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ORIGINAL ARTICLE

The microRNA expression signature of small cell lung cancer: tumor suppressors of *miR-27a-5p* and *miR-34b-3p* and their targeted oncogenes

Keiko Mizuno[1,4], Hiroko Mataki[1,4], Takayuki Arai[2], Atsushi Okato[2], Kazuto Kamikawaji[1], Tomohiro Kumamoto[1], Tsubasa Hiraki[3], Kazuhito Hatanaka[3], Hiromasa Inoue[1] and Naohiko Seki[2]

Small cell lung cancer (SCLC) constitutes approximately 15% of all diagnosed lung cancers. SCLC is a particularly lethal malignancy, as the 2-year survival rate after appropriate treatment is less than 5%. The patients with SCLC have not been received a benefit of the recently developed molecular targeted treatment. Therefore, a new treatment strategy is necessary for the patients. The molecular mechanisms underlying the aggressiveness of SCLC cells and their development of treatment-resistance are still ambiguous. In this study, we newly constructed a microRNA (miRNA) expression signature of SCLC by analysis of autopsy specimens. Based on the resultant signature, four miRNAs (*miR-27a-5p*, *miR-485-3p*, *miR-34-5p* and *miR-574-3p*) were found to be candidate anti-tumor miRNAs. To investigate their functional importance, we first validated the downregulation of *miR-27a-5p* and *miR-34b-3p* in SCLC clinical specimens. Next, we demonstrated that ectopic expression of both *miR-27a-5p* and *miR-34b-3p* significantly inhibited cancer cell aggressiveness. Our *in silico* analyses showed that four genes (topoisomerase 2 alpha (*TOP2A*), maternal embryonic leucine zipper kinase (*MELK*), centromere protein F (*CENPF*) and SRY-box 1 (*SOX1*) were identified as *miR-27a-5p*- and *miR-34b-3p*-regulated genes. Based on immunohistochemical analysis, TOP2A, MELK and CENPF were involved in SCLC pathogenesis. These genes might contribute to high proliferation and early metastatic spread of SCLC cells. Elucidation of differentially expressed miRNA-mediated cancer pathways based on SCLC signature may provide new insights into the mechanisms of SCLC pathogenesis.
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INTRODUCTION

Small cell lung cancer (SCLC) constitutes approximately 15–20% of all diagnosed lung cancers.[1,2] Owing to its aggressive nature, SCLC is a particularly lethal malignancy. SCLC cells characteristically acquire rapid cell proliferation and the ability to metastasize to distant sites. The majority of patients with SCLC present with metastatic disease, and the median survival time with combination chemotherapy is under 1 year for patients with extensive disease.[1,2] The conventional first-line treatment for SCLC with extensive disease is platinum-based chemotherapy.[3–5] Although the response rate of the treatment is good (70–80%), cancer cells acquire early resistance to conventional treatments. Median progression-free survival is 5–6 months,[3–5] and the disease escalates its aggressiveness. The patients with SCLC have not been received a benefit of the recently developed molecular targeted treatment. Therefore, understanding the molecular mechanisms of SCLC aggressiveness through current genomic approaches is needed.

The discovery of non-coding RNA in the human genome provided new directions for the study of human cancer pathogenesis.[6] MicroRNAs (miRNAs) belong to a member of non-coding RNAs that act as sequence-specific fine tuners of the expression levels of proteins and RNAs.[7,8] A single miRNA can regulate a large number of RNA transcripts in human cells.[9] Thus, aberrantly expressed miRNAs cause disruption of tightly regulated RNA networks, leading to pathologic behavior of cancer cells.[10,11] Currently, numerous studies have indicated that aberrantly expressed miRNAs are deeply involved in cancer pathogenesis.[10,11]

We have been revealed the anti-tumor miRNAs and their controlled cancer pathways by using miRNA expression signatures of several cancers, including lung cancer.[12–14] The next challenge in our miRNA studies is to identify key molecules and novel pathways involved in the resistance of cancer cells to current treatments. Based on this, we have constructed miRNA expression signatures by analyzing autopsy specimens from patients with prostate cancer and renal cell carcinoma.[15,16] Based on these signatures, we previously identified tumor-suppressive *miR-221/222*-mediated castration-resistant prostate cancer pathways and *miR-101*-mediated sunitinib-resistant pathways.[15,16]

[1]Department of Pulmonary Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan; [2]Department of Functional Genomics, Chiba University Graduate School of Medicine, Chiba, Japan and [3]Department of Pathology, Field of Oncology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan
[4]These authors contributed equally to this work.
Correspondence: Dr N Seki, Department of Functional Genomics, Chiba University Graduate School of Medicine, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan.
E-mail: naoseki@faculty.chiba-u.jp
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The molecular mechanisms underlying the aggressiveness of SCLC cells and the development of therapy-resistance are still ambiguous. Thus, we have investigated the molecular pathways contributing to treatment-resistant cancer cells in an effort to develop the new therapeutic strategies in SCLC. In the present study, we newly constructed miRNA expression signatures through analysis of primary and metastatic lesions (liver and brain). Based on the signatures, we identified two tumor-suppressive miRNAs (*miR-27a-5p* and *miR-34b-3p*) that are deeply involved in SCLC pathogenesis. Elucidation of the miRNA signature of SCLC may be useful for identification of novel molecular mechanisms of SCLC recurrence, metastasis and drug resistance.

