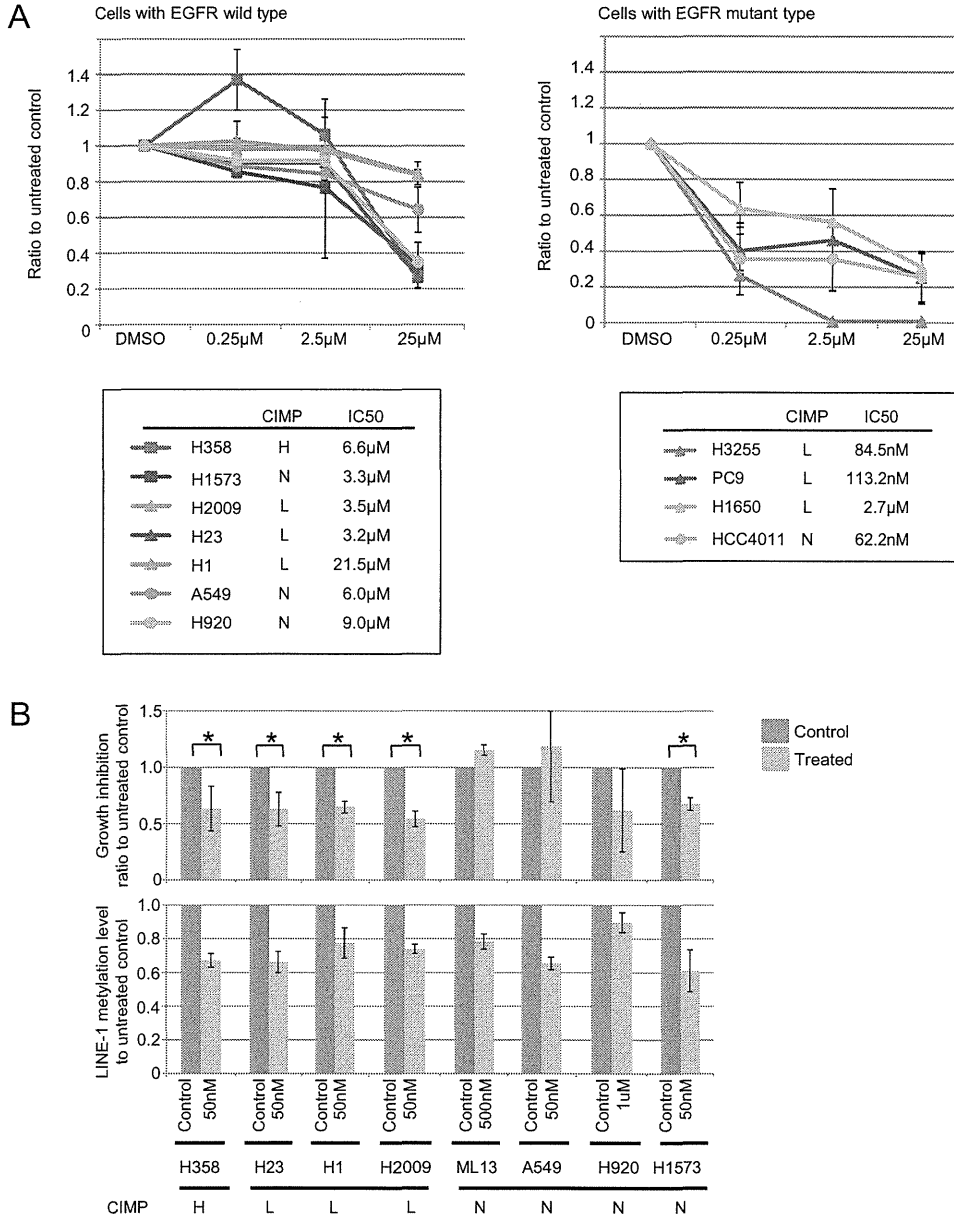

Fig. 4. Epigenetic treatment in AdCa cell lines. (**A**) Growth rate of AdCa cell lines with each *EGFR* status as measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-bdiphenyl tetrazolium bromide assay after 72 h of treatment of TKI (AG1478) in different doses. CIMP status of each cell line is indicated by H, L and N. (**B**) Growth inhibition of AdCas after 5-Aza-dC treatment. Growth inhibition was examined (upper panel) at the concentration where 20% of LINE-1 demethylation had occurred (lower panel). $^*P < 0.05$.

of genes critical for tumor progression and for response to chemotherapy.

We found another epigenomic subgroup (CIMP-L) of AdCas within the major subclasses classified by DNA methylation status. This subtype showed moderate accumulation of DNA methylation. Indeed, our cell line study showed that CIMP-L cells were sensitive to 5-Aza-dC treatment. These data suggest that tumorigenesis pathway of CIMP-L AdCas might also be affected by DNA methylation to a certain extent. However, we could not find any specific features of this subtype. This might be due to the lack of suitable markers to further classify CIMP-L, resulting in a mixture of subpopulations as was found in the colon cancer study (39,40). Sensitive and specific markers for CIMP-L in AdCas are needed to further characterize CIMP-L. Additional studies will be required to address this problem.

CIMP-positive lung AdCa cell lines appeared to be more sensitive to 5-Aza-dC treatment, in which demethylation effectively occurred even at low doses of 5-Aza-dC, regardless of *EGFR* mutation status. Epigenetic drugs targeting DNA methylation, such as 5-Aza-dC and 5-azacytidine, have shown clinical effectiveness in cancer treatment, especially for hematological malignancies (41,42). For the treatment of thoracic malignancies, studies showed that a certain population of patients with AdCas clinically benefit from 5-Aza-dC treatment (43,44). One of the important issues of research is the identification of biomarkers predictive of response to DNA methylation inhibitors (45). Our cell line analysis showed that CIMP status appeared to be associated with response to 5-Aza-dC, suggesting that epigenetic therapy might be a useful approach, especially for those individuals who have been diagnosed with CIMP. If this possibility was validated, our findings would be significant for the use of DNA methylation inhibitors in lung tumors.

In conclusion, we demonstrated here that six newly identified CIMP markers may be useful in the accurate and practical epigenomic

classifications of lung cancer. Our findings may enable the development of new molecular diagnostics tools for personalized medicine for lung cancers and confer a new paradigm for cancer treatment.

Supplementary material

Supplementary Figures 1–3 and Tables 1–4 can be found at http://carcin.oxfordjournals.org/.

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Malignant Pleural Mesothelioma Localized in the Thoracic Wall

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Malignant pleural mesothelioma (MPM) usually shows a diffuse pattern of growth over the pleural surfaces. We present an unusual case of MPM that was localized and had spread outside the thoracic wall.

CASE REPORT

A 65-year-old man was referred to our hospital because of dry cough. He was a never smoker and worked at a chemical fiber factory where he was exposed to asbestos for 40 years. Physical examination revealed that the skin over the left subclavian area was swollen but without tenderness or redness.

