Acta Med. Okayama, 2014
Vol. 68, No. 1, pp. 23–26

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Original Article

Preclinical Evaluation of MicroRNA-34b/c Delivery for Malignant Pleural Mesothelioma

Tsuyoshi Ueno[a], Shinichi Toyooka[a]*, Takuya Fukazawa[b], Takafumi Kubo[a], Junichi Soh[a], Hiroaki Asano[a], Takayuki Muraoka[a], Norimitsu Tanaka[a], Yuho Maki[a], Kazuhiko Shien[a], Masashi Furukawa[a], Masakiyo Sakaguchi[c], Hiromasa Yamamoto[a], Kazunori Tsukuda[a], and Shinichiro Miyoshi[a]

Departments of [a]Thoracic Surgery and [c]Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700–8558, Japan,
[b]Department of General Surgery, Kawasaki Medical School, Kurashiki, Okayama 700–8505, Japan

The microRNA-34s (miR-34s) have p53 response elements in their 5'-flanking regions and demonstrate tumor-suppressive functions. In malignant pleural mesothelioma (MPM), we previously reported that expression of miR-34b and miR-34c (miR-34b/c) was frequently downregulated by methylation in MPM cell lines and primary tumors. The forced overexpression of miR-34b/c showed significant antitumor effects with the induction of apoptosis in MPM cells. In this study, we examined the *in vivo* antitumor effects of miR-34b/c using adenovirus vector on MPM. We subcutaneously transplanted NCI-H290, a human MPM cell line, into BALB/C mice and injected adenovirus vector expressing miR-34b/c, luciferase driven by the cytomegalovirus promoter (Ad-miR-34b/c or Ad-Luc), or PBS control into tumors over 5 mm in diameter. A statistically significant growth inhibition of the tumor volume was observed in the Ad-miR-34b/c group from day 6 onward compared to the Ad-Luc group. The inhibition rate of Ad-miR-34b/c, compared to the tumor volume treated with Ad-Luc, was 58.6% on day 10 and 54.7% on day13. Our results indicate that adenovirus-mediated miR-34b/c gene therapy could be useful for the clinical treatment of MPM.

Key words: mesothelioma, microRNA, microRNA-34b/c, p53

Malignant pleural mesothelioma (MPM) is a neoplasm with highly invasive and aggressive clinical features, and exposure to asbestos is strongly associated with its etiology [1]. Curative modalities such as radiotherapy, conventional chemotherapy, or molecular targeting therapy have not yet been established for advanced MPM, so the development of new treatments is urgently needed [2].

Received February 13, 2013; accepted September 9, 2013.
*Corresponding author. Phone:+81–86–235–7265; Fax:+81–86–235–7269
E-mail:toyooka@md.okayama-u.ac.jp (S. Toyooka)

MicroRNAs (miRNAs) are a group of noncoding small RNAs that usually regulate their target mRNAs by posttranscriptional repression [3]. Recently, there has been intensive research into the role of miRNAs in human malignant tumors because of the ability of individual miRNAs to regulate multiple genes implicated in multiple pathways [4]. Similar to encoding genes, some miRNAs have been classified as oncogenic or tumor-suppressive according to their effects on cellular transformation [5]. Among the tumor-suppressive miRNAs are the miRNA-34 family members (miR-34s) with p53 response elements in their 5'-flank-

ing regions: the family includes miR-34a, miR-34b and miR-34c [6–8]. These members are direct transcriptional targets of p53 and constitute part of the p53 tumor suppressor network regulating cell cycle arrest, apoptosis and senescence [6, 9].

In MPM, mutations and deletions of the *TP53* gene are rare [10, 11], even though MPM is often associated with cell cycle alterations and anti-apoptosis, suggesting functional p53 deficiency [12]. We previously examined the methylation and expression status of miR-34s in thoracic malignancies such as lung cancer and MPM [13, 14]. We found that expression of miR-34b and miR-34c (miR-34b/c) was frequently downregulated by methylation in MPM cell lines and primary tumors, and that forced overexpression of miR-34b/c, but not of p53, had a significant antitumor effect that induced apoptosis in MPM cells *in vitro* [13].

In this study, we used an adenovirus vector containing miR-34b/c to examine the *in vivo* antitumor effects of miR-34b/c on the MPM cell line NCI-H290 (H290), in which miR-34b and miR-34c expression is downregulated by methylation.

Materials and Methods

Cell line. H290, a human MPM cell line, was a kind gift 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, USA).