MATERIALS AND METHODS

Patients and clinical lung cancer specimens

Clinical lung specimens were obtained from patients admitted to the Kagoshima University Hospital from 2011 to 2015. The patients' backgrounds and clinical characteristics are summarized in Table 1 and Supplementary Table S1. Normal tissues are summarized in Supplementary Table S2. Archival formalin-fixed, paraffin-embedded samples were used for expression analysis and immunohistochemistry. Clinical specimens were staged according to the International Association for the Study of Lung Cancer TNM classification.[17] This protocol was approved by the Institutional Review Board for Clinical Research of Kagoshima University School of Medicine.

Construction of the miRNA expression signature of SCLC based on autopsy specimens

A patient (64-year-old Japanese man) who died of SCLC underwent an autopsy. The patient had an excessive tobacco-smoking custom (90 pack years). His father had also died of lung cancer. Immunohistochemical examination demonstrated consistent expression of the neuron-specific antigen (synaptophysin and CD56). Specimens were obtained from primary lung lesions and metastatic liver and brain lesions. The clinical course of the patient is summarized in Figure 1.

Expression of miRNA patterns were analyzed by the TaqMan LDA Human microRNA Panel v2.0 (Applied Biosystems, Foster City, CA, USA). The assay procedure was described previously.[14] 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 carried out with *RNU48*.

Table 1 Characteristic of patients

	SCLC, n *(%)*	*NSCLC*, n *(%)*	*Non-cancerous tissue*, n *(%)*
Total number	11	52	27
Median age (range)	69 (64–86)	71 (50–88)	69 (50–88)
Gender			
Male	10 (90.9)	41 (78.8)	25 (92.0)
Female	1 (9.1)	11 (21.2)	2 (8.0)
Pathological tumor stage			
IA	1 (9.1)	6 (11.5)	
IB	1 (9.1)	13 (25.0)	
IIA	1 (9.1)	13 (25.0)	
IIB	1 (9.1)	7 (13.5)	
IIIA	0	12 (23.1)	
IIIB	0	1 (1.9)	
IV	7 (63.6)	0	

Cell lines, RNA isolation

Human SCLC cell lines (SBC-3 and NCI-H466 cells) were obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan) and the American Type Culture Collection (Manassas, VA, USA), respectively.

Total RNA was isolated from cultured cells using Isogen (Nippon Gene, Tokyo, Japan). Total RNA was obtained from formalin-fixed, paraffin-embedded human clinical specimens using Recover All Total Nucleic Acid Isolation kit (Ambion, Austin, TX, USA) as described previously.[18–20]

Quantitative real-time reverse transcription-PCR

The procedure for PCR quantification was described previously.[18–20] Stem–loop reverse transcription-PCRs for *miR-27a-5p* (P/N: 002445; Applied Biosystems), *miR-485-3p* (P/N: 001277), *miR-34b-3p* (P/N: 002102) and *miR-574-3p* (P/N: 002349) were used in this study. Human *GUSB* (P/N: Hs99999908_m1; Applied Biosystems) or *RNU48* (P/N: 001006; Applied Biosystems) were used to normalize the data for quantification of mRNA and miRNAs, respectively.

Transfection with miRNA mimic and cell proliferation, migration and invasion assays

The following mature miRNA species were used in the present study: Pre-miR miRNA precursors (*hsa-miR-27a-5p*, P/N: AM 13096; *hsa-miR-485-3p*, P/N: AM 10799; *hsa-miR-34b-3p*, P/N: AM 12727; *hsa-miR-574-3p*, P/N: AM 12848; Applied Biosystems) and negative control miRNA, P/N: AM 17111; Applied Biosystems. RNAs were incubated with OPTI-MEM (Invitrogen, Carlsbad, CA, USA) and Lipofectamine RNAiMAX reagent (Invitrogen) as described previously.[18–20]

Cells were transfected with 10 nM miRNAs by reverse transfection as described previously.[18–20] Cell migration assays and cell invasion assays were performed using modified Boyden chambers with 8 μm pores in 24-well tissue culture plates. Chambers for cell invasion assays consisted of Transwell-precoated Matrigel membrane filter inserts (BD Biosciences, Bedford, MA, USA). After 48 h of transfection, cells were plated in 24-well plates at 4×10^5 cells (SBC-3 cells) and 2×10^5 cells (NCI-H446 cells) per well, respectively. All experiments were performed in three independent trials.

Identification of oncogenic genes targeted by tumor-suppressive miRNAs

To identify genes putatively targeted by *miR-27a-5p* and *miR-34b-3p*, we performed *in silico* analysis using the TargetScan database and GEO expression data. First, we screened *miR-27a-5p*- and *miR-34b-3p*-targeted genes using the TargetScan database (release 7.1: http://www.targetscan.org/vert_71/). Next, we paired down the lists of genes based on a publicly available gene expression data set in a GEO database (accession number: GSE43346). Finally, we identified common genes targeted by *miR-27a-5p* and *miR-34b-3p*.

Immunohistochemistry

Three tissue autopsy specimens were used: SCLC lung tissue in the primary lesion and two metastatic tissues from the liver and brain. Specimens were immunostained following the manufacturer's protocol with the Ultra-Vision Detection System (Thermo Scientific, Fremont, CA, USA). For immunohistochemistry, we used primary rabbit polyclonal antibodies against the following: TOP2A (1:200, HPA006458; Sigma-Aldrich, St Louis, MO, USA), MELK (1:200, HPA017214; Sigma-Aldrich), CENPF (1:400, ab5; Abcam, Cambridge, UK) and SOX1 (1:500, ab87775; Abcam). The procedure was carried out as described previously.[18,19]

Statistics

The relationships between two groups and the numerical values obtained by real-time reverse transcription-PCR were analyzed using Mann–Whitney U-tests. The relationships among more than three variables and numerical values were analyzed using the Bonferroni-adjusted Mann–Whitney U-test.