A chest radiograph showed hypolucency on the left side. A computed tomographic scan of the chest showed a 7-cm diameter tumor on the left anterior chest wall surrounding the second rib (Fig. 1). No pleural plaque, effusion, or lymphadenopathy was detected. Fluorodeoxyglucose positron emission tomographic computed tomographic imaging showed the accumulation of fluorodeoxyglucose in the tumor with the maximum standardized uptake value of 15.1 without accumulation in any other organ. The diagnosis of MPM, epithelioid type, was made from a percutaneous needle-biopsy specimen. Thoracoscopic exploration showed that the tumor was covered with parietal pleura, and no dissemination was found on the pleura. The tumor and the involved first, second, and the third ribs were removed with curative intent. The resected specimen revealed cubiform tumor cells; some of which were forming a papillary tubular structure (Fig. 2*A*). Immunohistochemical analyses demonstrated that the tumor cells were positive for calretinin (Fig. 2*B*), D2–40, Wilms' tumor 1, thrombomodulin, cytokeratin 5/6, and epithelial membrane antigen, and negative for carcinoembryonic antigen (Fig. 2C), thyroid transcription factor, and napsin A. The diagnosis was confirmed as epithelioid type MPM. Detailed investigation of the specimen showed that the parietal pleura was involved with tumor cells (Fig. 3), indicating that the tumor originated from the parietal pleura. Six months later, he had a local recurrence and underwent radiotherapy and systemic chemotherapy.

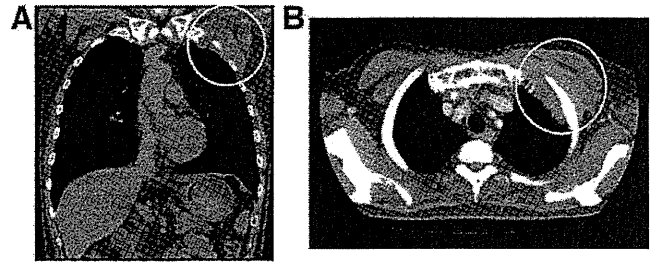

FIGURE 1. Computed tomographic scan of the chest at the time of diagnosis showing (*A*) coronal and (*B*) horizontal views of a tumor on the left anterior chest wall.

DISCUSSION

In the current case, a patient presented with MPM that localized and spread outside the thoracic wall. The tumor was initially suspected to be a soft-tissue neoplasm, osteoblastic metastatic tumor, or malignant lymphoma. The pathological diagnosis of MPM was determined from a percutaneous needle-biopsy specimen. Thoracoscopic exploration showed that the tumor was covered with parietal pleura without dissemination into the pleura, an unusual pattern of MPM progression.

MPM is classified in diffuse MPM or localized (LMPM). LMPM is uncommon and characterized by a sharply circumscribed tumor of the seromal membranes with the microscopic appearance of diffuse malignant mesothelioma, but without any evidence of diffuse spread.[1] It was formerly considered a benign variant of mesothelioma,[2] solitary fibrous tumor,[3] or other neoplasms, but is now defined as having the microscopic, histochemical, immunohistochemical, and ultrastructural features of diffuse MPM. However, little is known about the frequency and clinical behavior of LMPM.

The tumor in the current case was grossly localized in the thoracic wall. Detailed pathological analyses showed that the parietal pleura were involved with tumor cells, but diffuse pleural spread was not determined; therefore, we regard

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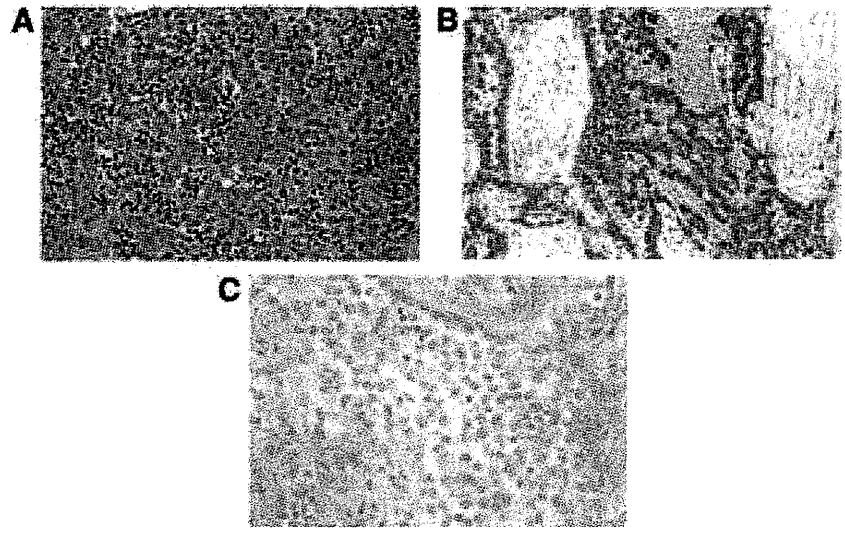

FIGURE 2. *A*, Microscopic examination of the biopsy specimen showed cubiform tumor cells; some of the cells formed papillary tubular structures consistent with malignant mesothelioma (hematoxylin-eosin, 40 ×). *B*, Immunohistochemical analysis indicated positive expression of calretinin (40 ×) and (C) negative expression of carcinoembryonic antigen (40 ×).

FIGURE 3. Microscopic examination of the biopsy specimen showed that the parietal pleural was involved with tumor cell (arrows), indicating that the tumor originated from the parietal pleura.

the current case as LMPM. LMPM should be considered a thoracic tumor that is localized and has spread outside the thoracic wall.

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MicroRNA miR-34b/c Enhances Cellular Radiosensitivity of Malignant Pleural Mesothelioma Cells

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Abstract. *Background: We previously reported that epigenetic silencing of microRNA-34b/c (miR-34b/c) plays an important role in the pathogenesis of malignant pleural mesothelioma (MPM). We examined the impact of miR-34b/c restoration on the radiosensitivity of MPM cells. Materials and Methods: We established stable miR-34b/c and scramble transfectants of two MPM cell lines, H2052 and H28. We examined these transfectants by clonogenic survival assay, phosphorylated histone H2AX (γH2AX) foci assay, cell cycle analysis, and western blotting. Results: Clonogenic survival assay revealed that miR-34b/c radiosensitized MPM cells. γH2AX foci assay showed DNA double-strand break repair was delayed in miR-34b/c transfectants. The proportion of sub-G1 phase cells was increased in miR-34b/c transfectants after irradiation. miR-34b/c inhibited expression of cyclin D1, cyclin-dependent kinase 4/6, B-cell lymphoma 2 (BCL-2) and increased cleaved poly (ADP-ribose) polymerase (cPARP) and cleaved caspase-3 after irradiation. Conclusion: Our results indicate that miR-34b/c enhances radiosensitivity by promoting radiation induced-apoptosis and suggested that miR-34b/c might be a useful therapeutic molecule to enhance radiotherapy in MPM.*

Malignant pleural mesothelioma (MPM) is an aggressive neoplasm arising from the pleura. The prognosis of MPM is dismal, as neither chemotherapy nor radiotherapy remarkably improves its prognosis (1). Thus, an urgent need to understand the molecular biology of MPM exists so that new therapeutic strategies for MPM can be developed (2).

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Key Words: Malignant pleural mesothelioma, microRNA, miR-34b/c, radiation, radiosensitivity.

MicroRNAs (miRs) are a class of 17-22 nucleotide small non-coding RNAs (3). Among them, miR-34b/c has an upstream p53-binding site and is directly induced by p53 in response to oncogenic stress and DNA damage (4, 5). The known targets for miR-34b/c include mesenchymal epithelial transition factor (c-MET), cyclin dependent kinase (CDK) 4/6, cyclin D1 (CCND1), cyclin E2 (CCNE2) (4), and BCL2 (6). Since miR-34b/c suppresses these targets, miR-34b/c is thought to play an important role in the p53 tumor suppressor network.

We previously reported that miR-34b/c was frequently methylated in MPM, compared with miR-34a (7). Aberrant methylation in the promoter region of miR-34b/c was present in 6 out of 6 MPM cell lines and 40 (85.1%) out of 47 primary MPMs, whereas methylation of miR-34a was present in 2 (33.3%) out of 6 MPM cell lines and 13 (27.7%) out of 47 primary MPMs. Interestingly, the introduction of miR-34b/c, but not p53, induced significant antitumor effects such as G_1 arrest, apoptosis, and the inhibition of migration, invasion and cell motility of MPM cells (7); these effects arose through the suppression of multiple targets of miR-34b/c, indicating the importance of miR-34b/c in MPM.

