6a and b). The XTT assay revealed significant inhibition of cell proliferation in each of the si-RNA transfectants in comparison with controls (% of cell viability, si-*ARPC5*; 55.6 ± 4.9, 35.6 ± 2.1 and 100.0 ± 9.3, respectively, $P<0.0001$; Figure 5c, si-*GSTP1*; 60.9 ± 4.6, 69.3 ± 7.6 and 100.0 ± 9.0, respectively, $P<0.0001$; Figure 6c).

DISCUSSION

MiRNAs are a new class of small RNAs that regulate the expression of many genes, and a large number of studies have shown that miRNAs are aberrantly expressed in many types of human cancers.[20] MiRNA expression profiles of NSCLC have also been reported by many researchers.[21,22] However, few studies describe similar analysis of lung-SCC, one of the most lethal malignancies in the world, with a 5-year survival rate of approximately 60% after curative surgery. Increased understanding of the molecular pathways involved in lung-SCC carcinogenesis would help improve diagnosis and therapy of the disease.

We screened 665 miRNAs, from lung-SCC and paired normal epithelial specimens, using stem-loop RT–PCR and identified 21 miRNAs downregulated in cancer tissues. To measure the effectiveness of our miRNA expression signature, we compared our data with the lists of downregulated miRNAs from past reports. Several genes (*miR-30a-3p*, *miR-126*, *miR-574*-3p and *miR-320*) were concordantly identi-

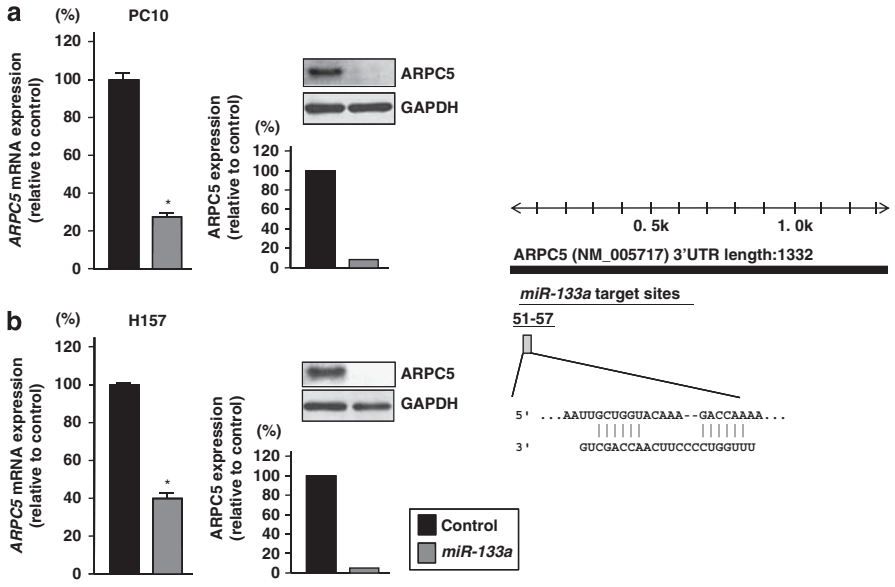

Figure 3 *miR-133a* regulates ARPC5 expression in lung-SCC cell lines. (**a**) Both mRNA and protein expression levels of *ARPC5* were significantly repressed in *miR-133a*-transfected PC10 cells. An internal control was *GUSB* for RT-PCR, and GAPDH for western blot analysis. (**b**) Both mRNA and protein expression levels of ARPC5 were significantly repressed in *miR-133a*-transfected H157 cells. $*P<0.05$. Predicted target sites of *miR-133a* in *ARPC5* in the 3′UTR region are shown (right).