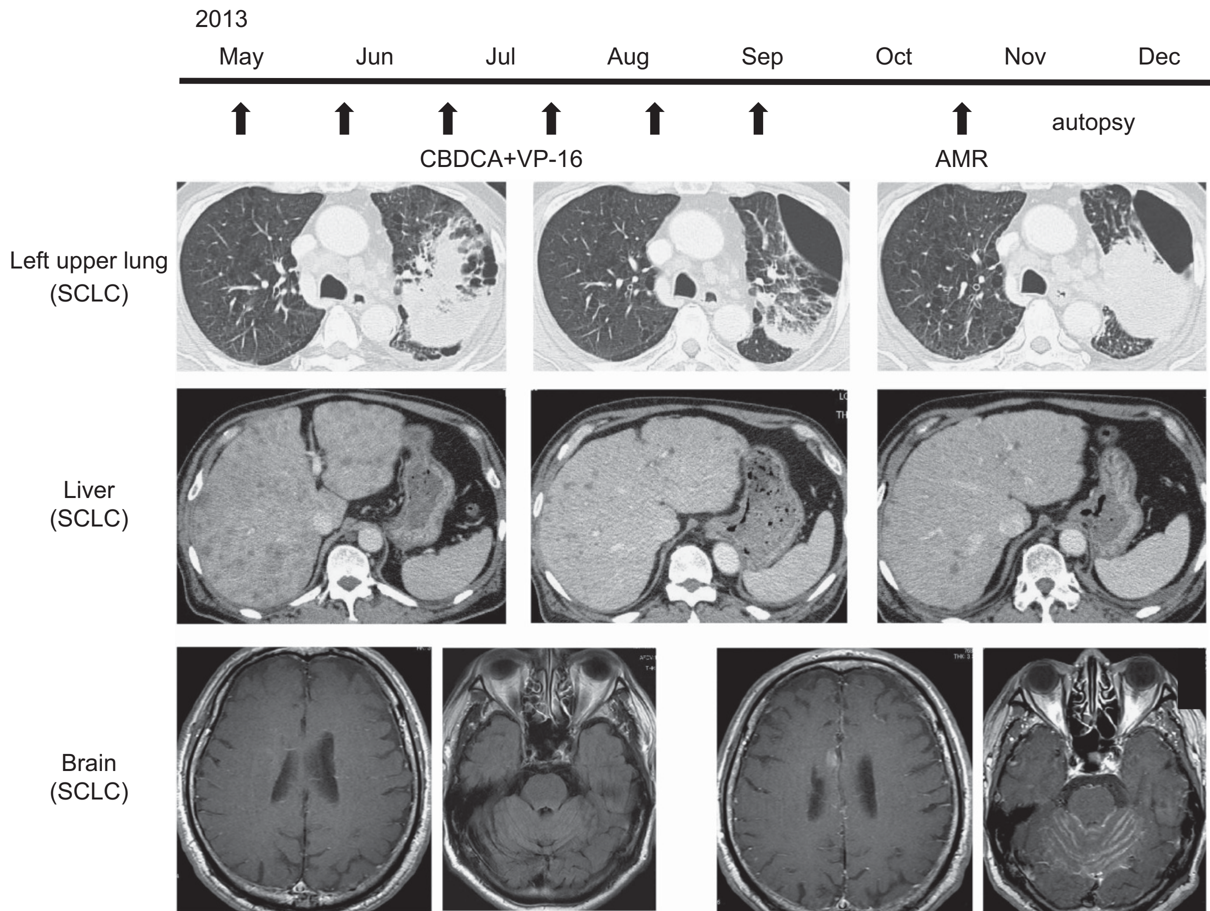

Figure 1 Clinical course of the SCLC patient who provided autopsy tissues. In May 2013, a 64-year-old Japanese man was admitted with increased shortness of breath. Consolidation was found in the left upper lung, and wall thickness of the pulmonary bulla was found in the right lower field in chest radiographs. He was diagnosed with lung synchronous carcinoma with multiple liver metastases. Bronchoscopic biopsy revealed small lung cell cancer (SCLC) from left B^{1+2} and squamous cell carcinoma from right B^6. These synchronous carcinomas responded to first-line chemotherapy CBDCA+VP-16 (a total of six courses). But consolidation of the left upper lung (SCLC) worsened only 3 weeks after the last course of chemotherapy. Chemotherapy was changed to amrubicin, but disturbance of consciousness gradually appeared and worsened. Magnetic resonance imaging (MRI) findings and cytology of the cerebrospinal fluid (SCLC) were consistent with brain metastasis and meningeal carcinomatosis. In November 2013, he died, and the patient underwent autopsy. We obtained SCLC tissues of the left upper lung, liver metastasis and brain metastasis.

RESULTS

Construction of the miRNA expression signature of SCLC specimens

Using a PCR-based array system, we analyzed differentially expressed miRNAs in the primary SCLC lesion and metastatic lesions (liver and brain) and compared them with non-cancerous lesions. Based on this analysis, we listed the top 35 downregulated miRNAs in SCLC tissues (Table 2). Among them, we focused on four miRNAs (*miR-27a-5p*, *miR-485-3p, miR-34b-3p* and *miR-574-3p*) that were significantly downregulated in SCLC specimens.