Although genetic alteration of p53 is rare in MPM (8), MPM biologically exhibit an apparent p53-deficiency, resulting in antiapoptosis effects and cell cycle alterations. CCND1, one of the targets of miR-34b/c, is an important component of the core cell cycle machinery. Recently, CCND1 was reported to be a therapeutic target for MPM (9). One study showed that the reduction of CCND1 enhances radiosensitivity of human cancer by suppressing DNA double-strand break (DSB) repair (10). Another report described that flavopiridol, a pan-CDK inhibitor, also enhances radiosensitivity and induces apoptosis in non-small cell lung cancer (11), as well as gastric and colon cancer (12).

After considering these points, we hypothesized that the introduction of miR-34b/c might restore p53-mediated tumor suppression in response to ionizing radiation (IR) in MPM

cells. In this study, we examined the impact of miR-34b/c on the cellular radiosensitivity of MPM cells.

Materials and Methods

Cell culture and establishment of stable miR-34b/c transfectants. Two MPM cell lines (H2052 and H28) in which the expressions of miR-34b and miR-34c were epigenetically suppressed were used in this study. The two MPM cell lines were kindly provided by Dr. Adi F. Gazdar (Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX, USA) and were maintained in RPMI-1640 (Sigma Chemical Co., Saint Louis, MO, USA) supplemented with 10% fetal bovine serum in 5% CO_2. Stable miR-34b/c and scramble RNA transfectants were established as previously described in our report (7). Briefly, a fragment of genomic DNA encoding miR-34b/c or scramble RNA fragment as a negative control was subcloned into a pSilencer 4.1-CMV neo plasmid vector (Ambion, Austin, TX, USA). The constructed plasmids were then transfected into MPM cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). Resistant clones were isolated by G418 selection for two weeks and were maintained in G418-containing medium.

Evaluation of miR-34b/c expression using quantitative real-time Polymerase Chain Reaction (PCR). microRNA was extracted from miR-34b/c-transfected and scramble RNA-transfected MPM cells using TaqMan MicroRNA Cells-to-CT™ Kit (Ambion). Real-time PCR for miR-34b and 34c was performed using TaqMan MicroRNA Assays (P/N4427975, Assay ID 000427 for has-miR-34b, Assay ID 000428 for has-miR-34c; Applied Biosystems, Foster City, CA) using the StepOnePlus™ Real-Time PCR system (Applied Biosystems). The expression of miR-374 was used as an endogenous control following the manufacturer's recommendation (www.appliedbiosystems.com). The relative expression ratio for miR-34b and miR-34c was calculated from triplicate PCR.

Clonogenic cell survival assay. miR-34b/c-transfected and scramble RNA-transfected H2052 and H28 cells were trypsinized to generate a single cell suspension, and a specified number of cells were seeded into each well in six-well tissue culture plates. After allowing the cells time to attach (6 hours), the plates were irradiated at 2, 4, 6 or 8 Gy. Fourteen days after IR, colonies were stained with crystal violet. The number of colonies containing at least 50 cells was determined, and survival curves were generated. The dose enhancement factor (DEF) was calculated at a surviving fraction of 0.1 to estimate the increase in radiosensitivity. Data presented are the mean±standard deviation (SD) from at least two independent experiments.

Immunofluorescent staining for phosphorylated histone H2AX (γH2AX). γH2AX has been established as a sensitive indicator of DNA DSB with the resolution of foci corresponding to DNA DSB repair (13). miR-34b/c-transfected and scramble RNA-transfected H2052 cells were grown in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, USA). The cells were fixed in 4% paraformaldehyde for 10 minutes at 1 hour, 6 hours, and 24 hours after IR (4 Gy) and permeabilized in Phosphate buffered saline (PBS) containing 0.2% NP40 for 15 minutes. The cells were then incubated overnight at 4°C with 1:200 diluted anti-γH2AX antibody (Millipore, Billerica, MA,USA). The cells were incubated with 1:50 diluted fluorescein isothiocyanate (FITC)-labeled secondary antibody (Jackson Immuno Research Labs, West Grove, PA, USA) for 1 hour and were then incubated in PBS containing 4',6-diamidino-2-phenylindole (1 μg/ml) for 30 minutes. Coverslips were mounted using an anti-fade solution (DAKO Corp., Carpinteria, CA, USA). The slides were examined under a fluorescent microscope (Keyence BZ-8000; Keyence, Osaka, Japan). The number of γH2AX foci was counted in each nucleus of at least 50 cells in each sample.

Cell cycle analysis. The cell cycle distribution was evaluated using flow cytometry in miR-34b/c-transfected and scramble RNA-transfected H2052 cells. Non-irradiated transfectants and 4 Gy-irradiated transfectants at 24 hours after IR were harvested and resuspended in PBS containing 0.2% Triton X-100 and 1 mg/ml RNase, then stained with propidium iodide and analyzed using a FACScan instrument as previously described (7). Doublets, cell debris, and fixation artifacts were gated out, and cell cycle analysis was performed using CellQuest version 3.1 software.

Western blot analysis. Cells were harvested and western blot analysis was carried out as previously described (7). We examined four molecules (CCND1, CDK4, CDK6, and BCL2) reported to be primary targets of miR-34b/c. We also examined BCL2 Associated X-protein (BAX), cPARP, pro-caspase 3, and cleaved caspase 3 to detect apoptosis. Actin was used as loading control. The primary antibodies used for the western blotting were as follows: anti-CCND1 (sc718, diluted 1000:1; Santa Cruz, Santa Cruz, CA, USA), anti-CDK4 (sc260, diluted 200:1; Santa Cruz), anti-CDK6 (#3136, diluted 2000:1; Cell Signaling), anti-BCL2 (sc7382, diluted 500:1; Santa Cruz), anti-BAX (#2774, diluted 1000:1; Cell Signaling Technology, Beverly, MA, USA), anti-cPARP (#9546, diluted 2000:1; Cell Signaling), anti-pro-caspase 3 (sc7272, diluted 2000:1; Santa Cruz), and anti-actin (MAB1501, diluted 20000:1; Millipore).

Statistical analysis. Data are represented as the mean±SD. An unpaired Student *t*-test was used to compare data between two groups. Probability values less than 0.05 were considered statistically significant. All the data were analyzed using JMP9.0 for Windows (SAS Institute, Cary, NC, USA).

Results

Evaluation of miR-34b/c expression using quantitative real-time PCR. The expressions of miR-34b and 34c were examined in miR-34b/c and scramble RNA transfectants. Compared with the scramble RNA transfectants, the expression of miR-34b was increased by 26.6-fold and 10.5-fold in miR-34b/c-transfected H2052 and H28 cells, respectively. The expression of miR-34c was increased by 68.7-fold and 31-fold in miR-34b/c-transfected H2052 and H28 cells, respectively. Compared with the increase of miR-34b and miR-34c in miR-34b/c transfectants, the expression of miR-34b and miR-34c in scramble RNA-transfected H2052 cells at 24 hours after 4 Gy IR was only increased by 1.2-fold and 1.4-fold, respectively. Thus, we confirmed that the expression of mature miR-34b and miR-34c were increased in stable miR-34b/c transfectants.

