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Frequent methylation and oncogenic role of microRNA-34b/c in small-cell lung cancer

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ABSTRACT

Small-cell lung cancer (SCLC) is an aggressive tumor with a dismal prognosis among primary lung cancers. MicroRNAs (miRNAs) can act as oncogenes or tumor-suppressor genes in human malignancy. The miR-34 family is comprised of tumor-suppressive miRNAs, and its reduced expression by methylation has been reported in various cancers, including non-small cell lung cancer (NSCLC). In this study, we investigated the alteration and tumor-suppressive impact of miR-34s in SCLC. The methylation of miR-34a and miR-34b/c was observed in 4 (36%) and 7 (64%) of 11 SCLC cell lines, respectively. Among the 27 SCLC clinical specimens, miR-34a and miR-34b/c were methylated in 4 (15%) and 18 (67%), respectively. In contrast, 13 (28%) miR-34a methylated cases and 12 (26%) miR-34b/c methylated cases were found in 47 NSCLC primary tumors. The frequency of miR-34b/c methylation was significantly higher in SCLC than in NSCLC ($p < 0.001$). The expressions of miR-34s were reduced in methylated cell lines and tumors and restored after 5-aza-2′-deoxycytidine treatment, indicating that methylation was responsible for the reduced expression of miR-34s. Because the frequency of methylation was higher in miR-34b/c, we focused on miR-34b/c for a functional analysis. We examined the effect of miR-34b/c introduction on cell proliferation, migration and invasion. The transfection of miR-34b/c to two SCLC cell lines (H1048 and SBC5) resulted in the significant inhibition of cell growth, migration, and invasion, compared with control transfectants. Our results indicate that the aberrant methylation of miR-34b/c plays an important role in the pathogenesis of SCLC, implying that miR-34b/c may be a useful therapeutic target for SCLC.

1. Introduction

Lung cancer is the leading cause of cancer-related death in the world at present. Small-cell lung cancer (SCLC) is an aggressive tumor with a dismal prognosis among primary lung cancers and accounts for approximately 13% of all lung cancers [1,2]. Chemotherapy or chemoradiotherapy is generally the treatment of choice for SCLC patients because the clinical features of SCLC include a high rate of metastasis to lymph nodes and distant organs beginning at an early stage. A strong initial response but early acquired resistance to chemotherapeutic agents is a unique feature of SCLC [3,4], resulting in a poor prognosis and a 5-year survival rate of only 5% [5]. Thus, the development of new therapeutic strategies is mandatory.

The understanding of molecular pathogenesis is crucial for the development of new therapeutic strategies for malignant tumors. Of note, the profile of molecular alterations is quite different among the histological subtypes of lung cancer [6–8]. For example, epidermal growth factor receptor (EGFR) mutations [9], *K-ras* mutations and *P16* methylation [8] are frequently found in non-small cell lung cancer (NSCLC), but not in SCLC [10]. In contrast, *TP53* mutations [8,11] or *RASSF1A* methylation [12] is frequently found in SCLC, compared with NSCLC. These results indicate that the molecular pathogenesis differs between SCLC and NSCLC.

Many studies have shown that microRNAs (miRNAs) can act as oncogenes or tumor suppressors and that the widespread alteration of miRNA expression patterns is highly relevant to various human malignancies [13]. In lung cancer, the reduced expression of let-7 [14] or the overexpression of miR-155 [15] is reportedly correlated with the clinical outcome of NSCLC. Among the miRNAs, miRNA-34 (miR-34) family members reportedly play important tumor-suppressive roles, as they are directly regulated by p53 and

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compose the p53 network [16]. The members of the miR-34 family consist of three miRNAs (miR-34a, 34b and 34c), the target genes of which are considered to be similar to one another but with some notable differences [17]. miR-34a is located on chromosome 1q36.22, while miR-34b and 34c (miR-34b/c) are located on chromosome 11q23 and are generated by the processing of a single transcript [17]. A previous study indicated that miR-34 methylation was present in NSCLC and was significantly related to an unfavorable clinical outcome [18]. In a functional analysis the introduction of miR-34b/c into NSCLC suppressed the cell proliferation of NSCLC [19]. However, these studies examining the roles of miRNA in lung cancer have been mainly performed for NSCLC, and miRNA alterations and their impact on SCLC have not been investigated.

In this study, we investigated miR-34 methylation in SCLC and found that miR-34b/c was frequently methylated in SCLC, compared with NSCLC. Subsequently, we investigated the biological impact of miR-34b/c methylation on SCLC to understand the role of miR-34b/c in the tumorigenesis of SCLC.