Expression levels of *miR-27a-5p, miR-485-3p, miR-34b-3p* and *miR-574-3p* in lung cancer clinical specimens and SCLC cell lines

We evaluated the expression levels of four downregulated miRNAs (*miR-27a-5p*, *miR-485-3p, miR-34b-3p* and *miR-574-3p*) in non-cancerous ($n=27$), SCLC ($n=11$) and NSCLC ($n=52$) clinical specimens and in cell lines (SBC-3 and NCI-H446). The expression levels of *miR-27a-5p* and *miR-34b-3p* were significantly reduced in SCLC specimens compared with non-cancerous specimens (Figure 2). These miRNAs expression levels were also low in both SCLC cell lines (SBC-3 and NCI-H446).

Effects of restoring *miR-27a-5p, miR-485-3p, miR-34b-3p* and *miR-574-3p* on cell proliferation, migration and invasion in SCLC cell lines

To investigate the functional roles of miRNAs in SCLC, we performed gain-of-function studies in SBC-3 and NCI-H446 cells by transfecting the cells with miRNA mimics. XTT assays showed that cell proliferation was significantly inhibited in *miR-27a-3p* transfectants of SBC-3 in comparison with the mock, although inhibition was not seen in NCI-H446. Additionally *miR-34b-3p* transfection significantly inhibited the proliferation of both cell lines in comparison with mock. Furthermore, cell proliferation was not inhibited in *miR-485-3p* and *miR-574-3p* transfectants of SBC-3 compared with mock, whereas it was significantly inhibited in NCI-H446 transfectants (Figure 3a).

In vitro assays showed that cell migration was inhibited in *miR-485-3p* transfectants of SBC-3 in comparison with the mock, whereas it was not inhibited in NCI-H446 transfectants. *miR-34b-3p* transfection significantly inhibited cell migration in both cell lines compared with mock. Cell migration activity was not inhibited in *miR-574-3p* transfectants of SBC-3 compared with mock, whereas it was significantly inhibited in NCI-H446 transfectants (Figure 3b).

Table 2 Downregulated miRNAs in SCLC tissues (versus normal lung tissues)

miRNA	*Log2 ratio (tumor/normal)*	*ΔΔCT*		P-value
		Normal lung tissues	*SCLC tissues*	
hsa-miR-15b-3p	−6.35	0.00051	6.26E-06	0.06680
hsa-miR-27a-5p	−4.70	0.00266	1.02E-04	0.03882
hsa-miR-663b	−4.63	0.00016	6.26E-06	0.27151
hsa-miR-519e-3p	−4.39	0.00013	6.26E-06	0.37846
hsa-miR-485-3p	−4.31	0.02718	1.37E-03	0.00437
hsa-miR-133a-3p	−3.96	0.05075	3.25E-03	0.15258
hsa-miR-34b-3p	−3.76	0.00877	6.47E-04	0.04624
hsa-miR-518e-3p	−3.57	0.00007	6.26E-06	0.31669
hsa-miR-486-5p	−3.41	0.09715	9.14E-03	0.23060
hsa-miR-566	−3.35	0.00006	6.26E-06	0.38202
hsa-miR-34c-5p	−2.92	0.00005	6.26E-06	0.05300
hsa-miR-1251	−2.81	0.00004	6.26E-06	0.25194
hsa-miR-618	−2.74	0.00094	1.40E-04	0.45331
hsa-miR-25-5p	−2.52	0.00004	6.26E-06	0.37351
hsa-miR-190b	−1.99	0.00002	6.26E-06	0.22934
hsa-miR-372	−1.98	0.00002	6.26E-06	0.22881
hsa-miR-489-3p	−1.98	0.00391	9.91E-04	0.12141
hsa-miR-1226-5p	−1.92	0.00004	9.84E-06	0.38976
hsa-miR-520g	−1.87	0.00002	6.26E-06	0.29067
hsa-miR-497-3p	−1.86	0.00002	6.26E-06	0.28960
hsa-miR-220b	−1.85	0.00002	6.26E-06	0.28853
hsa-miR-490-3p	−1.83	0.00002	6.26E-06	0.28672
hsa-miR-574-3p	−1.75	0.31271	9.31E-02	0.03250
hsa-miR-361-3p	−1.70	0.00002	6.26E-06	0.35344
hsa-miR-200c-5p	−1.70	0.00002	6.26E-06	0.35343
hsa-miR-1247-5p	−1.62	0.01007	3.28E-03	0.12521
hsa-miR-140-3p	−1.57	0.04628	1.56E-02	0.06092
hsa-miR-146b-5p	−1.50	0.50231	1.78E-01	0.16896
hsa-miR-522-3p	−1.39	0.00016	6.12E-05	0.16982
hsa-miR-10b-3p	−1.33	0.00071	2.84E-04	0.39806
hsa-miR-99b-3p	−1.25	0.00973	4.09E-03	0.11262
hsa-miR-517a-3p	−1.13	0.00010	4.60E-05	0.44499
hsa-miR-145-5p	−1.05	0.14148	6.81E-02	0.21012
hsa-miR-223-5p	−1.04	0.00092	4.48E-04	0.36009
hsa-miR-150-5p	−1.00	0.23346	1.17E-01	0.37179

Finally, Matrigel invasion assays demonstrated that cell invasive activity was significantly inhibited in *miR-27a-3p*, *miR-485-3p* and *miR-574-3p* transfectants of SBC-3 in comparison with the mock. Inhibition was not seen in NCI-H446 transfectants. Finally, *miR-34b-3p* transfection significantly inhibited cell invasion in both cell lines (Figure 3c).