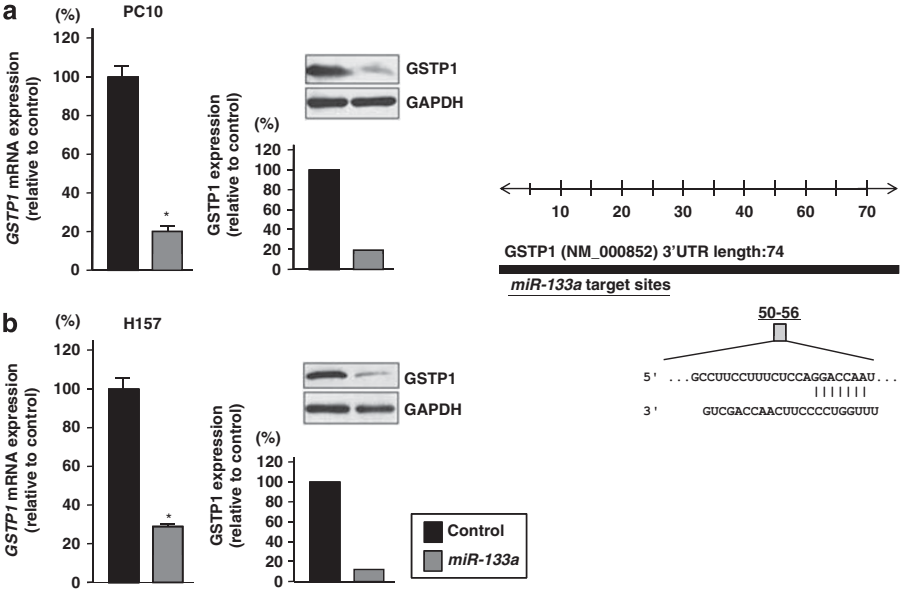

Figure 4 *miR-133a* regulates GSTP1 expression in lung-SCC cell lines. (**a**) Both mRNA and protein expression levels of GSTP1 were significantly repressed in *miR-133a*-transfected PC10 cells. An internal control was *GUSB* for RT-PCR, and GAPDH for western blot analysis. (**b**) Both mRNA and protein expression levels of GSTP1 were significantly repressed in *miR-133a*-transfected H157 cells. $*P<0.05$. Predicted target sites of *miR-133a* in *GSTP1* in the 3′UTR region are shown (right).

fied in our study and that of a recent report that analyzed miRNA profiles of lung-SCC.[23] Our data suggest that downregulated miRNAs can function as tumor suppressor genes and contribute to lung-SCC oncogenesis. Future experiments analyzing the function of miRNA regulation of gene expression in lung-SCC are likely to improve the understanding of mechanisms underlying this deadly disease.

In our expression signature, *miR-133a* is the most downregulated miRNA and expression studies revealed a reduction in miR-133a levels in many types of cancers.[14,18,24–27] Very interestingly, our recent reports of head and neck squamous cell carcinoma (SCC) and esophageal SCC showed that *miR-133a* is also significantly reduced in cancer tissues compared with normal epithelium.[14,17] The functional significance of *miR-133a* was investigated using head and neck SCC, esophageal SCC and bladder cell lines, and our data showed that restoration of *miR-133a* expression inhibited cancer cell proliferation, invasion and migration, and directly regulated actin-

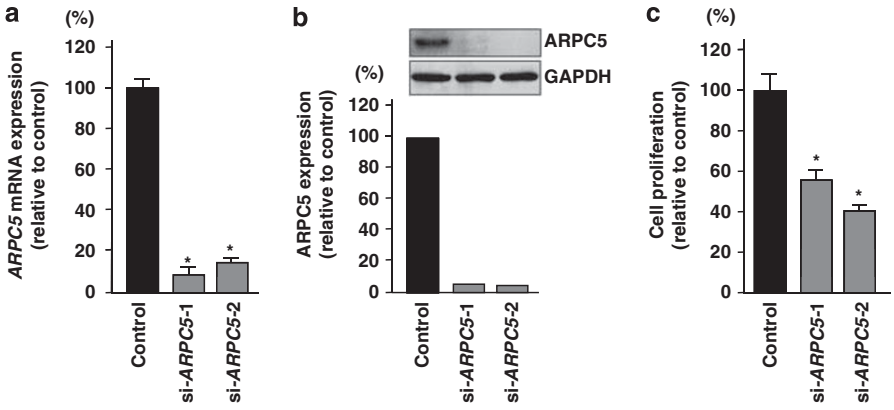

Figure 5 Effects of *ARPC5* silencing in lung-SCC cell line PC10. (**a**) Expression of *ARPC5* mRNA was significantly repressed by two si-*ARPC5* transfectants. *GUSB* was used as an internal control. (**b**) Expression of ARPC5 protein was repressed in two si-*ARPC5* transfectants. GAPDH was used as an internal control. (**c**) Cancer cell proliferation was determined with the XTT assay 72 h post transfection. The XTT assay showed significant inhibition of cell proliferation in two si-*ARPC5* transfectants. $*P<0.0001$.