Adenovirus vectors carrying microRNA-34b/c and Luciferase. Adenovirus vector expressing miR-34b/c driven by cytomegalovirus (CMV) promoter (Ad-miR-34b/c) was generated by homologous recombination and plaque purified. Adenovirus vector expressing luciferase driven by CMV promoter (Ad-Luc) was used as a control vector [13, 15]. Following the application manual Ver. 1.4 of AdEasy™ vector system (Agilent Technologies, CA, USA), Ad-miR-34b/c and Ad-Luc were amplified by infection with QBI-293A. Adenovirus was purified with CsCl and viral suspension was titrated with O.D. 260 nm [VP/mL].

Experimental animals. We used adult female mice (BALB/C nu/nu: 4 weeks) purchased from Charles River Laboratories Japan (Yokohama, Japan). They were maintained in a specific pathogen-free environment with free access to food and water at the Department of Animal Reserves, Shikata Laboratory, Advanced Science Research Center of Okayama University, and allowed to adapt to their environment for more than one week before beginning the experiments. The animals were housed and handled in accordance with the Okayama University Animal Research Committee Guidelines.

Treatment protocol. H290 human MPM cells [1.0×10^7 in 50 μl phosphate buffered saline (PBS)], mixed with 50 μl of Matrigel (BD Biosciences, San Jose, CA, USA), were subcutaneously injected into the thighs of mice on both sides. The tumors were permitted to grow to approximately 5 mm in diameter and the mice were then randomly assigned to one of three treatment groups: (1) intratumoral injection of Ad-Luc, (2) intratumoral injection of Ad-miR34b/c, or (3) intratumoral injection of PBS. The injections were targeted to the center and periphery of each tumor mass to deliver the agent diffusely. The first day of vector administration was taken as day 0. Animals were observed 3 times per week for physical appearance, activity levels and mortality patterns.

The transduction efficiency was checked after intratumoral vector injection. Two mice in each of the 3 groups were injected intratumorally with 100 μl of the same concentration of Ad-Luc or Ad-miR-34b/c, or 100 μl of PBS. They were sacrificed on day 3 and the intratumoral expression of miR-34b/c was detected using quantitative real-time PCR as described previously.

To examine the antitumor effects of Ad-miR-34b/c, we injected five mice per group with 100 μl of Ad-Luc, Ad-miR-34b/c or PBS on day 0, 3 and 6. The average concentration of Ad-Luc and Ad-miR-34b/c was 1.86 (range, $1.25-2.16) \times 10^{11}$ viral particles/tumor in 100 μl buffer with reference to a previous report [16].

Tumor sizes were measured with vernier calipers and tumor volume was calcu-lated using the following formula: $3.14 \times 1/2 \times$ (the shortest diameter)$^2 \times$ (the longest diameter). The day of sacrifice was day 20 after which tissues were frozen quickly by liquid nitrogen and stored at -80℃. The body weight of each mouse was monitored using a sensitive balance during the entire study period.

Statistical analysis. Data were represented as mean ± SD. Mann–Whitney U test was used to compare data between groups. The differences were considered statistically significant at $p < 0.05$.

Results

Confirmation of miR-34b/c expression after treatment. We evaluated the intratumoral expression of miR-34b and miR-34c after injection in the 3 groups of mice. miR-34b and miR-34c expression in the Ad-miR-34b/c group was 27.9 and 19.2 times higher than in the PBS group, respectively, while that in the Ad-Luc group was the same as the PBS group (Fig. 1). None of the mice died during the experiment in any of the treatment groups.

The growth inhibition of Ad-miR-34b/c treatment on mouse H290 xenografts. Subsequently, we evaluated the growth inhibition of implanted tumors with Ad-miR-34b/c treatment. There was no significant difference in the average tumor volume between the 3 groups on day 0: Ad-Luc $98.4 \pm 12.5\,mm^3$, Ad-miR-34b/c $97.1 \pm 14.9\,mm^3$, and PBS $108.7 \pm 7.1\,mm^3$. Time course and tumor volume after treatment are shown in Fig. 2. A statistically significant growth inhibition of tumor volume was observed in the Ad-miR-34b/c group compared to the Ad-Luc group from day 6 onward. The inhibition rate of Ad-miR-34b/c, compared to the tumor volume treated with Ad-Luc, was 58.6% on day 10 and 54.7% on day 13. Interestingly, the tumor volume in the Ad-miR-34b/c group did not change from day 6 to day 10, suggesting that Ad-miR-34b/c suppressed tumor growth during this period. After day 10, there was no difference in the rate of tumor growth between the Ad-miR-34b/c and Ad-Luc groups.

There was no significant difference in the average body weight of the mice between the 3 groups throughout the experiment (Fig. 3).