Identification of putative target genes regulated by *miR-27a-5p* and *miR-34b-3p* in SCLC

To identify putative target genes subjected to *miR-27a-5p* and *miR-34b-3p* regulation, we performed *in silico* analysis using the TargetScan database and GEO expression data. First, we screened *miR-27a-5p*- and *miR-34b-3p*-targeted genes using the TargetScan database (release 7.1: http://www.targetscan.org/vert_71/). We found 3238 and 4165 genes that had putative target sites in their 3′-UTRs for *miR-27a-5p* and for *miR-34b-3p*, respectively. Second, we paired down the lists of genes based on a publicly available gene expression data set in the GEO database (accession number: GSE43346) and identified 7 and 17 genes, respectively. Finally, we selected the following four common genes from those lists: *TOP2A*, *MELK*, *CENPF* and *SOX1*. The flow chart outlining our strategy for identification of putative target genes of *miR-27a-5p* and *miR-34b-3p* is shown in Figure 4.

Immunohistochemical staining of miR-targeted proteins (TOP2A, MELK, CENPF and SOX1) in SCLC clinical specimens

To validate expression of TOP2A, MELK, CENPF and SOX1 proteins, immunohistochemistry was used to assess SCLC autopsy specimens. Immunohistochemical staining demonstrated overexpression of TOP2A in the nuclei of the primary lesion and liver metastasis (Figure 5a). The expression of MELK was high in the cytoplasm of all sites (Figure 5b). Also, the expression of CENPF was relatively high in the nuclei of the primary lesion (Figure 5c). However the expression of SOX1 was not observed in any site. Therefore, it appeared that *TOP2A*, *MELK* and *CENPF* play key roles as oncogenes regulated by *miR-27a-5p* and *miR-34b-3p*. *TOP2A* and *MELK* appeared to be particularly important in the development of SCLC.

DISCUSSION

SCLC is a highly aggressive cancer with a poor prognosis because most patients are diagnosed with extensive disease.[1,2] Treatment strategies for late stage SCLC are commonly platinum-based chemotherapy.[3,4] Although SCLC cells are initially very sensitive to cytotoxic chemotherapy, in most cases, SCLC cells acquire resistance to these treatments.[5] No effective treatments are approved for recurrence and the distant metastases of the disease. To improve the dismal prognosis of SCLC, effective treatment strategies are urgently needed. Uncovering the genomic characteristics of the disease might add new therapeutic targets.

A growing body of evidence has shown that dysregulated miRNAs are deeply involved in human oncogenesis, metastasis and drug resistance.[11] Aberrantly expressed miRNAs can destroy tightly controlled RNA networks and promote oncogenic development. In this study, we first identified dysregulated miRNAs based on miRNA expression signatures of autopsy specimens from a patient with SCLC. The patient had undergone several therapeutic treatments, thus we considered that these specimens were likely treatment-resistant SCLC cells.

Our present data showed that several miRNAs, such as *miR-27a-5p*, *miR-485-3p*, *miR-34b-3p* and *miR-574-3p*, were markedly downregulated in cancerous tissues based on profiles of SCLC autopsy specimens. Among these miRNAs, clinical specimens confirmed that the expression levels of *miR-27a-5p* and *miR-34b-3p* were significantly reduced in SCLC tissues compared with non-cancerous tissues. Additionally, ectopic expression of these two miRNAs significantly inhibited cancer cell aggressiveness, suggesting that *miR-27a-5p* and *miR-34b-3p* functioned as tumor suppressors in SCLC cells. Based on these results, we focused on these two miRNAs and explored the molecular networks that they regulated.

By miRNA database searching (miRBase; http://www.mirbase.org/), pre-*miR-27a* produces two types of mature miRNAs, *miR-27a-5p* and *miR-27a-3p*. The *miR-27a-5p* is passenger strand of pre-*miR-27a*, whereas *miR-27a-3p* is guide strand of it. Most of past articles focused on the functional significance of the *miR-27a-3p* in several cancers. The functional significance of *miR-27a-3p* has confusion in cancer cells, including lung cancer. Previous studies have shown that *miR-27a-3p* is frequently upregulated and plays functional roles in multiple tumor types, including pancreatic cancer, breast cancer, ovarian cancer, esophageal cancer, renal cell carcinoma, hepatocellular carcinoma, glioma and gastric cancer.[21] The guide