2. Materials and methods

2.1. Clinical samples and cell lines

A total of lung cancer specimens comprising 12 SCLC, 47 NSCLC resected tumors, and 15 malignant pleural effusions in SCLC patients were retrieved from Okayama University Hospital, Okayama, Japan and NHO Yamaguchi-Ube Medical Center, Ube, Yamaguchi, Japan. Each resected tumor was not treated with preoperative chemotherapy or radiotherapy. Corresponding non-malignant tissue (peripheral lung or bronchial epithelium) of resected tumors were also available. Fifteen malignant pleural effusions were obtained from 8 patients who were treated by chemotherapy or radiotherapy and 7 patients who were not received any treatment. All the specimens were frozen with liquid nitrogen immediately after surgery and stored at −80 °C. Written informed consents were obtained from all the patients at two collection sites.

Eleven SCLC cell lines [NCI-H1048 (H1048), SBC5, HCC33, NCI-H211 (H211), NCI-H524 (H524), NCI-H841 (H841), NCI-H1688 (H1688), NCI-H1870 (H1870), NCI-H2141 (H2141), NCI-H82 (H82), NCI-H249 (H249)] and 14 NSCLC cell lines [PC-9, HCC827, NCI-H1975 (H1975), NCI-H3255 (H3255), A549, NCI-H1395 (H1395), NCI-H522 (H522), NCI-H838 (H838), HCC15, NCI-H125 (H125), NCI-H460 (H460), NCI-H661 (H661), NCI-H1299 (H1299), NCI-H358 (H358)] were used in this study. We also investigated the HBEC 5KT cell, established from non-malignant human bronchial cells. Cell lines whose prefix is NCI-H- (abbreviated as H-) or HCC-, and HBEC 5KT were kindly provided by Dr. Adi F. Gazdar (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX). SBC5 was obtained from Drs. M. Tanimoto and K. Kiura (Okayama University, Okayama, Japan). PC-9 was obtained from Immuno-Biological Laboratories (Takasaki, Gunma, Japan). A549 was obtained from American Type Culture Collection (Manassas, VA). All the cell lines except for HBEC 5KT were maintained in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. HBEC 5KT cell line was maintained in Keratinocyte-SFM (Invitrogen, Carlsbad, CA) with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF). H82, H249, H1048, and SBC5 were treated with 5-aza-2′-deoxycytidine (DAC) (Sigma–Aldrich) at a concentration of 5–10 μM for 6 days, with medium changes on days 1, 3, and 5, to restore gene expression that was reduced by methylation.

2.2. Methylation specific PCR (MSP) assay

Genomic DNA was extracted from cell lines and tissues by standard phenol–chloroform (1:1) extraction or by DNeasy Tissue Kit (Qiagen, Valencia, CA). DNA was subjected to bisulfate treatment using the EZ DNA Methylation Gold kit (Zymo Research Corp., Irvine, CA) according to the manufacturer's protocol. For malignant pleural effusions, genomic DNA was extracted using QIAamp Circulating Nucleic Acid kit (Qiagen) and bisulfite conversion was performed using Epitect Bisulfite kit (Qiagen) according to the manufacturer's protocol. The methylation statuses of miR-34a and 34b/c were determined by MSP assay using specific primers for the methylated and unmethylated forms [20]. The DNA from HBEC 5KT was treated with SssI methyltransferase (New England BioLabs, Beverly, MA) and was used as a positive control for methylated alleles. PCR products were analyzed in 2% agarose gels and stained with ethidium bromide.

2.3. miR-34s expression by quantitative RT-PCR

The miRNA was isolated from cell lines and tissue specimens with TaqMan MicroRNA Cells-to-CT™ Kit (Ambion, Austin, TX) and mirVana™ miRNA Isolation Kit (Ambion) and treated with DNase I (Ambion) to remove genomic DNA. Reverse transcriptional (RT) reaction was performed for extracted 0.5 μg miRNA with TaqMan MicroRNA Reverse Transcriptional Kit systems (Applied Biosystems, Foster City, CA) using TaqMan single RT primers for each miRNA (Applied Biosystems). The quantitative RT-PCR for miR-34a, 34b, and 34c using TaqMan MicroRNA Assays technology (Perkin Elmer Corp., Foster City, CA) with the Step One Plus Real Time PCR systems (Applied Biosystems). The miR-374 expression was used to normalize the expression of miR-34s as endogenous control of cell lines following manufacturer's recommendation (www.appliedbiosystems.com). After normalization of miR-34s expression to miR-374, all relative expression values were defined as the ratios of the normalized miR-34s expression value of cell lines or primary tumors to that of HBEC 5KT or individual non-malignant lung tissue, respectively.

2.4. Immunohistochemistry for p53 expression

Abnormality of p53 expression was examined using immunohistochemistry for primary tumors. To detect the p53 protein, we used a monoclonal antibody against human p53 (DAKO-p53, DAKO, Denmark). The routinely formalin-fixed, paraffin-embedded tissue blocks were sectioned at a 4 μm thickness. They were stained by hematoxylin and eosin stain to confirm the presence and intensity of tumor cells and processed for immunohistochemistry (IHC). Tissue sections were deparaffinized with xylene, rehydrated with graded ethanol, and immersed in Tris–buffered saline for IHC. For p53 scoring, more than 10 randomly chosen high power fields were evaluated under an optical microscope and a trained pathologist was defined blindly scored. In this study, p53 mutational status was defined as positive when more than 15% of cells were positive regardless of the intensity as previously reported [21,22].

