Epigenetic Silencing of MicroRNA-34b/c Plays an Important Role in the Pathogenesis of Malignant Pleural Mesothelioma

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Abstract

Purpose: Malignant pleural mesothelioma (MPM) is an aggressive tumor with a dismal prognosis. Unlike other malignancies, TP53 mutations are rare in MPM. Recent studies have showed that altered expression of microRNA (miRNA) is observed in human malignant tumors. In this study, we investigated the alterations of miR-34s, a direct transcriptional target of TP53, and the role of miR-34s on the pathogenesis of MPM.

Experimental Design: Aberrant methylation and expression of miR-34s were examined in MPM cell lines and tumors. miR-34b/c was transfected to MPM cells to estimate the protein expression, cell proliferation, invasion, and cell cycle.

Results: Aberrant methylation was present in 2 (33.3%) of 6 MPM cell lines and 13 (27.7%) of 47 tumors in miR-34a and in all 6 MPM cell lines (100%) and 40 (85.1%) of 47 tumors in miR-34b/c. Expression of miR-34a and 34b/c in all methylated cell lines was reduced and restored with 5-aza-2'-deoxycytidine treatment. Because epigenetic silencing was the major event in miR-34b/c, we investigated the functional role of miR-34b/c in MPM. miR-34b/c-transfected MPM cells with physiologic miR-34b/c expression exhibited antiproliferation with G1 cell cycle arrest and suppression of migration, invasion, and motility. The forced overexpression of miR-34b/c, but not p53, showed a significant antitumor effect with the induction of apoptosis in MPM cells.

Conclusions: We show that the epigenetic silencing of miR-34b/c by methylation is a crucial alteration and plays an important role in the tumorigenesis of MPM, suggesting potential therapeutic options for MPM. Clin Cancer Res; 17(15); 4965–74. ©2011 AACR.

Introduction

Malignant pleural mesothelioma (MPM) is a neoplasm with highly invasive and aggressive clinical features (1). Exposure to asbestos is strongly associated with the etiology of MPM. A curative modality such as radiotherapy, conventional chemotherapy, or molecular targeting therapy has not yet been established for advanced MPM and the development of new treatments is needed (2). An understanding of molecular pathogenesis is crucial for developing new therapeutic strategies. However, much less information about molecular alterations in MPM is available than for other neoplasms. Previous studies have revealed that the genetic alterations of MPM are quite different from that of other neoplasms. One of the unique molecular features of MPM is that mutations and deletions of the TP53 gene are rare (3, 4), even though MPM generally exhibits cell cycle alterations and antiapoptosis, which suggests functional p53 deficiency (5). Considering these observations, uncovering the molecular pathogenesis of MPM is likely to provide useful information.

MicroRNAs (miRNA) are a group of noncoding small RNAs that generally regulate their target mRNAs by post-transcriptional repression (6). In the recent half decade, intensive research about the role of miRNAs in human malignant tumors has been conducted because of the ability of individual miRNAs to regulate multiple genes implicated in multiple pathways (7). Similar to encoding genes, some miRNAs have been classified as oncogenic or...
miR-34s were downregulated in cell cycle arrest, apoptosis, and senescence (9, 12). Indeed, miR-34b/c can be a potential therapeutic target for MPM.

tumor-suppressive miRNAs according to their effects on cellular transformation (8). Among the tumor-suppressive miRNAs, the miR-34s, which have p53 response elements in their 5′ flanking regions, have recently been investigated (9–11). The members of the miR-34 family are composed of 3 miRNAs, miR-34a, miR-34b, and miR-34c, whose target genes are considered to be similar but with some notable differences (9). miR-34a is located on chromosome 1q36.22, whereas miR-34b and miR-34c (miR-34b/c) are located on chromosome 11q23 and are generated by the processing of a single transcript (9). The miR-34 family members are direct transcriptional targets of p53 and constitute a part of p53 tumor suppressor network regulating cell cycle arrest, apoptosis, and senescence (9, 12). Indeed, miR-34s were downregulated in TP53-null or TP53-mutant cells (9, 11).

Ablation of p53 in MPM cells (9, 10) and the loss of miR-34b/c expression (9, 11) is associated with transformation in MPM cells. This suggests that miR-34b/c could be a potential therapeutic target for MPM.