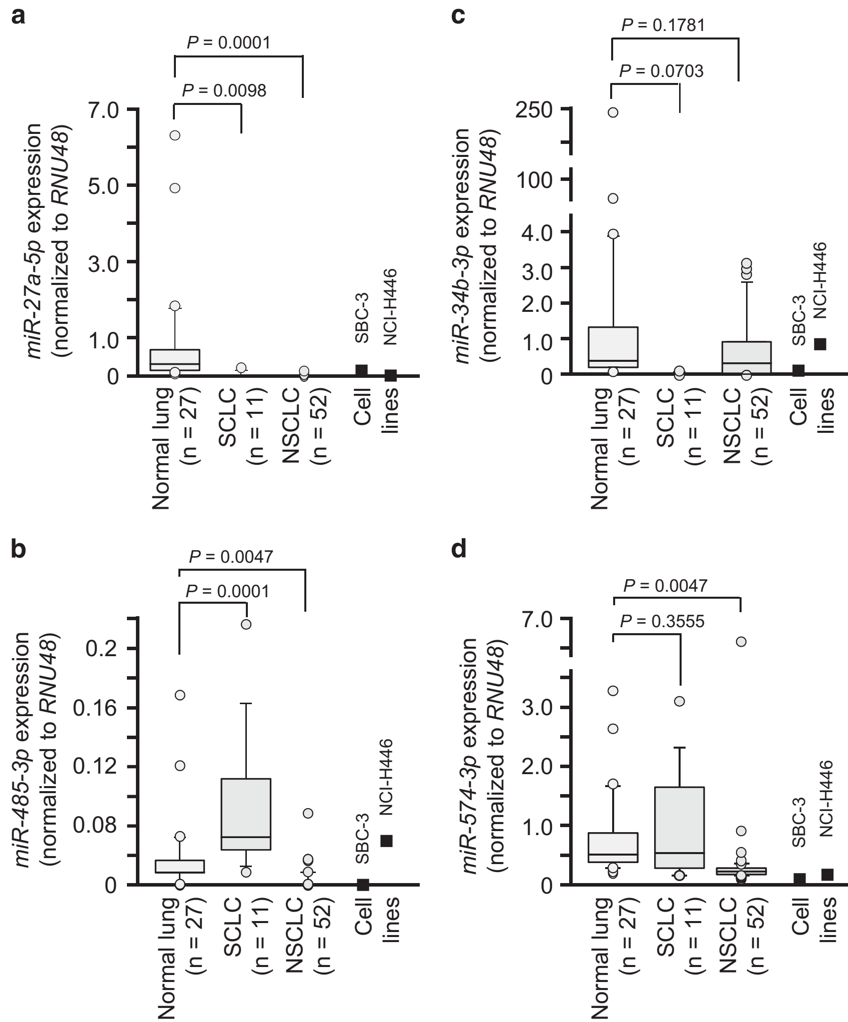

Figure 2 Expression levels of *miR-27a-5p*, *miR-485-3p*, *miR-34b-3p* and *miR-574-3p* in lung cancer clinical specimens and SCLC cell lines. RT-PCR showed that the expression levels of *miR-27a-5p* and *miR-34b-3p* were significantly lower in SCLC clinical specimens and cell lines than in non-cancerous lung tissues. *RNU48* was used as an internal control. Expression levels of (**a**) *miR-27a-5p*, (**b**) *miR-485-3p*, (**c**) *miR-34b-3p* and (**d**) *miR-574-3p*.

strand of *miR-27a-3p* promotes tumorigenesis in several types of cancer.[21,22] On the other hand, *miR-27a-3p* was directly regulated tyrosine kinase receptors, EGFR and MET in lung cancer.[23] Cancer stem cells is a promising target for cancer therapy in cases of cancer cell aggressiveness and drug resistance. Downregulation of *miR-27a-3p* was observed in sphere-forming cells in SCLC.[24] Inhibition of *miR-27a-3p* in parental cells enhanced stem-like properties of SCLC cells *in vitro*.[24]

The passenger strand of pre-*miR-27a* (*miR-27a-5p*) is downregulated in head and neck squamous cell carcinoma, and it acts as a tumor suppressor by targeting the EGFR signaling axis. *miR-27a-5p* simultaneously decreases expression of *EGFR*, *AKT1* and *mTOR*, leading to decreased solid tumor viability.[22] In the past established theory of miRNA biogenesis, the passenger strand of miRNA is degradation and not incorporated into RNA-induced silencing complex.[7] Surprisingly, our recent studies showed that *miR-145-3p* (passenger stand of pre-*miR-145*) actually functioned as anti-tumor miRNA in lung cancer and bladder cancer.[25,26] Similarly, we confirmed the anti-tumor function of *miR-139-3p* (passenger strand of pre-*miR-139*) in bladder cancer.[27] These findings indicate that some passenger strand of miRNAs have biological function in cells. The involvement of passenger strand miRNAs in the regulation of cellular processes is a novel concept in RNA research.

It is an important study theme to investigate molecular mechanisms of transcriptional control of miRNAs in cancer cells. Silencing mechanisms of *miR-27a-5p* remain largely undefined. A recent study showed that Twist-1, a transcription factor of epithelial–mesenchymal transition regulation, was negatively controlled by *miR-27a-3p* expression in hepatocellular carcinoma.[28] Other study showed that hepatocyte growth factor-mediated MET signal induced *miR-27a-3p* expression in lung cancer cells.[23] The further study is necessary to elucidate expression control of *miR-27a-5p* in SCLC.

The expression level of *miR-34b-3p* is decreased in several cancers, such as neuroblastoma and cervical cancer.[29,30] In neuroblastoma, *miR-34b-3p* functions as a tumor suppressor by targeting *CCNE2* and *E2F3*.[29] A representative tumor suppressor p53 regulated the expression control of several miRNAs. The *miR-34*-family (*miR-34a/b/c*) was direct targets of p53 regulation.[31] Expression of the *miR-34*-family caused antitumor effects, such as inducing apoptosis and cell cycle arrest. Therefore, cancer cells enhance inactivation of *miR-34*-family expression through CpG methylation.[31]

However, the functions of these miRNAs are still not fully understood. Based on those studies and our findings, we investigated