2.5. Plasmid construction and gene transfection

The miR-34b/c or scramble fragment as control was subcloned into pSilencer 4.1-CMV neo Plasmid Vector (Ambion). Four μg of pSilencer 4.1-CMV neo Vector was introduced into SCLC cell lines using Lipofectamine™ 2000 Reagent (Invitrogen). For experiments of transient transfection, cells were collected 72 h after the transfection. In order to establish stable transfectants, selection of the

cells was started 48 h after the transfection in 6 cm dish with G418 (Invitrogen) antibiotics. Resistant clones were cloned after 3 weeks of selection.

2.6. Colony formation assay for cell proliferation

The *in vitro* cell proliferation was tested by liquid colony formation assay. Fifty viable cells were plated onto 6-well plates in triplicate. Cells were cultured and counted 14 days later after staining with 0.1% crystal violet in 20% ethanol for 5 min at room temperature. The number of visible colonies (>50 cells) was counted.

2.7. Cell migration and invasion assays

Cell migration and invasion ability were established using a Boyden chamber assay with filter inserts (pore size, 8 μm) in 6-well plates (BD Biosciences Discovery Labware, Bedford, MA). The cells in 2 ml serum-free RPMI1640 medium (300 μl containing 2.5×10^5 cells for transwell migration assay and 5×10^5 for Matrigel invasion assay) were added to the top chamber. The bottom chamber was prepared with 10% FBS as a chemoattractant. Non-invasive cells were removed by scrubbing with a cotton swab after 24–48 and 48–96 h of incubation for migration assay and invasion assay, respectively. The cells that had migrated through the membrane and stuck to the lower surface of the membrane were fixed and stained using Diff-Quik stain (Sysmex, Kobe, Japan). For quantification of migration and invasion, the cells were counted under a microscope in 5 predetermined fields at ×100 magnifications representing the average of 3 independent experiments.

2.8. Western blot analysis

Cells were grown to 80% confluency and harvested in lysis buffer [20 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/ml leupeptin] (Cell signaling Technology, Beverly, MA) supplemented with Complete, Mini (Roche, Basel, Switzerland) to extract protein. Total 30 μg of protein were separated by SDS-PAGE and transferred to PVDF membranes. The proteins on membranes were incubated overnight at 4 °C with the primary antibodies. We selected two molecules (c-MET and CDK6). The primary antibodies for Western blotting are as follows: anti-MET (25H2, Cell Signaling), anti-phospho-MET (3D7, Tyr1234/1235; Cell Signaling), and anti-CDK6 (DCS83, Cell Signaling). The following secondary antibodies were used: goat anti-rabbit or anti-mouse IgG-conjugated horseradish peroxidase (HRP) (Santa Cruz). To detect specific signals, the membranes were detected by ECL plus Western Blotting Detection Reagents (Amersham Biosciences UK Limited, Buckinghamshire, UK).

2.9. Statistical analysis

Statistical analysis was performed using SPSS for Windows version 17.0 (SPSS, Chicago, IL, USA). All of the *in vitro* experiments were performed at least three times. Data were represented as mean ± standard deviation. The significance of the differences between two groups was determined using the chi-square test and the Mann–Whitney *U*-test, as appropriate. A 5% significance level ($p < 0.05$) was considered statistically significant.