Malignant pleural mesothelioma (MPM) is an aggressive tumor with a dismal prognosis. Unlike other malignancies, mutations of TP53 are rare in MPM. Here, we found that the methylation of miR-34b/c, direct transcriptional targets of the p53, was often present resulting in reduced expression in MPM. The miR-34b/c-transfected MPM cells showed anti-proliferation with G1 arrest and inhibition of migration, invasion, and motility. Overexpression of miR-34b/c, but not p53, showed significant anti-tumor effect by apoptosis in MPM cells. Our results show that epigenetic silencing of miR-34b/c plays a pivotal role in pathogenesis of MPM and suggest that miR-34b/c can be a potential therapeutic target for MPM.

### Translational Relevance

Malignant pleural mesothelioma (MPM) is an aggressive tumor with a dismal prognosis. Unlike other malignancies, mutations of TP53 are rare in MPM. Here, we found that the methylation of miR-34b/c, direct transcriptional targets of the p53, was often present resulting in reduced expression in MPM. The miR-34b/c-transfected MPM cells showed anti-proliferation with G1 arrest and inhibition of migration, invasion, and motility. Overexpression of miR-34b/c, but not p53, showed significant anti-tumor effect by apoptosis in MPM cells. Our results show that epigenetic silencing of miR-34b/c plays a pivotal role in pathogenesis of MPM and suggest that miR-34b/c can be a potential therapeutic target for MPM.

### Clinical samples and cell lines

Surgically resected specimens of 47 MPMs were obtained from Okayama University Hospital, Okayama, Japan (5 cases), and from Karmanos Cancer Institute, Detroit, MI (42 cases). Written informed consents were obtained from all patients at 2 collection sites. The histologic subtypes of primary MPM consisted of 32 epithelial, 10 biphasic, 4 sarcomatoid, and 1 lymphohistiocytic variant types. Ten nonneoplastic pleura from lung cancer patients were obtained from surgically resected pulmonary specimens and were used for the methylation assay. In addition, 2 nonmalignant mesothelial primary cultures (mesothelial cells) were established from pleural effusions that arose in patients free of cancer, as described in our previous report (22), and these cells were used as controls. All the tissues were frozen in liquid nitrogen immediately after surgery and stored at −80°C. Six MPM cell lines [NCI-H28 (H28), NCI-H290 (H290), NCI-H2052 (H2052), NCI-H2452 (H2452), H1, and MSTO-211H], a lung cancer cell line [NCI-H125 (H125)], and 1 human bronchial epithelial cell line (HBEC 5KT) were used in this study. Six cell lines (H28, H290, H2052, H2452, H125, and HBEC 5KT) were kind gifts from Dr. Adi F. Gazdar (Hamon Center for Therapeutic Oncology Research and Department of Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX). These cell lines were proven to have individual genetic origins by the PowerPlex 1.2 System (Promega) at University of Texas Southwestern Medical Center at Dallas (23). The H1 cell line was established by H.I. Pass. MSTO-211H was obtained from American Type Culture Collection. The cells were maintained in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% FBS and incubated in 5% CO2 except for HBEC 5KT, which was maintained in Keratinocyte-SFM (Invitrogen) with bovine pituitary extract and human recombinant epidermal growth factor (24). The cell lines were treated with 5-aza-2′-deoxycytidine (DAC; Sigma-Aldrich Co.) at a concentration of 5 μmol/L for 6 days to restore the gene expression that was reduced by methylation.

### Methylation-specific PCR assay and bisulfite DNA sequencing

Genomic DNA was extracted from cell lines and tissues, and DNA was subjected to bisulfite treatment. The methylation status of miR-34s was determined by methylation-specific PCR (MSP) assay and bisulfite DNA sequencing as previously reported (17, 18, 25). The extent of miR-34b/c methylation was determined by real-time quantitative MSP (q-MSP) assay using Power SYBR Green PCR Master Mix (Applied Biosystems). Further details are provided in Supplementary Methods.

### Evaluation of miR-34s expression using quantitative reverse transcription-PCR

miRNA was isolated from cell lines and tissue specimens and the reverse transcription reaction was carried out. The
lines were infected at a MOI of 50 pfu per cell.