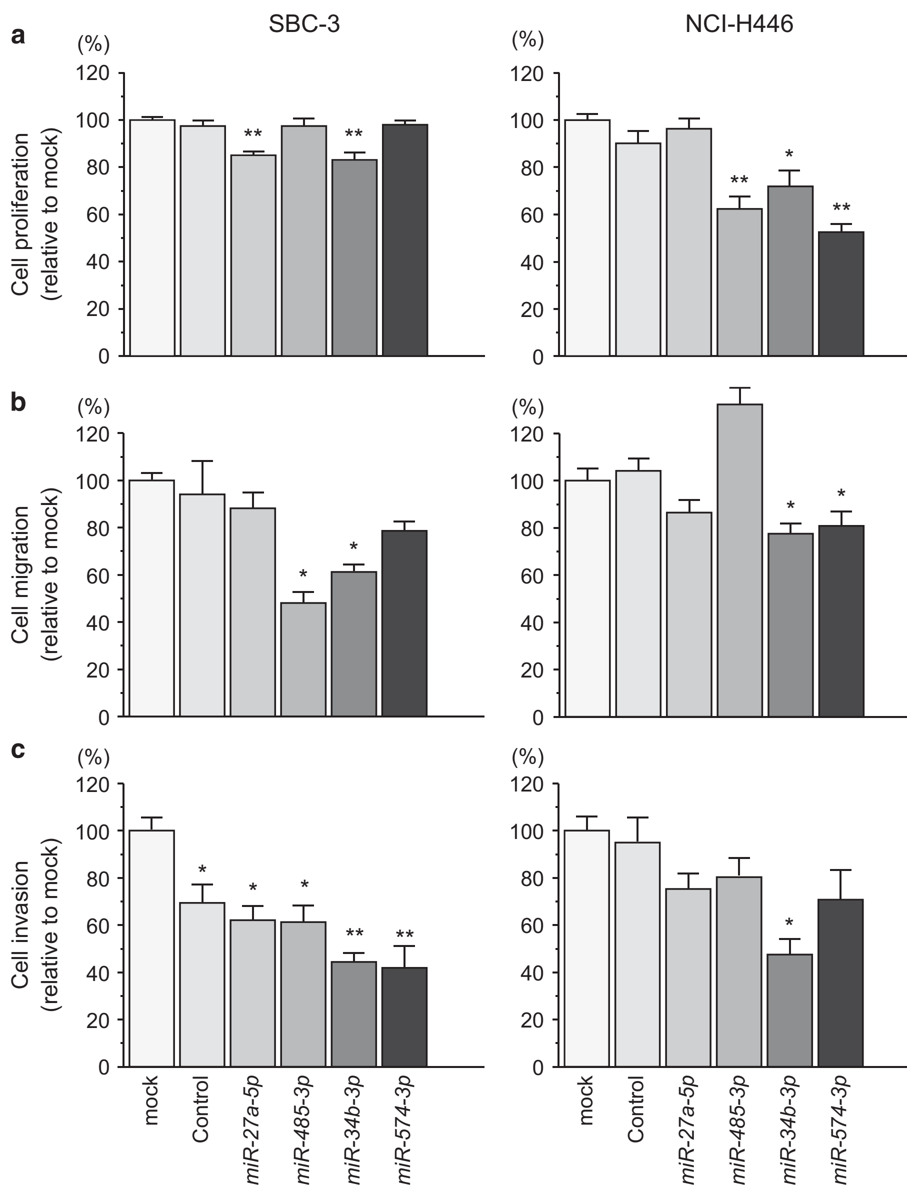

Figure 3 Functional significance of *miR-27a-5p*, *miR-485-3p*, *miR-34b-3p* and *miR-574-3p* in SCLC cell lines. (**a**) Cell proliferation was determined by XTT assays 96 h after transfection with four miRNAs at 10 nmol l^{-1} (*miR-27a-5p*, *miR-485-3p*, *miR-34b-3p* and *miR-574-3p*), miR-control or mock transfection. (**b**) Migration assays conducted 48 h after transfection. (**c**) Cell invasion assays conducted 48 h after transfection. $*P<0.0083$ and $**P<0.0001$.

the molecular networks regulated by the miRNAs that we identified to better understand the etiology of SCLC. We hypothesized that *miR-27a-5p* and *miR-34b-3p* might coordinately regulate target genes associated with SCLC pathogenesis. Therefore, we performed *in silico* analysis and identified four genes (*TOP2A*, *MELK*, *CENPF* and *SOX1*) that were potential targets of *miR-27a-5p* and *miR-34b-3p*. Immunohistochemistry indicated that MELK, TOP2A and CENPF play key roles in promoting oncogenesis. We suggest that TOP2A and MELK are particularly important in SCLC.

MELK is classified as a member of the SLK/AMPK serine–threonine kinase family and known as an embryonic and neural stem cell marker. It is associated with cell survival, proliferation and apoptosis in various cancers.[32,33] Several studies have reported a correlation between *MELK* gene expression and tumor malignancy grade for astrocytoma and breast cancer.[34,35] It was reported that *MELK* could play roles in cell cycle regulation (possibly in G0–G1 and S phases) as well as in responses against radiation and 5-FU treatment in colorectal cancer cells.[32] Inoue *et al.*[36] demonstrated that *MELK* was highly expressed in most SCLC cell lines and primary SCLC tumors. In this study, we demonstrated that the cancerous tissues of autopsy specimens that were presumably resistant to drug therapies stained strongly for MELK, suggesting that *MELK* was involved in resistance to chemotherapy in SCLC.

TOP2A is a subfamily of DNA topoisomerase type II that controls and alters the topologic states of DNA during transcription. Abnormal alterations of *TOP2A* include changes in gene copy number and gene expression level in cancer cells.[37] Aberrant expression of *TOP2A* is generally associated with poor prognosis in breast cancer, ovarian cancer, oral cancer, esophageal cancer and lung cancer.[38–40] In particular, multidrug resistance protein and TOP2A are involved in drug resistance in NSCLC.[41] We confirmed the upregulation of *TOP2A* in SCLC autopsy specimens by immunohistochemistry.