Effect of miR-34b/c on cellular radiosensitivity. A clonogenic survival assay was performed to examine the effect of miR-

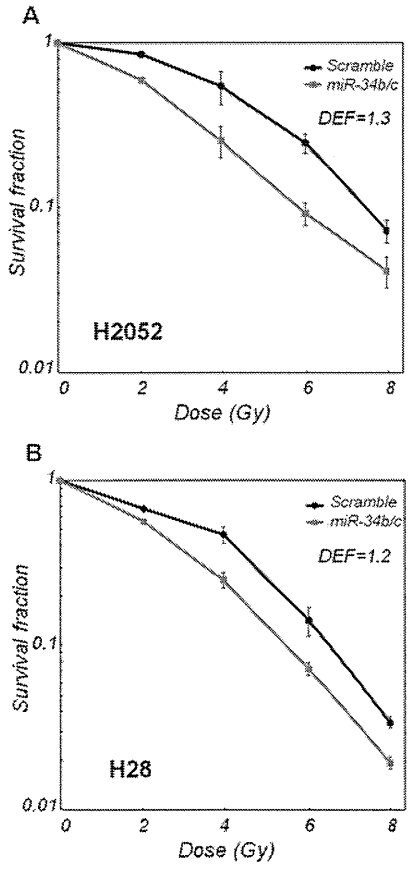

Figure 1. *Effect of miR-34b/c on cellular radiosensitivity in malignant pleural mesothelioma (MPM) cells. miR-34b/c transfected and scramble RNA-transfected MPM cells, H2052 (A) and H28 (B), were irradiated with graded doses of X-rays. The colony-forming efficiency was determined at 14 days after irradiation, and survival curves were generated. Data are the mean±SD of two independent experiments. DEF, Dose-enhancement factor.*

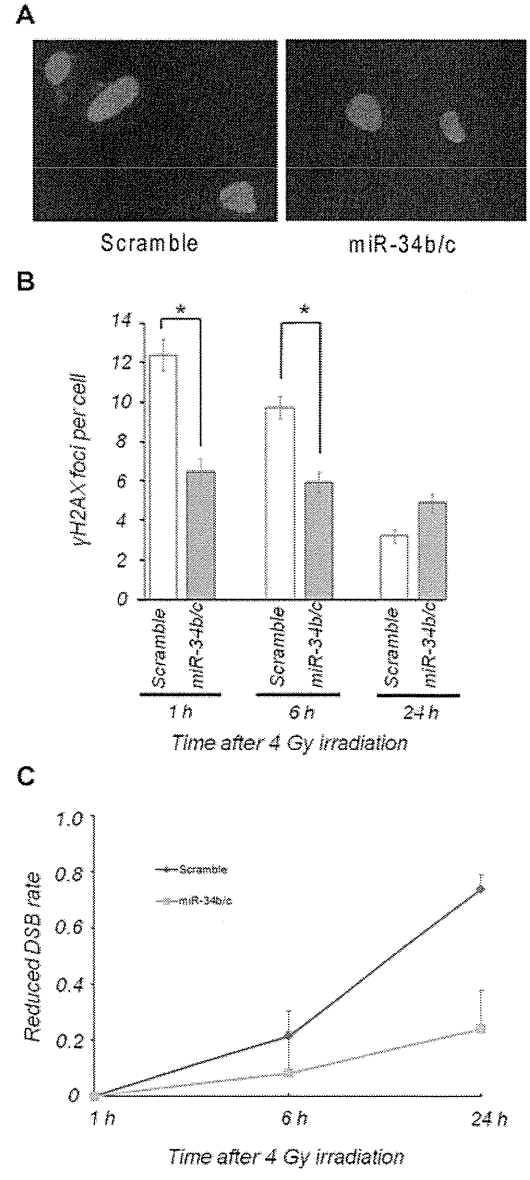

Figure 2. *Effect of miR-34b/c on radiation-induced phosphorylated histone H2AX (γH2AX) foci in H2052 cells. A: Representative micrographs obtained from scramble RNA-transfected H2052 cells (left) and miR-34b/c-transfected H2052 cells (right) at 1 hour after irradiation (4 Gy). B: miR-34b/c-transfected and scramble RNA-transfected H2052 cells on growing chamber slides were exposed to irradiation (4 Gy) and fixed at a specific time for use in immunocytochemical analyses of the nuclear γH2AX foci. Foci were evaluated in 50 nuclei per treatment for each sample. Data are the mean±SEM. *p<0.05. C: Reduced double strand break (DSB) rate in miR-34b/c-transfected and scramble RNA-transfected H2052 cells at 1, 6 and 24 hours after 4 Gy irradiation. Data are the mean+SEM of two independent experiments.*

34b/c on radiosensitivity in miR-34b/c transfectants, compared with the scramble control. As shown in Figure 1, miR-34b/c enhanced radiosensitivity of both miR-34b/c-transfected H2052 and H28 cells, with a DEF of 1.3 and 1.2, respectively. Because the radiosensitizing effect of miR-34b/c was stronger in H2052 cells than that in H28 cells, subsequent experiments were performed using H2052-derived transfectants.

Effect of miR-34b/c on radiation-induced γH2AX foci. We examined the DNA damage repair potential using a γH2AX foci assay. Representative micrographs are shown in Figure 2A. Compared with the scramble RNA transfectant, the number of γH2AX foci in miR-34b/c-transfected H2052 was significantly lower at 1 and 6 hours (considered to be early phase) after IR but was higher at 24 hours (considered to be late phase) (Figure 2B). We also calculated the rate of reduced γH2AX foci at 6 hours and 24 hours compared with 1 hour, *i.e.* the named reduced DSB rate (NDR). The formula to calculate the NDR is as followed: (γH2AX foci per cell at 1 hour - γH2AX foci per cell at 24 hours)/γH2AX foci per

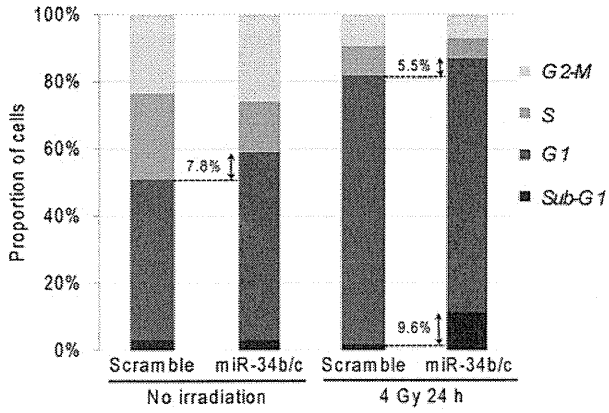

Figure 3. *Influence of miR-34b/c on the cell cycle distribution. miR-34b/c-transfected and scramble RNA-transfected H2052 cells were irradiated at 4 Gy. The cells were collected at 24 hours after irradiation. The cell cycle distribution was determined using flow cytometry according to an analysis of propidium iodide-stained cells. Data shown are representative of two independent experiments.*