Plasmid construction, gene transfection, and colony formation assay
The miR-34b/c or scramble sequence fragment as control was subcloned into pSilencer 4.1-CMV neo Plasmid Vector Ambion; refs. 17, 18, 25. Four micrograms of constructed plasmids was introduced into MPM cells using Lipofectamine 2000 Reagent (Invitrogen). For experiments of transient transfection, cells were collected 72 hours after transfection. To establish stable transfectants, selection of the cells was started 48 hours after transfection in 6-well plates with G418 (Gibco) antibiotics. Resistant clones were cloned by ring isolation after 3 weeks of selection. In vitro cell proliferation was tested by liquid colony formation assay (details in Supplementary Methods).

Western blot analysis
Preparation of total cell lysates and Western blot analysis were done as described in Supplementary Methods. We selected 7 molecules (c-MET, CDK4, CDK6, CCND1, CCNE2, Bcl-2, c-MYC, and E2F3) that had been reported as primary targets of miR-34s (9, 11, 26).

Flow cytometric analysis
Cells were harvested and resuspended in PBS containing 0.2% Triton X-100 and 1 mg/mL RNase for 5 minutes at room temperature and then stained with propidium iodide at 50 μg/mL to determine subdiploid DNA content using a FACScan. Doublets, cell debris, and fixation artifacts were gated out, and cell cycle analysis was done using CellQuest version 3.3 software.

Cell migration, invasion, and motility assays
The cell migration and invasion ability were estimated using a Boyden chamber assay with filter inserts (pore size: 8 μm) in 6-well dishes (BD Biosciences Discovery Labware). The motility of MPM cells was estimated using time lapse video microscopy using a Keyence BZ-8000 (Keyence). Further details are provided in Supplementary Methods.

Recombinant adenoviral vector construction
Ad-miR-34b/c driven by cytomegalovirus (CMV) promoter (Ad-miR-34b/c) was generated by homologous recombination and plaque purified (27). Adenoviral vector expressing p53 driven by CMV promoter (Ad-p53) and adenoviral vector expressing luciferase driven by CMV promoter (Ad-Luc) were used as control vectors. The optimal multiplicity of infection (MOI) was determined by infecting each cell line with Ad-CMV/GFP and assessing the expression of green fluorescent protein (GFP) by flow cytometric analysis. H28, H290, and H2052 MPM cell lines were infected with the adenoviral vectors at an MOI of 200 plaque-forming units (pfu) per cell. All other cancer cell lines were infected at a MOI of 50 pfu per cell.

MTS assay for cell viability in adenoviral-infected cells
Cells were plated in 96-well plates at a density of 1.0 × 10^3 cells per well 24 hours before infections and treated with PBS, Ad-Luc, Ad-p53, or Ad-miR-34b/c. Cell viability was evaluated at 0, 1, 2, 3, and 4 days following the adenoviral infection by MTS assay with CellTiter 96 AQueous One Solution Reagent (Promega).

Statistical analysis
Data were represented as mean ± SD. Mann–Whitney U test was used to compare data between 2 groups. P < 0.05 was considered as being statistically significant.