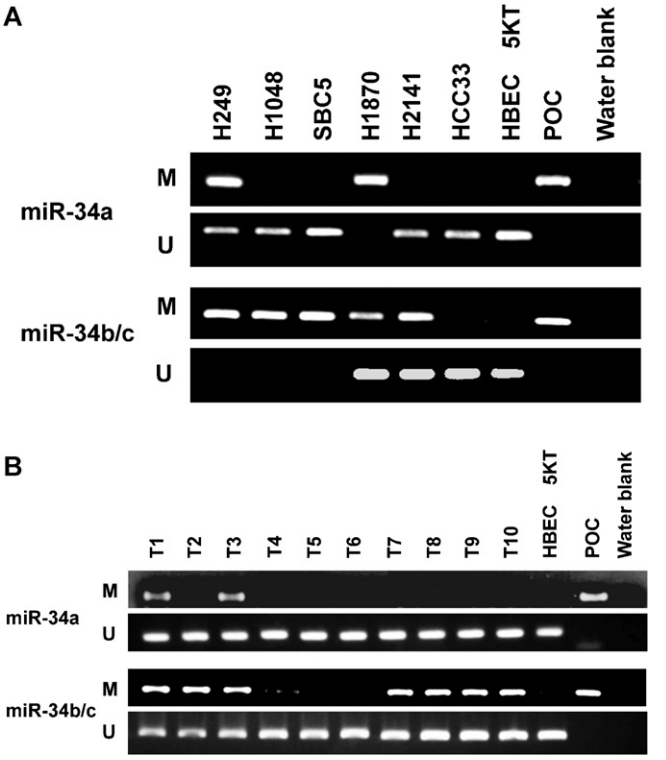

Fig. 1. Methylation status of miR-34a and 34b/c in SCLCs. Representative examples of conventional methylation specific PCR for miR-34a and 34b/c in SCLC cell lines (A) and primary tumors (B). The unmethylated form of miR-34s was always found in primary tumors that had been grossly dissected and thus had at least some contamination with normal cells. M, methylated; U, unmethylated; POC, positive control (Sss1 treated DNA).

3. Results

3.1. Methylation and expression status of miR-34s

We examined the methylation frequencies of miR-34a and b/c in 11 SCLC cell lines, 27 clinical samples consisting of 12 primary tumors and 15 malignant pleural effusions. Usefulness of malignant pleural effusions to detect DNA methylation has been previously reported [23]. For comparison purposes, the methylation frequencies of miR-34a and b/c were also examined in 14 NSCLC cell lines and 47 primary tumors. Representative images of the electrophoresis results are shown in Fig. 1. MSP of the cell lines showed three PCR band patterns: only a methylated band, both a methylated and an unmethylated band, and only a methylated band. The presence of both methylated and unmethylated bands indicated partial methylation. In this study, cell lines with partial methylation were classified as methylated cell lines. The methylation of miR-34a and miR-34b/c was observed in 4 (36%) and 7 (64%) out of 11 SCLC cell lines, respectively. Among the 27 SCLC clinical specimens, miR-34a and miR-34b/c were methylated in 4 (15%) and 18 (67%), respectively. Stratified by specimens, miR-34a methylation was found in 3 of 12 SCLC tumors and 1 of 15 malignant pleural effusions and miR-34b/c methylation was found in 9 of 12 tumors and 9 of 15 malignant pleural effusions. There was no significant difference in frequency of methylation between resected tumors and malignant pleural effusion specimens. In addition, no significant difference was found in the frequencies of miR-34 methylation between previously treated 8 pleural effusions (0% in miR-34a and 75% in miR-34b/c) and 7 pleural effusions without any treatment (14% in miR-34a and 57% in miR-34b/c), respectively. Among the NSCLC tumors, miR-34a and miR-34b/c methylation were observed

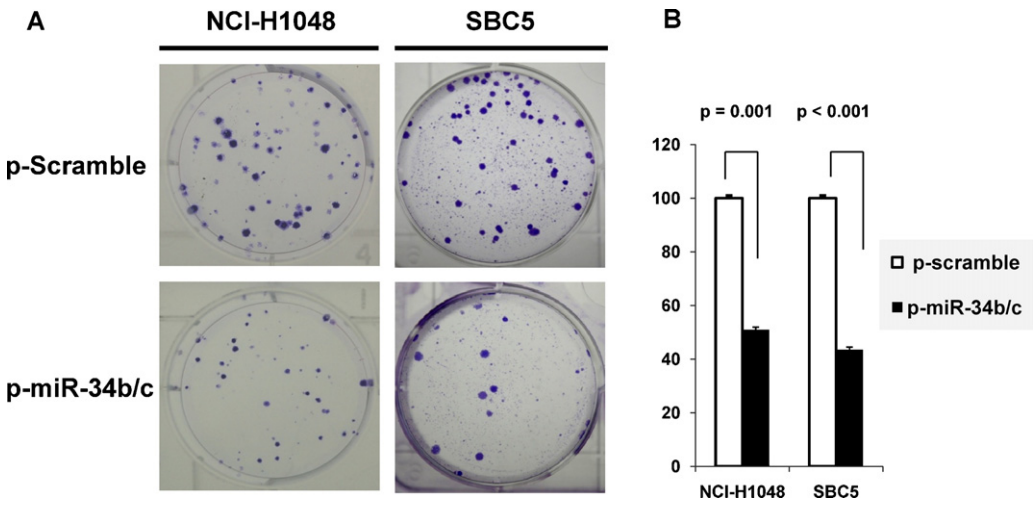

Fig. 2. Colony formation assays of SCLC cell lines stably transfected with miR-34b/c (p-miR-34b/c) or control (p-scramble) plasmid vectors. (A) Representative results of colony formation assays carried out using the indicated SCLC cell lines are shown. (B) Relative colony formation efficiencies are presented. Shown are means of three replications; error bars represent standard deviations.

in 5 (36%) and 3 (21%) cell lines and in 13 (28%) and 12 (26%) primary tumors, respectively. The frequency of miR-34b/c methylation was significantly higher among the SCLC primary tumors than among the NSCLC samples ($p < 0.001$).