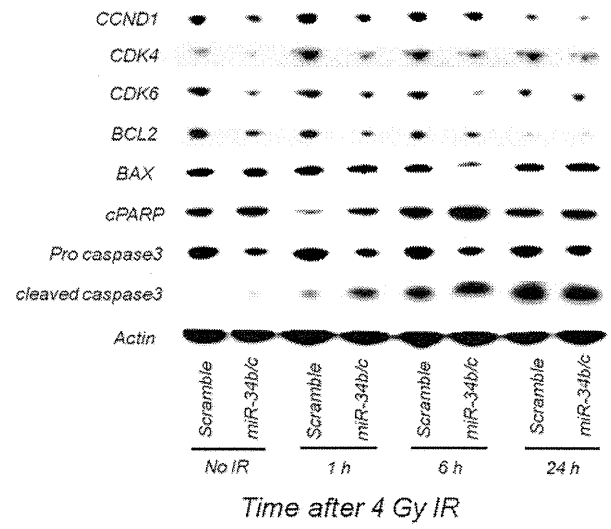

Figure 4. *Effect of miR-34b/c on primary target proteins and apoptosis-related proteins. miR-34b/c-transfected and scramble RNA-transfected H2052 cells were irradiated (4 Gy) and harvested at the specified times. The putative target proteins of miR-34b/c namely (cyclin D1 CCND1), cyclin dependent kinase 4 (CDK4), cyclin dependent kinase 6 (CDK6), and B-cell lymphoma 2 (BCL2) and apoptosis-related proteins BCL2 Associated X-protein (BAX), cleaved poly (ADP-ribose) polymerase (cPARP), procaspase 3, and cleaved caspase 3 were examined. Each blot is representative of two independent experiments. IR: Irradiation.*

cell at 1 hour. NDR was lower in miR-34b/c transfectants (24.0%) than that in scramble transfectants (73.8%) at 24 hours after IR (Figure 2C). This result suggests that DNA DSB repair potential was impaired in miR-34b/c transfectants compared with the scramble transfectants.

miR-34b/c induced G_1 arrest and apoptosis. To further investigate the mechanism responsible for the radiosensitization effect of miR-34b/c, we performed a cell cycle analysis on H2052 cells. The percentage of sub-G_1 and G_1 phase miR-34b/c transfectants was increased by 7.8% in non-irradiated H2052 cells and by 5.5% in 4 Gy-irradiated H2052 cells at 24 hours after IR (Figure 3). Focusing on the apoptotic fraction, the sub-G_1 phase was increased by 9.6% in miR-34b/c-transfected H2052 cells at 24 hours after IR, suggesting that miR-34b/c enhanced radiation-induced apoptosis.

Impact of miR-34b/c on primary target proteins and apoptosis-related proteins. To examine the effect of miR-34b/c on its target proteins and apoptosis-related proteins, we focused on CCND1, CDK4/6, and BCL2 as targets of miR-34b/c and BAX, cPARP, pro-caspase 3, and cleaved caspase 3 for detecting apoptosis. The expressions of CCND1, CDK4/6 and BCL2 in miR-34b/c transfected H2052 cells were down regulated at almost all the time points after IR that were examined (Figure 4). The expression of pro-caspase 3 was decreased in miR-34b/c transfectant. In contrast, the expressions of cleaved Caspase3 and cPARP was increased in miR-34b/c transfectants and gradually increased after IR. These results suggested that miR-34b/c suppressed its target proteins and that IR enhanced apoptosis of miR-34b/c transfectants.

Discussion

In this study, a clonogenic survival assay revealed that the restoration of miR-34b/c enhanced the radiosensitivity of MPM cells. This result is the essential finding of the present study. To understand the mechanism of radiosensitization, we focused on DNA DSB repair, cell cycle distribution, and expression of the target proteins of miR-34b/c and apoptosis-related proteins.

Firstly, we estimated the DNA DSB status by counting γH2AX foci. The number of γH2AX foci after IR was lower at the early phase but higher at the late phase and the NDR was low in miR-34b/c transfectants compared with the scramble transfectants. As translation of this result, two possibilities should be considered: i) DNA DSB repair was impaired in miR-34b/c transfectants in the early phase and DSBs accumulated at the late phase, ii) miR-34b/c transfectants contained a low amount of DNA because miR-34b/c itself influenced the cell cycle, resulting in low rate of cells entering the S phase. To understand how miR-34b/c impaired DNA DSB repair, CCND1, a putative target of miR-34b/c, seems to play a crucial role. Reportedly, CCND1 binds directly to RAD51 and is recruited to sites of DNA damage, and a reduction in CCND1 impairs the recruitment of RAD51 to damaged DNA and increases the cellular radiosensitivity (10). We confirmed that miR-34b/c suppresses CCND1 with

or without IR and that the suppression was greater in miR-34b/c transfectants at 24 hours after IR. Accordingly, miR-34b/c is assumed to impair DNA DSB repair by suppressing CCND1. This is considered to be a part of the mechanism by which miR-34b/c enhances radiosensitivity.

Secondly, we estimated the cell cycle distribution and related proteins. We observed that the proportion of sub-G_1 phase miR-34b/c transfectants at 24 hours after IR was significantly greater than that for scramble transfectants, indicating that radiation-induced apoptosis took place in miR-34b/c transfectants. Of note, BCL2 expression was inhibited and that of cPARP and cleaved caspase 3 were increased in miR-34b/c transfectants after IR. As other cell cycle-related targets of miR-34b/c, CCND1, CCNE2, CDK4 and CDK6 are known as key molecules for the G_1 checkpoint (4, 6, 14), and some of these targets were confirmed to be down-regulated in miR-34b/c transfectants in the present study. Indeed, G_1 arrest occurred in miR-34b/c transfectants. However, a question arises here. Generally cells in the G_1 phase are considered to be relatively radioresistant compared with those in other phases (15). There are several reports that explain this contradiction. Kodym *et al.* reported that flavopiridol, a pan-CDK inhibitor, was reported to increase the radiosensitivity of both proliferating and quiescent lung cancer cells by suppressing DNA DSB repair thorough a cell cycle-unrelated mechanism (11). Other studies have demonstrated CDK inhibitor-mediated G_1 arrest and radiation-induced apoptosis in breast cancer (16), as well as colonic and gastric cancer (12). We observed that miR-34b/c suppressed CDK4/6, which indicates that miR-34b/c has similar effects to a CDK inhibitor. Considering these, the effects of miR-34b/c on impairing DNA DSB repair and suppressing cell cycle-related proteins resulted in enhancement of radiosensitivity of MPM cells.

In conclusion, we revealed that the restoration of miR-34b/c in MPM cells enhanced radiosensitivity and promoted radiation-induced apoptosis, indicating that miR-34b/c used in combination with IR may be a potential therapeutic target for MPM.

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悪性中皮腫の血清診断における可溶性メソテリン関連ペプチド（SMRP：Soluble Mesothelin-related Peptides）の有用性に関する多施設共同試験

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太田　三徳[5]・松村　晃秀[6]・岡田　守人[7]・岸本　卓巳[8]
中野喜久雄[9]・中野　孝司[1]

要　旨

悪性中皮腫の血清診断における可溶性メソテリン関連ペプチド（soluble mesothelin-related peptides：SMRP）の診断性能を多施設共同試験によって検討した。対象は，悪性中皮腫85例，比較対照疾患としての肺癌240例，良性呼吸器疾患（石綿非関連良性疾患136例，石綿関連良性疾患157例），高血圧・慢性心疾患74例および石綿曝露歴のない健常者110名。Chemiluminescent enzyme immunoassay（CLEIA）法による血清SMRP濃度測定キット（ルミパルス® メソテリン）を用いて血清SMRP濃度を測定した。参考基準値を1.5 nmol/Lに設定した場合の各対象の陽性率は，悪性中皮腫66%，肺癌21%，石綿非関連良性疾患18%，石綿関連良性疾患15%，高血圧・慢性心疾患9%，健常者1%であった。また，悪性中皮腫における血清SMRP濃度は，比較対照群および健常者に比較して有意に高値であった。原発部位別の陽性率は，胸膜65%，腹膜86%であった。悪性胸膜中皮腫の各病期におけるSMRP陽性率は，I期（64%），II期（67%），III期（55%），IV期（69%）であった。また，各組織型におけるSMRP陽性率は，上皮型（65%），二相型（60%），肉腫型（75%），であった。今回のルミパルス® メソテリンを用いた試験結果では，悪性中皮腫の血清診断におけるSMRPの診断性能は，これまでの報告と同等であった。さらに，悪性胸膜中皮腫早期例，肉腫型および腹膜中皮腫における診断的有用性も示唆された。