Results
Methylation and expression status of miR-34s in MPM and other cancers
The methylation status of miR-34s was determined by MSP assay and bisulfite DNA sequencing as previously reported (17, 18, 25). Representative examples of MSP assays are shown in Figure 1A and B. In the 6 MPM cell lines examined, miR-34a was methylated in 2 (33.3%) cell lines (H28 and H290) and miR-34b/c was methylated in all the 6 cell lines (100%). In 47 MPM tumors, miR-34a methylation was present in 13 (27.7%) cases: 10 (31.2%) of the 32 epithelial-type tumors, 1 (10%) of the 10 biphasic-type tumors, 2 (50%) of the 4 sarcomatoid-type tumors, and none of the 1 lymphohistiocytic variant–type tumor. miR-34b/c methylation was present in 40 (85.1%) cases: 29 (90.6%) epithelial-type tumors, 7 (70%) biphasic-type tumors, 3 (75%) sarcomatoid-type tumors, and 1 (100%) lymphohistiocytic variant–type tumors. No methylation was found in 10 nonneoplastic pleura specimens and 2 nonmalignant mesothelial cells. All the MPMs that had miR-34a methylation also had miR-34b/c methylation. We quantified the extent of methylation of miR-34b/c using q-MSP assay in 3 MPM cell lines. NCI-H2052 and NCI-H290 showed 2 ± 0.2-fold and 5 ± 0.6-fold increase of the extent of miR-34b/c methylation compared with NCI-H28 by quantitative MSP assay, respectively (not shown as a table or a figure). We also evaluated the degree of methylation of miR-34b/c using subcloning technique of bisulfate sequencing, and heavy methylation at the CpG sites in the 5' flanking region of miR-34b/c was observed (Fig. 1C). Of note, the percentage of methylated CpG sites that was reported (17, 18, 25). Representative examples of MSP assays are shown in Figure 1A and B. In the 6 MPM cell lines examined, miR-34a was methylated in 2 (33.3%) cell lines (H28 and H290) and miR-34b/c was methylated in all the 6 cell lines (100%). In 47 MPM tumors, miR-34a methylation was present in 13 (27.7%) cases: 10 (31.2%) of the 32 epithelial-type tumors, 1 (10%) of the 10 biphasic-type tumors, 2 (50%) of the 4 sarcomatoid-type tumors, and none of the 1 lymphohistiocytic variant–type tumor. miR-34b/c methylation was present in 40 (85.1%) cases: 29 (90.6%) epithelial-type tumors, 7 (70%) biphasic-type tumors, 3 (75%) sarcomatoid-type tumors, and 1 (100%) lymphohistiocytic variant–type tumors. No methylation was found in 10 nonneoplastic pleura specimens and 2 nonmalignant mesothelial cells. All the MPMs that had miR-34a methylation also had miR-34b/c methylation. We quantified the extent of methylation of miR-34b/c using q-MSP assay in 3 MPM cell lines. NCI-H2052 and NCI-H290 showed 2 ± 0.2-fold and 5 ± 0.6-fold increase of the extent of miR-34b/c methylation compared with NCI-H28 by quantitative MSP assay, respectively (not shown as a table or a figure). We also evaluated the degree of methylation of miR-34b/c using subcloning technique of bisulfate sequencing, and heavy methylation at the CpG sites in the 5' flanking region of miR-34b/c was observed (Fig. 1C). Of note, the percentage of methylated CpG sites that was evaluated by subcloning technique was higher in NCI- H2052 (93.9%) and NCI-H290 (97.7%) than in NCI-H28 (74.2%; Fig. 1C). In contrast, methylation at the CpG sites was rarely observed in nonmalignant mesothelial cells. The expression of miR-34a, miR-34b, and miR-34c in MPM cell lines and primary tumors was examined using quantitative RT-PCR. Because the expression levels of the 2 nonmalignant mesothelial cells were similar, we mixed them and used them as standards for expression of nonmalignant mesothelial cells. The expression value of miR-34s in the cells was defined as the ratio of the expression in individual cell lines to that of nonmalignant mesothelial cells and was arbitrarily assigned a value of 100. We
arbitrarily considered the cell lines whose miR-34s expression values were less than 10 as being MPM cell lines with reduced expression. These expression values were reduced in all the methylated MPM cell lines and were not reduced in the unmethylated MPM cell lines. The expression values of cell lines are shown in Table 1. To confirm the results

![Figure 1. Methylation status of miR-34a and miR-34b/c in MPMs. A and B, representative examples of conventional MSP for miR-34a and miR-34b/c in MPM cells (A) and primary tumors (B). The unmethylated form of miR-34a was always found in primary tumors that had some contamination with normal cells. M, methylated; U, unmethylated; POC, positive control (SssI-treated DNA). C, methylation status of individual cloned DNA fragments of 3 MPM cell lines and 2 nonmalignant mesothelial cells is shown. Each row represents 1 sequenced allele. Each circle represents a CpG dinucleotide (closed circle, methylated; open circle, unmethylated). Clonal numbers are indicated by prefix C to the left. The numbers at the top indicate the CpG dinucleotide in the amplicon (5' to 3'). The positions of CpG dinucleotides for MSP primers reported previously are indicated by horizontal arrows. The values in parentheses were expression values of miR-34b/c in each cell line compared with the nonmalignant mesothelial cells whose miR-34b/c expression was defined as 100. %M, the rate of methylated CpG dinucleotides.]