Discussion

In this study, we showed the direct antitumor effect of miR-34b/c introduced by adenovirus vector on miR-34b/c silenced MPM cells *in vivo*. To the best

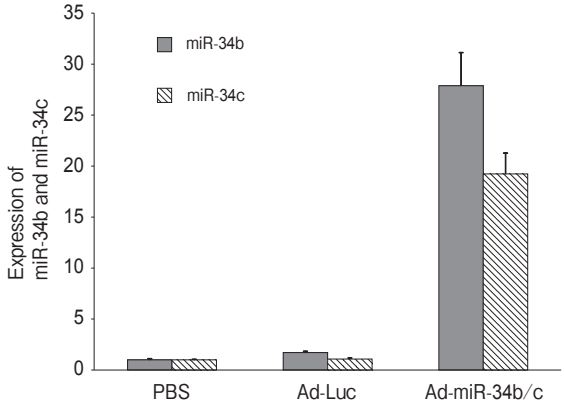

Fig. 1 The transduction efficiency after intratumoral adenovirus vector injection. The intratumoral expression of miR-34b and miR-34c was detected for 2 mice in each group with quantitative real-time PCR. miR-34b and miR-34c expression in the Ad-miR-34b/c group was higher than in the PBS group ($p < 0.05$), while that in the Ad-Luc group was the same as the PBS group. The expression in PBS group was considered the control.

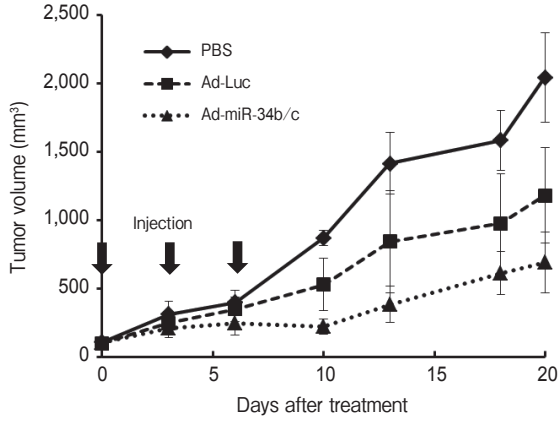

Fig. 2 The growth inhibition of Ad-miR-34b/c treatment on mouse H290 xenografts. The average tumor volume was calculated for 5 mice per group and the time course of tumor volumes after treatment is shown. A statistically significant growth inhibition of tumor volume was observed in the Ad-miR-34b/c group from day 6 on compared to the Ad-Luc group ($p < 0.05$).

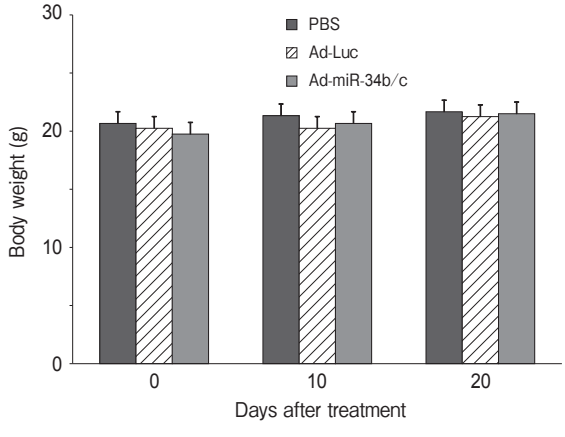

Fig. 3 The body weight in mice on day 0, 10 and 20 after each treatment. There was no significant difference in the average body weight of the mice between the 3 groups.

of our knowledge, the *in vivo* therapeutic effect of miR-34b/c has not previously been reported in any form of malignant disease. In addition, there have been no reports of miRNA use in MPM *in vivo*. However, the therapeutic impact of miR-34a was previously shown following lentivirus-mediated transfer. Furthermore, a lipid-based delivery of chemically synthesized miR-34a in lung cancer cells has also been demonstrated [17, 18].

Our previous study revealed the anti-proliferative effect of Ad-miR-34b/c on 3 different miR-34b/c methylated MPM cell lines (H28, H290 and H2052). However, stable xenografts could only be generated from H290 cells in the present study. Although our previous study demonstrated the effect of Ad-miR-34b/c on MPM even in cell H2052 with preserved miR-34a expression, the solo xenograft model here may be considered a limitation in this study.

The tumor growth curve in this study revealed that the antitumor effect was observed until day 10, 4 days after the last injection of Ad-miR-34b/c. There are a number of possible explanations for this, including the heterogeneous tumor infection of Ad-miR-34b/c or the cytostatic effect of Ad-miR-34b/c *in vivo*.