3.2. Relationship between methylation and expression in SCLC

The expressions of miR-34a, 34b, and 34c in 11 SCLC cell lines and 6 available SCLC primary tumors (4 methylated and 2 unmethylated cases) were examined using quantitative RT-PCR (Tables 1 and 2). The expression values of the miR-34s were defined as the ratio of the normalized expression value of SCLC cell lines to that of HBEC 5KT and that of the primary SCLC tumors to that of individual non-malignant lung tissue with arbitrarily assigned values of 1, respectively. As shown in Tables 1 and 2, all expression values of miR-34s in SCLC cell lines and primary tumors were lower than their normal control samples (HBEC 5KT and non-malignant lung tissue). The expressions of the miR-34b and 34c were significantly lower in the methylated cell lines than in the unmethylated cell lines (miR-34b, $p = 0.011$; miR-34c, $p = 0.008$). Regarding miR-34a, no significant difference or tendency was detected ($p = 0.107$). This finding may reflect the small sample sizes or the presence of partially methylated cells. One cell line (H2141) with unmethylated miR-34a had very low expression value (0.05) for miR-34a, suggesting the presence of an alternative mechanism for gene silencing.

We also treated 4 cell lines with DAC and found that the expressions of the miR-34s were restored in the methylated SCLC cell lines but not in the unmethylated cell lines (Table 3).

3.3. p53 status in SCLC

p53 is a transcriptional factor for miR-34s. Thus, we examined the correlation between the p53 status and the expressions of miR-34s (Table 2). Genotyping data for the *TP53* gene in H1048, SBC5, H211, H524, H841, H2141, H82, and H249 were queried from the database of the International Agency for Research on Cancer (IARC) version 15 (www-p53.iarc.fr) to find that all cell lines harbor *TP53* mutation. HCC33 and H1870 also harbor mutation (HCC33, C242Y and H1870, Y234C) and these data were provided by Dr. Adi F. Gazdar (University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA). The mutation status of H1688 was not available. As a result, 10 out of 11 cell lines were found to carry *TP53* mutations. The p53 status of the primary tumors was determined using immunohistochemistry [21,22]. The aberrant positive expression of p53 protein was regarded as a p53 abnormality (mutation) in the primary cancers. Four (67%) out of the 6 primary SCLC tumors exhibited positive p53 immunohistochemistry results suggesting the presence of p53 mutation. The relationship between the p53 alterations and the expressions of miR-34s was examined, but no correlation between the p53 status and the expressions of miR-34s was found.

3.4. Impact of miR-34b/c on cell proliferation

Because the frequency of methylation was higher in miR-34b/c and because miR-34a methylation in SCLC cell lines was mainly partial methylation but miR-34b/c was heavily methylation, we focused on miR-34b/c for a functional analysis. For this purpose, we established stable transfectants with miR-34b/c and scramble controls for H1048 and SBC5. To examine the anti-proliferative effect of miR-34b/c induction, we performed a colony formation assay for stable transfectants. Cell proliferation was significantly inhibited in the SCLC cells transfected with miR-34b/c, compared with that in the cells that were transfected with a scramble control (50% inhibition for H1048, $p = 0.001$; 44% for SBC5, $p < 0.001$) (Fig. 2).

3.5. Impact of miR-34b/c on cell migration and invasion

To estimate the effect of introducing miR-34b/c on the migration and invasion potential of SCLC, cell migration and invasion potential were examined using a Boyden chamber. Microscopy images of the Boyden chamber assay are shown in Fig. 3. Migration and invasion were significantly suppressed in miR-34b/c transfectants, compared with control transfectants (migration: 50% inhibition for H1048, $p = 0.051$; 54% for SBC5, $p = 0.044$; invasion: 33% inhibition for H1048, $p = 0.002$; 30% inhibition for SBC5, $p = 0.012$).

3.6. Protein expression of stable transfectants

To examine the effect of miR-34b/c introduction, we focused on c-MET (both total and phosphorylated types) and CDK6 which were the putative target of miR-34b/c. Western blotting was carried out in SCLC stable transfectants. Total and phosphorylated c-MET and CDK6 expressions were down-regulated in the SCLC cell lines examined. CDK6 tended to be down-regulated by the miR-34b/c in cell lines whose native protein expression was present (Fig. 4).

Table 1
The relative expressions and methylation status of miR-34s in 11 SCLC cell lines.