Table 1. The expression and methylation status of miR-34s in cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Histology</th>
<th>Methylation status</th>
<th>miR expression value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H28</td>
<td>MPM (sarcomatoid)</td>
<td>M</td>
<td>0.5</td>
</tr>
<tr>
<td>NCI-H290</td>
<td>MPM (epithelial)</td>
<td>M</td>
<td>2.1</td>
</tr>
<tr>
<td>NCI-H2052</td>
<td>MPM (epithelial)</td>
<td>M</td>
<td>9.5</td>
</tr>
<tr>
<td>NCI-H2452</td>
<td>MPM (biphasic)</td>
<td>U</td>
<td>4.5</td>
</tr>
<tr>
<td>HP-1</td>
<td>MPM (biphasic)</td>
<td>M</td>
<td>4.5</td>
</tr>
<tr>
<td>MSTO-211H</td>
<td>MPM (biphasic)</td>
<td>U</td>
<td>4.5</td>
</tr>
<tr>
<td>NCI-H125</td>
<td>Lung cancer (adenosquamous carcinoma)</td>
<td>U</td>
<td>323</td>
</tr>
<tr>
<td>HBEC 5KT</td>
<td></td>
<td>U</td>
<td>695</td>
</tr>
<tr>
<td>Mesothelial cells</td>
<td></td>
<td>U</td>
<td>100</td>
</tr>
</tbody>
</table>

NOTE: miR-34s expression values are relative expression values compared with those of nonmalignant mesothelial cells, which are defined as 100.
obtained from cell lines, we assessed the relationship between methylation and expression status for miR-34b/c in primary tumors. We randomly chose 10 samples (different sets from the ones shown in Fig. 1B) and examined the expression of miR-34b and miR-34c. In the 10 samples tested, 2 samples were unmethylated and we set the expression of miR-34b/c in one of them as 100 and compared the other samples with it. Similar results were obtained in 10 clinical samples (Supplementary Table S3). We treated 6 MPM cell lines with DAC and found that the expression of the miR-34s was restored in the methylated MPM cell lines. The degree of upregulation in the expression of miR-34s after DAC treatment ranged from 4- to 80.9-fold in methylated genes (Supplementary Table S1).

Impact of miR-34b/c on cell proliferation

To screen for the antiproliferative effect of miR-34b/c, a colony formation assay was conducted with transient transfection. Four MPM cell lines (H28, H290, H2052, and H2452) were transiently transfected with miR-34b/c or a scrambled control. Colony formation was remarkably inhibited in 3 MPM cell lines with miR-34b/c transfection, compared with that in cells transfected with the scramble control (55% inhibition in H28, P < 0.01; 42% in H290, P < 0.01; and 64% in H2052, P < 0.01; Supplementary Fig. S1A and B). No colonies were formed in H2452 transfected with the scramble control or miR-34b/c (data not shown). Of note, although a colony formation assay after transient transfection showed antiproliferation in MPM cell lines, we found significant cell toxicity, probably caused by the transfection itself, in MPM cell lines transfected with scramble or miR-34b/c plasmid vectors. Thus, we established stable transfectants to investigate the various cellular effects of miR-34b/c on MPM.

Establishment of stable transfectants

We established stable transfectants with miR-34b/c and the scramble control in H28, H290, and H2052. The expression values of the miR-34b/c stable transfectants ranged from 25.6 to 131, shown in Table 2, part A. These values were not so different from that of nonmalignant mesothelial cells; therefore, the expression of miR-34b and miR-34c in the stable transfectants was considered to be within the physiologic range of nonmalignant mesothelial cells. A colony formation assay of stable transfectants confirmed that cell proliferation was significantly inhibited in MPM cells transfected with miR-34b/c, compared with that in cells transfected with the scramble control (Fig. 2A and B).

Protein expression and cell cycle analysis of stable transfectants

To examine the effect of miR-34b/c introduction, we focused on c-MET, CDK4, CDK6, CCND1, CCNE2, Bcl-2, c-MYC, and E2F3, which have been reported as putative targets of miR-34b/c (9, 25). Western blotting was carried out in MPM stable transfectants. Total and phosphorylated c-MET expression, in particular, was strongly downregulated in the 3 MPM cell lines examined. No difference in Bcl-2 expression between the miR-34b/c and control transfectants. We chose p-c-MET and c-MYC as a representative and quantified the protein expression level