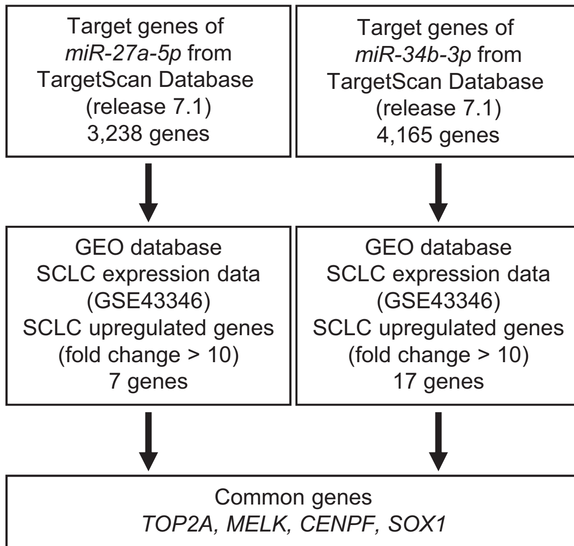

Figure 4 Strategy for identification of putative candidate genes controlled by *miR-27a-5p* and *miR-34b-3p* in SCLC. Outline of the identification of *miR-27a-5p* and *miR-34b-3p* target genes by *in silico* analysis of the TargetScan database and genome-wide gene expression analysis using a publicly available gene expression data set in the GEO database.

CENPF encodes a protein that associates with the centromere–kinetochore complex. This protein is a member of the centromere protein family and acts in a critical chromosomal segregation process, including kinetochore assembly and spindle checkpoint signaling during mitosis.[42] Overexpression of *CENPF* has been observed in prostate cancer and breast cancer.[42,43] Our recent study showed that downregulated tumor-suppressive *miR-205* enhanced prostate cancer aggressiveness through direct regulation of *CENPF*.[42]

In conclusion, the expression of *miR-27a-5p* and *miR-34b-3p* was downregulated in SCLC autopsy specimens. They both act as tumor suppressors in SCLC cells. Oncogenic *MELK*, *TOP2A* and *CENPF* are regulated by these miRNAs, and high expression of those oncogenes in SCLC autopsy specimens indicates clinical importance. Elucidation of *miR-27a-5p*- and *miR-34b-3p*-mediated molecular networks may lead to a better understanding of SCLC aggressiveness and the development of new treatment strategies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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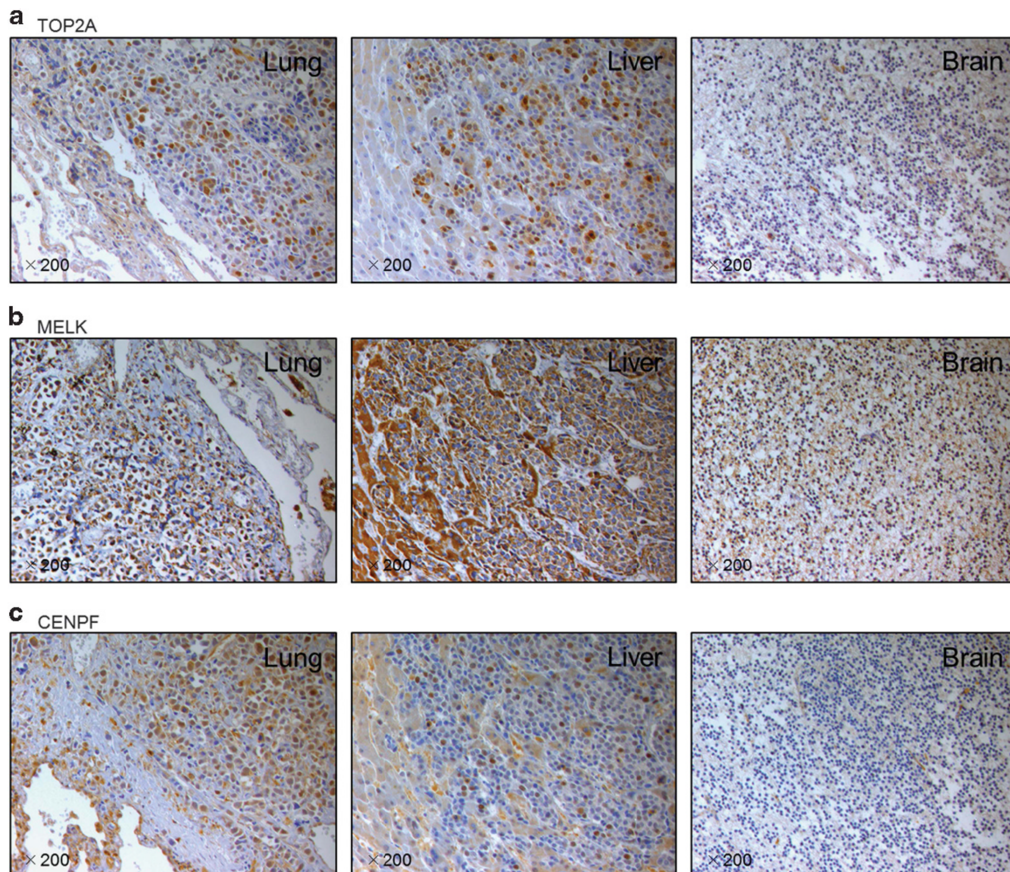

Figure 5 Immunohistochemical staining of TOP2A, MELK and CENPF in SCLC autopsy specimens. (**a**) Overexpression of TOP2A in the nucleus was observed in the primary lesion and liver metastasis. (**b**) The expression of MELK was high in the cytoplasm. (**c**) The expression of CENPF was relatively high in the nuclei of the primary lesion.

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