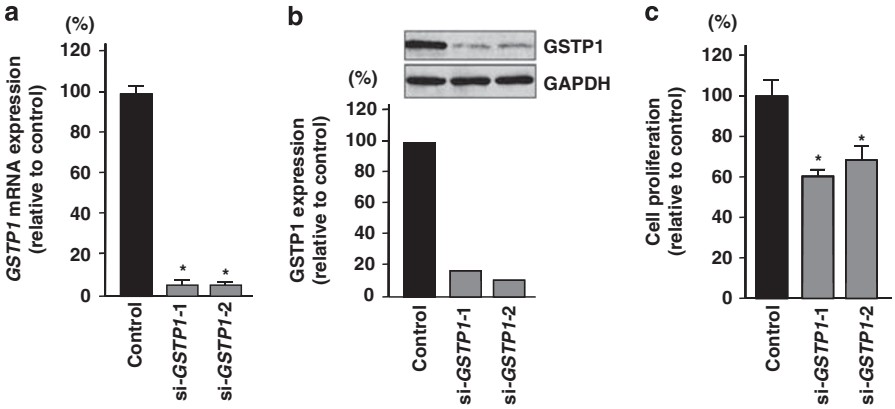

Figure 6 Effects of *GSTP1* silencing in lung-SCC cell line PC10. (**a**) Expression of *GSTP1* mRNA was significantly repressed in two si-*GSTP1* transfectants. *GUSB* was used as an internal control. (**b**) Expression of GSTP1 protein was repressed in two si-*GSTP1* transfectants. GAPDH was used as an internal control. (**c**) Cancer cell proliferation was determined with the XTT assay after 72 h post transfection. The XTT assay showed significant inhibition of cell proliferation in two si-*GSTP1* transfectants. $*P<0.0001$.

related genes such as *FSCN1*, *LASP1* and *TAGLN2*.[13,14,18,28] The identification of *miR-133a* regulation of cancer pathways could provide new insights into potential mechanisms of human carcinogenesis.

In this study, we focused on the functional significance of *miR-133a*, because the role of this miRNA in lung-SCC is not well understood. First, we confirmed the reduction of *miR-133a* expression in clinical specimens. Furthermore, we show that restoration of *miR-133a* in cancer cells significantly inhibited cell proliferation, suggesting that *miR-133a* functions as a tumor suppressor in lung-SCC as well as other cancers. To investigate the messenger RNA–miRNA networks in lung-SCC, we adopted a method of genome-wide gene expression analysis in PC10 and H157 cells, using *miR-133a* transfectants to identify oncogenic targets. We selected four candidate target genes, *transgelin 2* (*TAGLN2*), *actin-related protein2/3 complex, subunit 5, 16kDa* (*ARPC5*), *LAG1 homolog, ceramide synthase 2* (*LASS2*) and *glutathione S-transferase pi 1* (*GSTP1*), from our expression profile. Two genes (*ARPC5* and *GSTP1*) are upregulated in cancer tissues and were analyzed in detail.

This is the first report that *ARPC5* is regulated by *miR-133a* in lung-SCC cells. Actin is rapidly formed in response to specific cellular signals that converge on the Arp2/3 complex to regulate assembly.[29] The human Arp2/3 complex is composed of seven subunits including ARPC5, the smallest subunit.[30] We found that increased expression of *ARPC5* gene in lung-SCC correlated with reduced levels of *miR-133a*. A TargetScan database search revealed that *ARPC5* is a candidate target of *miR-133a*. ARPC5 mRNA and protein expression is reduced in *miR-133a* transfectant cell lines, indicating that *miR-133a* regulates *ARPC5* levels in lung-SCC cells. Silencing of *ARPC5* reduced cancer cell proliferation in PC10 cells, suggesting that *ARPC5* might contribute to lung-SCC cancer development. Thus, *ARPC5* functions as an oncogene and may represent a new diagnostic marker and therapeutic target for the treatment of lung-SCC. In addition, we previously performed genome-wide gene expression analysis using *miR-133a* transfectants derived from head and neck SCC and bladder cancer cell lines. According to the expression signatures, *ARPC5* is a candidate of *miR-133a* targets in cancer cells. This result suggests that *miR-133a–ARPC5* pathway is universally important to cancer cell. These microarray data are registered in Gene Expression Omnibus database, accession numbers GSE 19717, 20028 and 26032.

GSTP1, a member of the GST enzyme superfamily, catalyzes the conjugation of electrophiles to glutathione in the process of detoxification.[31] GSTP1 protein has several critical roles in both normal and neoplastic cells, including phase II xenobiotic metabolism, stress

responses, signaling and apoptosis. Over-expression of GSTP1 has been observed in many types of cancer, including lung cancer.[32–34] More recently, we demonstrated that *miR-133a* functions as a tumor suppressor and directly regulates *GSTP1* in head and neck SCC[16] and bladder cancer.[35] This analysis suggests that a similar regulation occurs in lung-SCC and may indicate that regulation of *GSTP1* by the tumor suppressor *miR-133a* is a common feature of human SCC.

In conclusion, the reduction of *miR-133a* was a frequent event in lung-SCC cancer cells. *miR-133a* may function as a tumor suppressor and it regulates *ARPC5* and *GSTP1*. Both of these genes were upregulated in lung-SCC specimens. The *miR-133a*-regulated novel cancer pathways could provide new insights into molecular mechanisms in lung-SCC and might contribute to the development of new therapeutic strategy for the disease.

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