| Table 2. Expression values of miR-34s in MPM cells with miR-34b/c transfection |
|---------------------|---------------------|---------------------|
| Cell lines          | miR expression value |
|                     | miR-34a | miR-34b | miR-34c |
| A. Plasmid stable transfectants |
| NCI-H28             | p-Scramble | 0.7 | 1.8 | 2.5 |
|                     | p-miR-34b/c | 0.8 | 131 | 94.7 |
| NCI-H290            | p-Scramble | 1.4 | 3 | 5 |
|                     | p-miR-34b/c | 1.3 | 25.6 | 28.8 |
| NCI-H2052           | p-Scramble | 47.8 | 9.3 | 4.5 |
|                     | p-miR-34b/c | 52.6 | 62.6 | 66.8 |
| Mesothelial cell    | 100 | 100 | 100 |
| B. Adenoviral-transfected cells |
| NCI-H28             | Ad-Luc | 0.6 | 2.1 | 2.5 |
|                     | Ad-miR-34b/c | 0.9 | 1,470 | 6,540 |
| NCI-H290            | Ad-Luc | 1.2 | 4.3 | 5.5 |
|                     | Ad-miR-34b/c | 1.1 | 501 | 2,170 |
| NCI-H2052           | Ad-Luc | 50.1 | 10.5 | 5.2 |
|                     | Ad-miR-34b/c | 54.2 | 1,640 | 3,430 |
| Mesothelial cell    | 100 | 100 | 100 |
of them by densitometry analysis using NIH ImageJ software tentatively (Supplementary Fig. S7). Although there may not be much point in quantifying the protein expression obtained by Western blot, the results seem to be consistent with an effect by the miR-34b/c.

A cell cycle analysis was conducted for MPM stable miR-34b/c and control transfectants. All 3 MPM stable miR-34b/c transfectants significantly showed an increase in the G0–G1 fraction (P < 0.002) and a decrease in the G2–M and S fractions (P < 0.002), indicating that miR-34b/c induced G1 cell cycle arrest (Fig. 2D).

MPM stable transfectants and migration, invasion, and motility assay

To estimate the effect of miR-34b/c on migration and invasion potential in MPM, cell migration and invasion was examined using a Boyden chamber. Microscopic images of the Boyden chamber assay are shown in Figure 3A and B. Migration and invasion were significantly suppressed in miR-34b/c stable transfectants, compared with control transfectants. In addition, the effect of miR-34b/c on the motility of MPM cells was estimated using time lapse video microscopy (Fig. 3C). Representative videos are shown in Supplementary Data S1 and S2. The migration velocity was slower in miR-34b/c stable transfectants of H28 and H2052, compared with control transfectants. However, no remarkable difference between miR-34b/c and control transfectants was noted in H290. Although there may be exceptions, on the whole, these results indicated that miR-34b/c was associated with the migration, invasion, and motility of MPM cells.

Adenoviral-mediated p53 and miR-34b/c transfer into cell lines

p53 and p21 were expressed in the 5 MPM cell lines tested (Supplementary Fig. S2). Genotyping data for the TP53 gene were queried from the database of the Cancer Genome Project, Sanger Institute, Cambridge, UK (www.sanger.ac.uk) to confirm that no TP53 mutations existed in all MPM cell lines we used. These data indicate that the wild-type TP53 gene is present and that p53 is likely functional in MPM cell lines.

To examine the impact of p53 on MPM, we transferred the TP53 gene using an adenovirus vector (Ad-p53) into MPM (H28, H290, and H2052) and a lung cancer cell line (H125), with mutant TP53 gene. Western blotting
confirmed that p53 and p21 were upregulated after Ad-p53 transfer (Supplementary Fig. S3: data not shown in H125). Cell viability was examined using an MTS assay. Ad-p53 transfer did not significantly influence the cell viability of the H28, H290, and H2052 cell lines whereas cell viability of H125 was influenced by Ad-p53 (Fig. 4). We also examined the effect of p53 on the expression of miR-34s in MPM and lung cancer cell lines. After Ad-p53 transfer, miR-34b and miR-34c were upregulated in lung cancer cells without miR-34b/c methylation but not in MPM cells with miR-34b/c methylation (Supplementary Table S2).