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Evaluation of the usefulness of SMRP (Soluble Mesothelin-related Peptides) for the diagnosis of malignant mesothelioma

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Key words：malignant mesothelioma（悪性中皮腫），SMRP，soluble mesothelin-related peptides（可溶性メソテリン関連ペプチド），biomarker（バイオマーカー），early detection of mesothelioma（悪性中皮腫の早期診断）

はじめに

悪性中皮腫（malignant mesothelioma, MM）は体腔内面を広く覆う漿膜に発生する腫瘍で，胸膜，腹膜，心膜，および非常にまれではあるが，精巣鞘膜からも発生する。この中で，胸膜由来の悪性胸膜中皮腫（malignant pleural mesothelioma：MPM）が最も多い。これまで，MMは非常にまれな疾患とされてきたが，本疾患と石綿曝露との密接な関連性が報告されてから半世紀が経過した現在，その罹患者数および死亡者数は世界的に急激な増加を辿っている[1)2)]。MPMの予後は不良であり，生存期間中央値は9～17カ月とされている[3)]。MPMの早期発見は非常に困難であり，確定診断が得られた時点では，すでに進行期である症例が大多数を占める。MPMに対する標準的治療法はいまだに確立されておらず，手術療法によって腫瘍が肉眼的に完全切除されても治癒に至る症例は極めて少ない。したがって，治療成績の向上には，疾患の早期発見と手術療法に化学療法や放射線療法などを組み合わせた集学的治療を確立することが極めて重要な役割を果たす[4)]。

MPMの早期診断には，画像検査や胸腔鏡検査の進歩とともに，血清や体腔液診断に有用なバイオマーカーの開発が必要不可欠である。現在，MPMの血清診断における新規バイオマーカーとして最も注目されている分子が，可溶性メソテリン関連ペプチド（soluble mesothelin-related peptides：SMRP）である[5)]。SMRP本体の蛋白であるメソテリンは，膵臓癌，卵巣癌，中皮腫やその他の癌に過剰発現する40 kDaの細胞膜表面に存在する糖蛋白である。メソテリンは癌特異的抗原ではなく，胸膜，腹膜および心膜の正常中皮細胞にも発現する分化抗原と考えられている。メソテリンの遺伝子は69 kDaの前駆蛋白（mesothelin/megakaryocyte potentiating factor：MPF family proteins）をコードし，この糖蛋白はfurin-like proteinaseで切断後N末端側は31 kDaのMPFとして血中に放出される。C末端側40 kDaの糖蛋白はメソテリンとして細胞膜に結合している。メソテリンには3種類のvariant formが知られおり，そのうちのひとつは修飾されたカルボキシル基終末をもち，GPIアンカーを欠如するために細胞膜から遊離する。このsoluble isoformがSMRPに相当するとされる[6)]。2003年，Robinsonらは血清SMRPのMPMにおける診断的意義に関する最初の報告を行った[7)]。近年，SMRPを認識する2種類のモノクローナル抗体（OV569と4H3）を用いた新しい定量的enzyme-linked immunosorbent assay（ELISA）キットであるMESOMARK™の開発によって，血清SMRP濃度の測定が可能となった。このキットを用いて，SMRPのMMに対する診断性能を検討した研究が欧米や豪州から報告されている[8)～16)]。

今回，われわれは，MESOMARK™と同一の抗体を用いたchemiluminescent enzyme immunoassay（CLEIA）法による血清SMRP濃度測定キット（ルミパルス® メソテリン）を用いて，MM血清診断におけるSMRPの診断性能を多施設共同試験によって検討した。

Ⅰ．試験実施施設および対象

本試験は，愛知県がんセンター，大阪府立呼吸器・アレルギー医療センター，国立病院機構近畿中央胸部疾患センター，兵庫医科大学，兵庫県立成人病センター，労働者健康福祉機構岡山労災病院，国立病院機構呉医療センターの合計7施設にて，各施設の倫理委員会の審査・承認を受けて，2005年から2006年に多施設共同試験として実施した。

対象は，各施設を受診したMM患者および対照疾患患者のうち，試験参加に関して文書で同意が得られた被験者および健常者（石綿曝露歴のない院内ボランティア）の計802例である。MM85例のうち，MPMは77例で，7例が悪性腹膜中皮腫，1例が精巣鞘膜由来の中皮腫であった。比較対照疾患の内訳は，肺癌，良性呼吸器疾患（石綿非関連良性疾患および石綿関連良性疾患），石綿曝露歴のない高血圧・慢性心疾

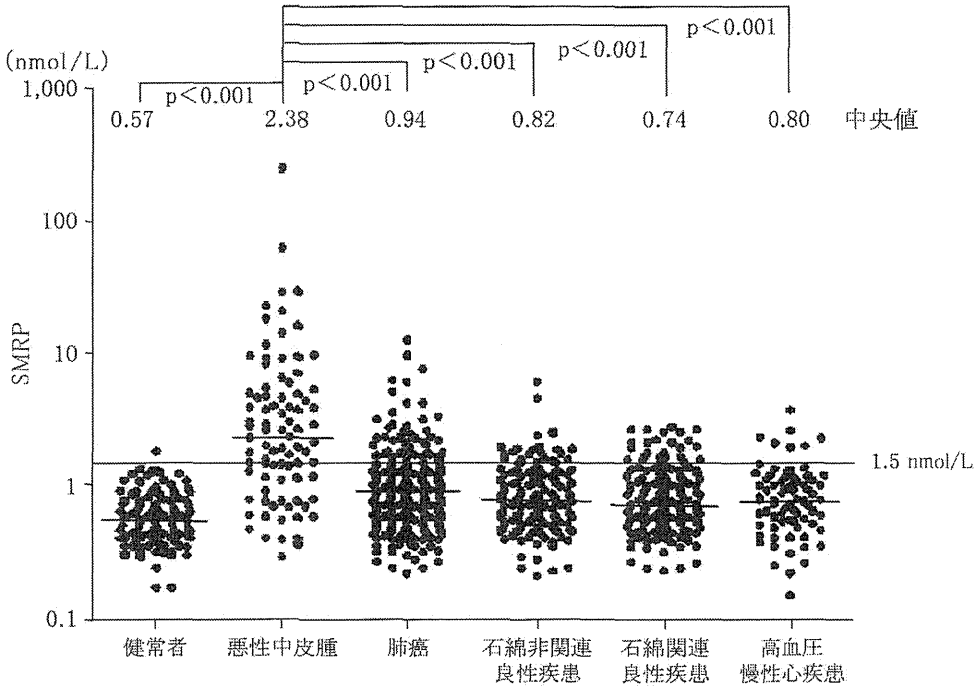

図 1　悪性中皮腫および対照疾患における血清 SMRP 濃度の分布

表 1　悪性中皮腫および対照疾患における血清 SMRP 濃度および陽性率

	健常者	悪性中皮腫	肺癌	石綿非関連良性疾患	石綿関連良性疾患	高血圧・慢性心疾患
症例総数	110	85	240	136	157	74
中央値（nmol/L）	0.57	2.38	0.94	0.82	0.74	0.80
SD（nmol/L）	0.30	28.57	1.36	0.74	0.58	0.62
陽性数	1	56	51	24	24	7
陽性率	1%	66%	21%	18%	15%	9%