In addition, miR-34b/c is known to enhance the effects of radiation *in vitro*, so its use in combination with chemotherapeutic agents or radiation might enhance the antitumor effects of a single treatment [19]. This merits further investigation in future studies.

In summary, we show that miR-34b/c suppresses MPM tumor growth *in vivo*, which supports the findings of our previous *in vitro* study. Preclinical studies such as this are an important part of the process of clinical application to assess target molecule and delivery systems *in vivo*. Thus, our study is relevant in that it shows the feasibility of miRNA MPM therapy using a virus vector, and suggests that Ad-miR-34b/c in particular would be a promising therapeutic agent for MPM.

References

1. Spirtas R, Heineman EF, Bernstein L, Beebe GW, Keehn RJ, Stark A, Harlow BL and Benichou J: Malignant mesothelioma: attributable risk of asbestos exposure. Occup Environ Med (1994) 51: 804–811.
2. Robinson BW, Musk AW and Lake RA: Malignant mesothelioma. Lancet (2005) 366: 397–408.
3. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell (2004) 116: 281–297.
4. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR and Golub TR: MicroRNA expression profiles classify human cancers. Nature (2005) 435: 834–838.
5. Garzon R, Calin GA and Croce CM: MicroRNAs in Cancer. Annu Rev Med (2009) 60: 167–179.
6. He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, Xue W, Zender L, Magnus J, Ridzon D, Jackson AL, Linsley PS, Chen C, Lowe SW, Cleary MA and Hannon GJ. A: microRNA component of the p53 tumour suppressor network. Nature (2007) 447: 1130–1134.
7. Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, Zhai Y, Giordano TJ, Qin ZS, Moore BB, MacDougald OA, Cho KR and Fearon ER: p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Curr Biol (2007) 17: 1298–1307.
8. Corney DC, Flesken-Nikitin A, Godwin AK, Wang W and Nikitin AY: MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. Cancer Res (2007) 67: 8433–8438.
9. Hermeking H: p53 enters the microRNA world. Cancer Cell (2007) 12: 414–418.
10. Cote RJ, Jhanwar SC, Novick S and Pellicer A: Genetic alterations of the p53 gene are a feature of malignant mesotheliomas. Cancer Res (1991) 51: 5410–5416.
11. Toyooka S, Kishimoto T and Date H: Advances in the molecular biology of malignant mesothelioma. Acta Med Okayama (2008) 62: 1–7.
12. Shimamura A and Fisher DE: p53 in life and death. Clin Cancer Res (1996) 2: 435–440.
13. Kubo T, Toyooka S, Tsukuda K, Sakaguchi M, Fukazawa T, Soh J, Asano H, Ueno T, Muraoka T, Yamamoto H, Nasu Y, Kishimoto T, Pass HI, Matsui H, Huh NH and Miyoshi S: Epigenetic silencing of microRNA-34b/c plays an important role in the pathogenesis of malignant pleural mesothelioma. Clin Cancer Res (2011) 17: 4965–4974.
14. Tanaka N, Toyooka S, Soh J, Kubo T, Yamamoto H, Maki Y, Muraoka T, Shien K, Furukawa M, Ueno T, Asano H, Tsukuda K, Aoe K and Miyoshi S: Frequent methylation and oncogenic role of microRNA-34b/c in small-cell lung cancer. Lung Cancer (2012) 76: 32–38.
15. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW and Vogelstein B: A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A (1998) 95: 2509–2514.
16. Kawauchi K, Watanabe M, Kaku H, Huang P, Sasaki K, Sakaguchi M, Ochiai K, Huh NH, Nasu Y and Kumon H: Preclinical safety and efficacy of in situ REIC/Dkk-3 gene therapy for prostate cancer. Acta Med Okayama (2012) 66: 7–16.
17. Kasinski AL and Slack FJ: miRNA-34 prevents cancer initiation and progression in a therapeutically resistant K-ras and p53-induced mouse model of lung adenocarcinoma. Cancer Res (2012) 72: 5576–5587.
18. Wiggins JF, Ruffino L, Kelnar K, Omotola M, Patrawala L, Brown D and Bader AG: Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. Cancer Res (2010) 70: 5923–5930.
19. Maki Y, Asano H, Toyooka S, Soh J, Kubo T, Katsui K, Ueno T, Shien K, Muraoka T, Tanaka N, Yamamoto H, Tsukuda K, Kishimoto T, Kanazawa S and Miyoshi S: MicroRNA miR-34b/c enhances cellular radiosensitivity of malignant pleural mesothelioma cells. Anticancer Res (2012) 32: 4871–4875.