Next, we transferred Ad-miR-34b/c into cells. Seventy-two hours after Ad-miR-34b/c infection, miR-34b and miR-34c expression were evaluated and the increased expression of both miR-34b and miR-34c was confirmed. The expression values of Ad-miR-34b/c–infected MPMs ranged from 501 to 6,540, as shown in Table 2, part B. These values were much higher than that in nonmalignant mesothelial cells, indicating that adenoviral-mediated miR-34b/c introduction induced miR-34b and miR-34c overexpression. An MTS assay showed that MPM cells infected with Ad-miR-34b/c revealed a significant decrease in cell viability, compared with those infected with Ad-Luc (H28, \( P < 0.01 \); H290, \( P < 0.01 \); and H2052, \( P < 0.01 \); Fig. 4).

**Ad-miR-34b/c or Ad-p53 transfer and apoptosis**

Western blotting was carried out in MPM cells after Ad-miR-34b/c infection to examine the effect of miR-34b/c overexpression on protein expression. Whereas the results were similar to those in stable transfectants, in which the miR-34b and miR-34c expressions were comparable to physiologic levels, Bcl-2 protein expression was strongly downregulated in Ad-miR-34b/c–infected MPM cell lines (Supplementary Fig. S4). On the basis of these results, cell cycle analysis was conducted to examine whether apoptosis is induced by miR-34b/c using flow cytometry 72 hours after infection. In the H28, H290, and H2052 cell lines, infection with Ad-miR-34b/c caused an increase in the sub-G0–G1 DNA content, compared with that in cells infected with Ad-Luc (H28, 5.6%–47.0%; H290, 6.0%–39.9%; H2052, 4.0%–11.5%), indicating the induction of apoptosis (Supplementary Fig. S5). In contrast, Ad-p53 did not induce a drastic change in the

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**Figure 3.** The impact of miR-34b/c on MPM cell migration, invasion, and motility. Representative examples of migration (A) or invasion (B) assay are shown above. The quantitative values expressed as the means ± SD of 5 microscopic fields are representative of 3 experiments (below). C, cell motility was observed under a time-lapse video microscopy. The position of cell nucleus was measured and tracked every minute (0–5 hours) and plotted (red lines; top). The distance that the cell nucleus transversed and velocity for each minute were calculated to determine the speed of the movement in control (p-Scramble) or miR-34b/c–transfected MPM (p-miR-34b/c) cells (bottom).
sub-G₀–G₁ DNA content (H28, 5.6%–7.6%; H290, 6.0%–11.6%; H2052, 4.0%–6.7%).

**Discussion**

Genetic inactivation of p53 arises in approximately 50% of malignant tumors (28), alteration of \( \text{TP53} \) is rare in MPM (29–31). Indeed, the expression of wild-type p53 and p21, the best-characterized downstream targets of p53, was intact in the MPM cell lines that were used in this study, suggesting that p53 is functional in MPM as previously reported (32, 33). Preclinical experience with mesothelioma cell lines has shown a resistance to \( \text{TP53} \) gene transfer in a number of cell lines (34), indicating that most MPM cells already have functional p53. Also in the present study, \( \text{TP53} \) gene transfer had a minimal effect in MPM cells, whereas miR-34b/c transfer induced apoptosis, producing a significant antitumor effect.

We validated that several genes that have been identified as targets of miR-34b/c, including \( c\text{- MET} \), \( \text{CDK4} \), \( \text{CDK6} \), \( \text{CCND1} \), \( \text{CCNE2} \), \( \text{Bcl-2} \), \( \text{c-MYC} \), and \( \text{E2F3} \), were downregulated after the introduction of miR-34b/c. Among the genes that are miR-34b/c targets, \( c\text{- MET} \) was recently reported to be activated in MPM by overexpression or mutation. The suppression of \( c\text{- MET} \) using MET inhibitors revealed the potent inhibition of proliferation, invasion, and migration in some MPM cell lines (35). Thus, the inhibition of \( c\text{- MET} \) may contribute to the tumor-suppressive function of miR-34b/c in MPM. Of note, the H2052 cell line, in which \( c\text{- MET} \) was expressed, was not inhibited by the \( c\text{- MET} \) inhibitor (35). We confirmed that H2052 was not inhibited by the knockdown of \( c\text{- MET} \) using siRNA, whereas H28 and H290 were significantly inhibited (Supplementary Fig. S6). However, miR-34b/c introduction inhibited proliferation of H2052. This result suggested that the tumor-suppressive effect of miR-34b/c on MPM resulted from the downregulation of multiple oncogenic genes that are directly or indirectly regulated by miR-34b/c, which implies that miRNA such as miR-34b/c may be much more efficient and effective for a therapeutic target rather than an individual gene such as \( c\text{- MET} \).