SD：standard deviation

患であった（**表 1**）。

II. 血清 SMRP 濃度の測定

すべての被験者に対して採血を実施し，速やかに血清を分離後，−80℃にて凍結保存した。

血清 SMRP 濃度は，体外診断用医薬品として 2010 年 10 月に認可されたルミパルス® メソテリン（富士レビオ株式会社製造）を用いて測定した。本キットは，米国，カナダ，ヨーロッパおよびオーストラリア等で販売されている ELISA 法による SMRP 測定キットである MESOMARK™ と同一の抗体を用いた，2 ステップサンドイッチ法に基づく CLEIA 法による SMRP 測定試薬である。本試薬は，全自動化学発光酵素免疫測定システム用試薬であり，約 30 分間で測定が完了し，測定範囲は 0.1 から 100 nmol/L に及ぶ。MESOMARK™ との相関性は，ほぼ 1：1 であり，同等の測定結果の得られることが報告されており，参考基準値は両者ともに 1.5 nmol/L に設定されている[9)17)]。

統計学的解析には，statistical package for social science（SPSS）を使用し，2 群間の有意

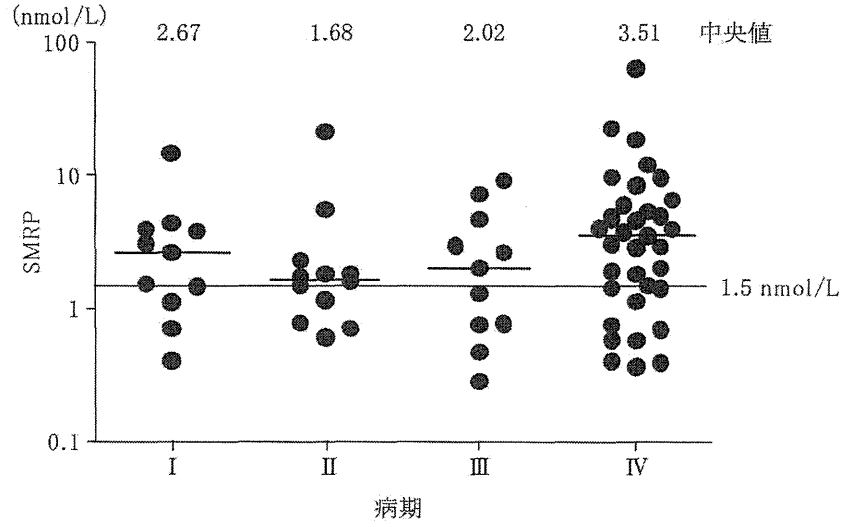

図 2　悪性胸膜中皮腫の各病期における血清 SMRP 濃度の分布

表 2　悪性胸膜中皮腫の各病期における血清 SMRP 濃度および陽性率

	I	II	III	IV
症例総数	11	12	11	35
中央値（nmol/L）	2.67	1.68	2.02	3.51
SD（nmol/L）	3.92	5.67	2.93	11.05
陽性数	7	8	6	24
陽性率	64%	67%	55%	69%

SD：standard deviation

差検定は Mann-Whitney 検定を用いて行った。各図中には測定値の中央値を示した（単位は nmol/L）。

III. 結　果

1．健常者における血清 SMRP 濃度

石綿曝露歴のない健常者 110 例の血清 SMRP 濃度を測定し，測定結果を対数変換した結果，SMRP 測定値の正規分布が確認された。SMRP 測定値は，1 例（1.86 nmol/L）を除き，すべて参考基準値（1.5 nmol/L）未満であった（陰性率 99.1%）。

2．MM および比較対照疾患における血清 SMRP 濃度および陽性率（図 1，表 1）

血清 SMRP 濃度の参考基準値を 1.5 nmol/L とした場合，この値よりも高値を示す症例の割合（陽性率）は，それぞれ MM66%（56/85 例），肺癌 21%（51/240 例），石綿非関連良性疾患 18%（24/136 例），石綿関連良性疾患 15%（24/157 例），高血圧・慢性心疾患 9%（7/74 例）であった。MM における血清 SMRP 濃度は，比較対照群および健常者に比較して有意に高値であった。MM の原発部位別の陽性率は，胸膜 65%（50/77 例），腹膜 86%（6/7 例）であった。

3．MPM 各病期における血清 SMRP 濃度および陽性率（図 2，表 2）

病期が確定した MPM69 例について，各病期における血清 SMRP 濃度および陽性率を検討した結果，各病期間での血清 SMRP 濃度に有意差はなく，陽性率は，それぞれ I 期 64%（7/

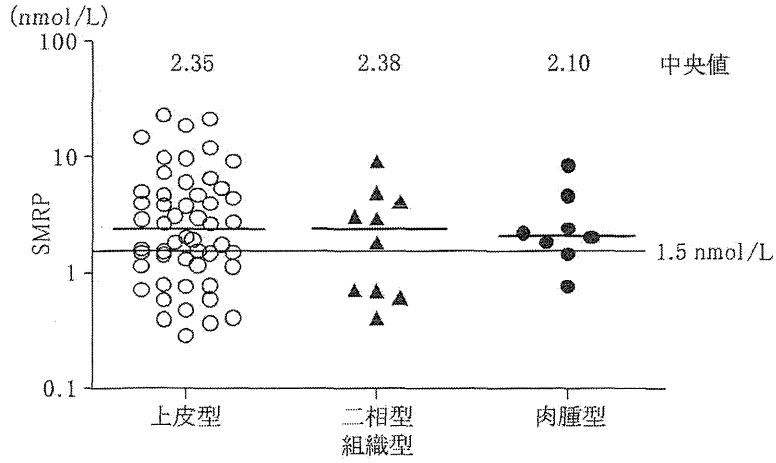

図 3　悪性胸膜中皮腫の各組織型における血清 SMRP 濃度の分布

表 3　悪性胸膜中皮腫の各組織型における血清 SMRP 濃度および陽性率

	上皮型	二相型	肉腫型
症例総数	52	10	8
中央値（nmol/L）	2.35	2.38	2.10
SD（nmol/L）	5.19	2.72	2.48
陽性数	34	6	6
陽性率	65%	60%	75%

SD：standard deviation

11例），II期67%（8/12例），III期55%（6/11例），IV期69%（24/35例）であった。

4．MPM各組織型における血清SMRP濃度および陽性率（図3，表3）

組織型の確定し得たMPM70例の内訳は，上皮型52例，二相型10例，肉腫型8例であった。各組織型間における血清SMRP濃度に有意差はなく，陽性率は，それぞれ上皮型65%（34/52例），二相型60%（6/10例），肉腫型75%（6/8例）であった。

IV．考　察

近年，SMRPを認識する2種類のモノクローナル抗体（OV569と4H3）を用いた新しい定量的ELISAキットであるMESOMARK™の開発によって，血清SMRP濃度の測定が可能となった[8)~16)]。これらの報告では，血清SMRP濃度は健常者および石綿曝露者，石綿関連良性胸膜疾患，肺癌などの対照疾患に比較して，MMにおいて有意に上昇していた[8)~16)]。MESOMARK™と同一の抗体を使用したCLEIAキットであるルミパルス® メソテリンを用いた本試験においても，血清SMRP濃度は，健常者および肺癌，良性呼吸器疾患，高血圧・慢性心疾患に比較してMMにおいて有意に上昇していた。この結果は，MM血清診断におけるSMRPの高い診断性能と測定結果の普遍性および再現性とを意味するものと考えられた。これまでのMESOMARK™を用いた測定報告では，MMにおける血清SMRP濃度中央値は0.79から2.39 nmol/Lの範囲に分布していた[8)~16)]。本試験におけるMMの血清SMRP