Cell lines	p53 status	Methylation status		miR relative expression value[a]		
		miR-34a	miR-34b/c	miR-34a ($\times10^{-3}$)	miR-34b ($\times10^{-3}$)	miR-34c ($\times10^{-3}$)
HCC33	Mut	U	U	9.6	8.3	10.6.
NCI-H211	Mut	M/U	U	0.3	5.2	8.7
NCI-H524	Mut	U	U	7.1	11.4	130
NCI-H841	Mut	M/U	U	3.4	4.3	6.7
NCI-H1688	NA	U	M/U	17.1	2.7	0.69
NCI-H1870	Mut	M	M/U	0.01	1.2	2.7
NCI-H2141	Mut	U	M/U	0.05	1.56	3.5
NCI-H82	Mut	U	M	0.3	2.6	0.8
NCI-H249	Mut	M/U	M	3.4	1.0	0.8
NCI-H1048	Mut	U	M	36.2	4.3	3.6
SBC5	Mut	U	M	31.2	1.3	1.0
HBEC 5KT	Wt	U	U	–	–	–

Mut, mutation; Wt, wild type; NA, not available; M, methylated; U, unmethylated; M/U, partially methylated.
[a] miR-34s relative expression values are relative expression values compared with those of HBEC 5KT, which are defined as 1.

Table 2
The relative expressions and methylation status of miR-34s in 6 available SCLC primary tumors.

Sample	Age (year)	Gender	Stage	p53 mutational status[a]	Methylation status		miR relative expression value[b]		
					miR-34a	miR-34b/c	miR-34a ($\times10^{-1}$)	miR-34b ($\times10^{-1}$)	miR-34c ($\times10^{-1}$)
T1	60	Male	LD	Mut	U	M	2.4	0.022	0.034
T8	80	Female	LD	Wt	U	M	2.1	0.61	0.068
T9	69	Male	LD	Mut	U	M	0.19	1.1	0.36
T10	69	Male	ED	Mut	U	M	1.5	0.77	0.53
T4	66	Male	LD	Mut	U	U	0.17	7.3	4.4
T5	56	Male	LD	NA	U	U	1.2	3.1	1.7
Non-malignant tissue				Wt	U	U	–	–	–

LD, limited disease; ED, extensive disease; Mut, mutation; Wt, wild type; NA, not available; M, methylated; U, unmethylated.
[a] p53 mutational status is as positive if more than 15% of nuclei are stained.
[b] miR-34s relative expression values are relative expression values compared with those of each non-malignant tissue, which are defined as 1.

4. Discussion

In this study, we found that miR-34b/c methylation was a frequent alteration of SCLC. As a result, miR-34b/c expression was reduced in SCLC, causing tumor cell proliferation and invasiveness. Our results also indicated that miR-34b/c methylation is the early event for tumorigenesis of SCLC because there was no difference in frequency between surgically resected tumors supposed to be early stage and malignant pleural effusions considered to be advanced stage. In addition to lung cancer, miR-34b/c methylation has been reported in various kinds of malignant tumors including colorectal and ovarian cancers [20,24]. We previously reported the methylation and function of miR-34b/c in malignant pleural mesothelioma [25]; the same established methodology was also used in the present study. p53 is a transcriptional factor for miR-34s. The p53 mutation has been reported in various cancers, including lung cancer. However, the effect of p53 mutation on the expression and methylation of miR-34b/c remains uncertain. In ovarian cancer, miR-34a methylation and p53 mutation are not associated. Corney et al. reported that no direct correlation was observed between miR-34 methylation status and miR-34 expression levels. In addition, p53 mutation has no effect on miR-34b/c methylation in ovarian cancer [24]. In the SCLC cell lines that were examined in the present study, 10 out of 11 cell lines carried a p53 mutation, but the expression levels of the miR-34s varied and were correlated with the methylation status. In primary tumors, while the p53 mutational status was determined using immunohistochemistry [21,22], the relationship among the expression and methylation of miR-34b/c and p53 mutation was similar to that in the cell lines. Our data suggest that miR-34 expression was not completely silenced in the p53-altered cases and that methylation may contribute more strongly to the expressions of miR-34s.

Antitumor effects, including the inhibition of cell proliferation, migration, and invasion, were observed with the introduction of miR-34b/c. In general, miRNA has multiple target mRNAs, and c-Met is a well-known target molecule of miR-34b/c [16,17]. c-Met and its ligand hepatocyte growth factor (HGF) have been shown to be involved in cell proliferation, invasion, and angiogenesis [26,27]. A previous study found that the c-Met/HGF pathway was functional in SCLC, indicating *in vitro* that the c-MET/HGF axis may

Table 3
The fold change of miR-34s expression after DAC treatment.

DAC treated cell lines	Methylation status		Fold change after DAC		
	miR-34a	miR-34b/c	miR-34a	miR-34b	miR-34c
NCI-H82	U	M	1.2	3.9	279.8
NCI-H249	M/U	M	3.8	1.9	20.5
NCI-H1048	U	M	1.1	4.2	52.6
SBC5	U	M	1.8	2.8	2.2

M, methylated; U, unmethylated; M/U, partially methylated. Increase of miR-34a and miR-34b/c expression in SCLC cell lines with methylation after 5-aza-2′-deoxycytidine (DAC) treatment. The expression ratio shows the relative miR-34s expression values in DAC-treated cell lines compared with the miR-34s expression values in non-treated cell lines.