Our results also showed that cell cycle arrest and induction of apoptosis were caused by miR-34b/c. A subset of genes known as cell cycle and transcriptional regulators were also downregulated with miR-34b/c introduction (9, 12). \( \text{CDK4} \), \( \text{CDK6} \), \( \text{CCND1} \), and \( \text{CCNE2} \) are regulators of cell cycle and are required for the transition from G₁–S phase (36). Among them, \( \text{CDK4} \) is negatively regulated by \( p16\text{INK4A} \), which is deleted in the majority of MPMs (29). Frizzle and colleagues showed that the reexpression of \( p16\text{INK4A} \) induced G₁ cell cycle arrest and apoptosis in MPM (37). Thus, the inhibition of CDK4 along with other cell cycle regulators is considered to lead to the induction of cell cycle arrest and the subsequent inhibition of proliferation in MPM.

The antiapoptotic potential of MPM makes it highly resistant to chemotherapeutic agents and radiation (2). Wild-type p53 causes G₁ cell cycle arrest, allowing damage to be repaired before replication or triggering apoptosis if the damage cannot be repaired (38). In MPM, which has wild-type \( \text{TP53} \) gene, resistance to apoptosis has been considered to arise downstream of p53. Bcl-2 is an antiapoptotic protein located downstream of p53. Overexpression of Bcl-2 in MPM tumor has been reported from 8% to 40% (39, 40) and 4 of 6 MPM cell lines seem to exhibit Bcl-2 overexpression in our study (Supplementary Fig. S2). In miR-34b/c stable transfectants, Bcl-2 did not seem to be downregulated, compared with control transfectants. Because stable transfectants are derived from selected clones that survive after miR-34b/c transfection, stable transfectants are not appropriate for examining the effect of miR-34b/c on apoptosis. Thus, whereas Ad-miR-34b/c...
transfer led to overexpression of miR-34b and miR-34c and induced apoptosis with the downregulation of Bcl-2, whether physiologic levels of miR-34b/c expression are capable of downregulating Bcl-2 in MPM remains unknown.

In this study, we used stable transfectants and adenoviral-infected cells. Our stable transfectants with physiologic expression values of miR-34b and miR-34c would be appropriate models for investigating the pathogenic role of miR-34b/c in MPM. Using this system, the restoration of the miR-34b and miR-34c suppressed oncologic features of MPM, including cell proliferation and invasiveness, strongly suggests that the silencing of miR-34b/c by methylation is a key alteration in MPM. On the other hand, the adenoviral system induced elevated miR-34b and miR-34c expression possibly induced apoptosis, suggesting the therapeutic possibility of miR-34b/c transfer for MPM.

Many functionally validated miRNAs target oncogenes and tumor suppressors. Moreover, gain or loss of function of individual miRNA has been reported to affect tumor cell proliferation, apoptosis, and invasion (41, 42). Therefore, it is thought that normalization of miRNA expression could be a potential method of therapeutic intervention. As miR-34b/c acts as a tumor suppressor, its expression should be restored in targeted tumor cells by the delivery. Delivery systems for miRNA to access tumor cells are available, including viral or liposomal delivery (43, 44).

Because MPM is one of the most difficult malignancies to treat, it is significant that the current study suggests a potential utility of miR-34b/c as a therapeutic option. Combination of miR-34b/c delivery and existing conventional therapies could be synergistic. Because miR-34b/c recovers several crucial functions in p53 pathway, miR-34b/c may increase the sensitivity of tumor cells for conventional antitumor agents or radiation. Further investigations are warranted for applying the miR-34b/c to novel therapeutic strategies.

In conclusion, our results show that miR-34b/c is frequently downregulated by aberrant methylation in MPM, resulting in the loss of tumor-suppressive p53 function and the acquisition of a malignant phenotype. miR-34b/c plays an important role in the pathogenesis of MPM, and the epigenetic silencing of miR-34b/c might explain why p53 functions are impaired in MPM despite the presence of intact p53 in the majority of MPM. Our study provides new insights into the molecular pathogenesis of MPM and suggests that miR-34b/c can be a potential therapeutic target for MPM.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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Epigenetic Silencing of MicroRNA-34b/c Plays an Important Role in the Pathogenesis of Malignant Pleural Mesothelioma

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