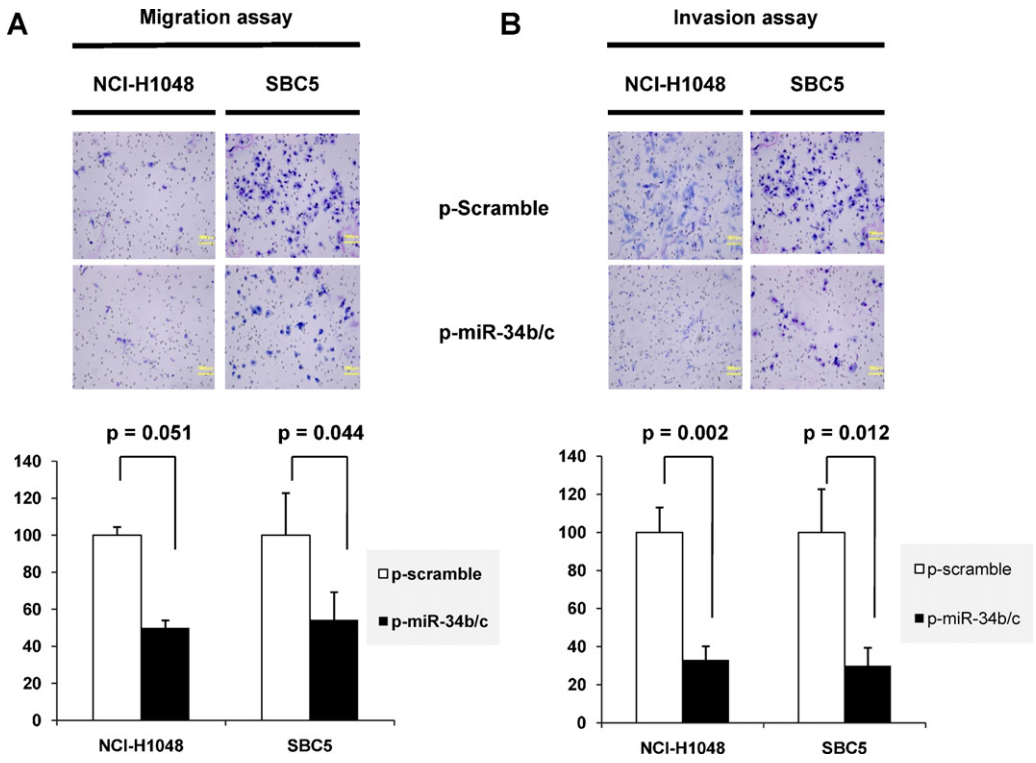

Fig. 3. The impact of miR-34b/c introduction on SCLC cell migration and invasion potential. The miR-34b/c inhibits cell migration (A) and invasion (B) of the SCLC cells. Migrated or invaded cells were fixed and stained, and representative examples are shown above. The quantitative values expressed as the means ± SD of five microscopic fields are representative of two separate experiments (below).

be a promising target for SCLC [27,28]. Our results, together with those of previous reports, strongly suggest that miR-34b/c plays an important role in the pathogenesis of SCLC and may be a useful therapeutic target for SCLC.

In SCLC, the methylation of miR-34a was not a frequent event compared with that of miR-34b/c, but its expression was low compared with that in non-malignant lung tissue. The degree of reduction in miR-34a expression was similar to that of miR-34b/c in some SCLC cell lines, suggesting that miR-34a silencing may also be involved in the pathogenesis of SCLC, although the mechanism of silencing has not been clearly elucidated. Further study on the role of miR-34a in SCLC is warranted.

In conclusion, our findings showed that miR-34b/c is more frequently methylated in SCLC than in NSCLC, resulting in the down-regulation of miR-34b/c. As this alteration confers a growth and invasion advantage to cancer cells, targeting miR-34b/c is a potential therapeutic option for the treatment of SCLCs. The targeting of SCLCs is of particular interest, as recent trials of targeted drugs for lung cancer have focused mainly on NSCLCs.

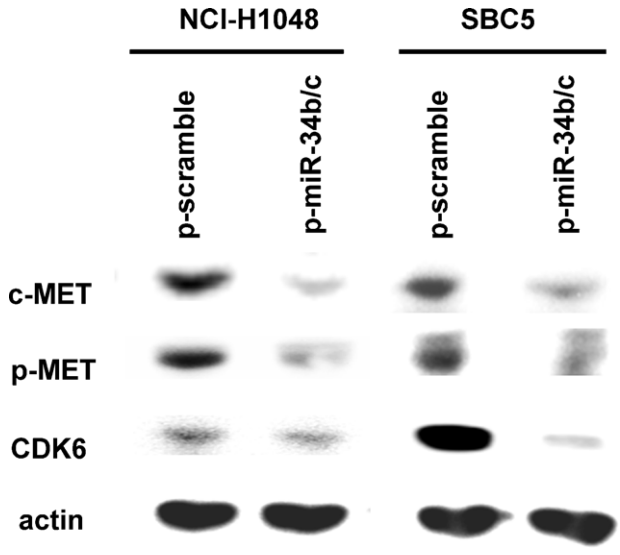

Fig. 4. Protein expression profile of SCLC cell lines in stable transfection of miR-34b/c (p-miR-34b/c) or control (p-scramble) plasmid vectors.

Conflict of interest statement

None declared.

Acknowledgement

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Erratum

Erratum to "Frequent methylation and oncogenic role of microRNA-34b/c in small-cell lung cancer" [Lung Cancer 76 (1) (2012) 32–38]

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The publisher regrets errors were introduced in Fig. 3A of the article described above. The images for both p-Scramble and p-miR-34b/c in SBC5 were incorrect. An amended version of Fig. 3 is shown below. Note that the analyses for both migration and invasion assays were appropriate and thus it is not necessary to correct the bar graphs in Fig. 3 as well as the legends for Fig. 3. The authors also consider that there is no need to correct any other parts of this article. The publisher would like to apologise for any inconvenience caused.

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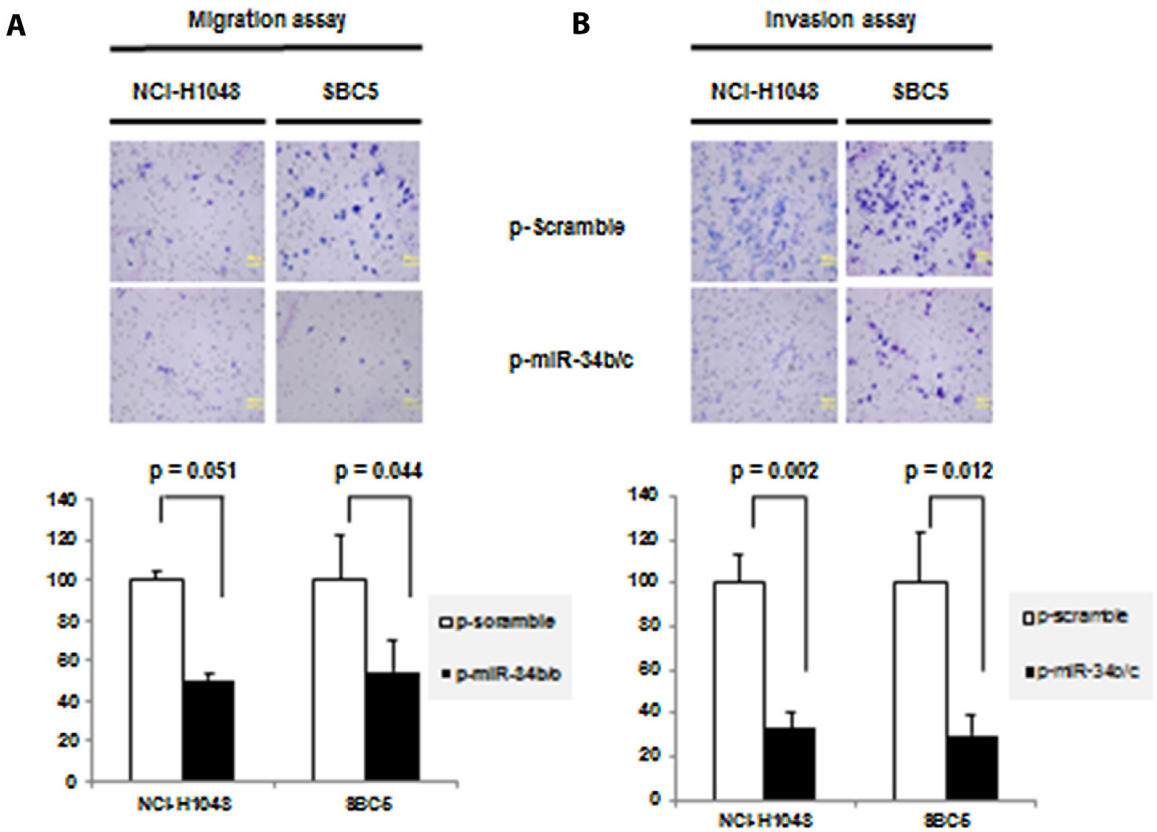
A
Migration assay
NCI-H1048
SBC5
p-Scramble
p-miR-34b/c
p = 0.051
p = 0.044
140
120
100
80
60
40
20
0
p-scramble
p-miR-34b/c
NCI-H1048
SBC5
B
Invasion assay
NCI-H1048
SBC5
p = 0.002
p = 0.012
140
120
100
80
60
40
20
0
p-scramble
p-miR-34b/c
NCI-H1048
SBC5

Fig. 3.