濃度中央値も2.38 nmol/Lであり，この範囲内に含まれていた。参考基準値（カットオフ値）に関しては，健常者を対象とした結果から，MESOMARK™およびルミパルス® メソテリンともに，1.5 nmol/Lに設定されている[9)17)]。本試験において，健常者110名のうち99%が，血清SMRP濃度1.5 nmol/L（参考基準値）未満であった。この参考基準値の条件下で，MMにおけるSMRPの陽性率（感度）は66%であった。一方，これまでのMESOMARK™を用いた測定報告では，SMRPの感度は48%から80%（中央値68.2%）と報告されており，ルミパルス® メソテリンの感度もこれらの測定報告に匹敵するものと考えられる。

MPMにおけるSMRP陽性率は65%であり，腹膜中皮腫を含むMM全症例の陽性率と概ね同等であった。MPM各病期間での血清SMRP濃度と陽性率に有意差はなく，I-II期においても，III-IV期に匹敵する陽性率が得られた。このことは，本キットが高感度の測定系であることから，早期例においても血清SMRPの検出が可能であることが推察された。Passら[13)]，II期以上の症例はI期症例に比較して血清SMRP濃度が有意に上昇することを報告しているが，その他の報告では病期と血清SMRP濃度との明らかな関連性は指摘されなかった[9)10)14)]。今後，MPM早期診断マーカーとしてのSMRPの意義を明らかにするために，早期例を中心とした検討が必要になるものと考える。

MPMの各組織型と血清SMRP濃度との関連性に関して，本試験では，各組織型間での血清SMRP濃度と陽性率に有意差はなく，上皮型のみならず肉腫型においても75%が陽性を示した。これまでのMESOMARK™を用いた測定報告では，組織型と血清SMRP濃度との関連性については一定の見解が得られておらず，上皮型が肉腫型に比較して有意に高値であるという報告[7)8)10)15)]と，本試験と同様，各組織型間で有意差はないとする報告[9)11)13)14)]とに分かれている。

MMの診断には，石綿曝露歴，臨床症状，血液生化学検査，画像および内視鏡検査，体腔液細胞診，生検病理組織診などを駆使した総合的なアプローチが必要とされる[4)]。しかしながら，高齢者や全身状態不良例では，侵襲的検査の適応外となることも多く，診断に苦慮する場合も少なくない。このような症例では，MMに対して診断性能の高いバイオマーカーによる血清診断が重要な役割を担う。ルミパルス® メソテリンによる血清SMRP濃度測定は，一般の血液検査と同様，患者から採取した血清または血漿を用いて全自動分析装置にて約30分間で測定が完了する。したがって，患者への侵襲もなく，技術的にも容易に短時間で測定結果を得ることが可能であることから，今後，本キットは，MMを疑う患者に対して汎用されていくことが予想される。また，MM発症リスクの高い石綿曝露者を対象とした血液スクリーニング検査のひとつとして用いられることも想定される。

おわりに

現状では，石綿曝露者に発症した胸水貯留が良性石綿胸水であるのか，MPMの初期症状であるのかを鑑別することは困難である。しかし，この時期での診断機会を逸することは早期MPMの発見を見逃すこととなる。今回の検討では，SMRP測定がMPMの早期診断に有用なバイオマーカーとなることを示唆する結果が得られており，今後のさらなる研究の成果に期待が寄せられる。

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PLOS ONE

An Anilinoquinazoline Derivative Inhibits Tumor Growth through Interaction with hCAP-G2, a Subunit of Condensin II

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Abstract

We screened 46 novel anilinoquinazoline derivatives for activity to inhibit proliferation of a panel of human cancer cell lines. Among them, Q15 showed potent *in vitro* growth-inhibitory activity towards cancer cell lines derived from colorectal cancer, lung cancer and multiple myeloma. It also showed antitumor activity towards multiple myeloma KMS34 tumor xenografts in Icr/scid mice *in vivo*. Unlike the known anilinoquinazoline derivative gefitinib, Q15 did not inhibit cytokine-mediated intracellular tyrosine phosphorylation. Using our mRNA display technology, we identified hCAP-G2, a subunit of condensin II complex, which is regarded as a key player in mitotic chromosome condensation, as a Q15 binding partner. Immunofluorescence study indicated that Q15 compromises normal segregation of chromosomes, and therefore might induce apoptosis. Thus, our results indicate that hCAP-G2 is a novel therapeutic target for development of drugs active against currently intractable neoplasms.

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☯ These authors contributed equally to this work.

Introduction

Although advances in treatment, such as combination chemotherapy and chemoradiation, have slightly improved the outcome of tumor therapy over the last several decades [1], tumors are the leading cause of death in economically developed countries and the second leading cause of death in developing countries [2]. Colorectal tumors, lung tumors, and multiple myeloma (a hematopoietic tumor) are particularly intractable. Therefore, novel drugs having potent activity are required to treat such tumors, and in order to develop them, it is important to identify novel molecular targets related to the pathogenesis of these intractable tumors.

Anilinoquinazoline derivatives such as gefitinib and erlotinib, selective tyrosine kinase inhibitors, have been reported to be effective against recurrent non-small-cell lung tumor [3,4]. Here, we screened 46 anilinoquinazoline derivatives, which are structurally similar to gefitinib or erlotinib, for growth-inhibitory activity towards a panel of intractable tumor cell lines. Among these compounds, we identified Q15 as a potent proliferation inhibitor and apoptosis inducer of the colon tumor, lung tumor and multiple myeloma cell lines examined. We further confirmed that Q15 showed higher antitumor activity than gefitinib towards multiple myeloma KMS34 tumor xenografts in Icr/scid mice *in*

Table 1. IC_{50} values (μM) of anilinoquinazoline derivatives for inhibiting proliferation of different human tumor cell lines.

Cell lines	Compounds		
	Q15	Q16	Q17
KMS11	5.1	13.5	10.1
KMS27	14.5	19.1	20.9
KMS34	1.1	5.8	6.1
KMM1	4.7	22.1	20.3
RPMI8662	2.3	22.4	6.1
SW480	2.0	6.4	17.4
HeLa	3.3	7.3	13.5

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vivo. Surprisingly, however, Q15 did not inhibit intracellular signaling or the phosphorylation status of ERK1/2, indicating that the mechanism of its antitumor effect is different from that of gefitinib. Therefore, we next focused on the possible mechanism of action of Q15.

Previously we have developed an mRNA display system named *in vitro* virus (IVV) [5–7], in which an *in vitro*-translated full-length protein (phenotype) is covalently attached to its encoding mRNA (genotype) through puromycin [8]. Here, we employed this mRNA display method to search for the target of Q15 for induction of apoptosis, and identified hCAP-G2, which is a subunit of condensin II [9,10], as a Q15 binding partner. We further confirmed that Q15 binds to the condensin II complex through direct interaction with hCAP-G2, and therefore may affect chromosomal segregation in mitosis, resulting in abnormal cell division and subsequent apoptosis. These results indicate that Q15 is a promising candidate drug for treatment of high-risk multiple myeloma. Further, hCAP-G2 appears to be a novel therapeutic target for development of drugs active against currently intractable neoplasms.

Results

Identification of a Potent Proliferation Inhibitor of Several Multiple Myeloma Cell Lines from a Library of Anilinoquinazoline Derivatives

We screened a compound library consisting of 46 anilinoquinazoline derivatives for activity to inhibit proliferation of various sorts of intractable tumor cell lines. Five multiple myeloma cell lines (KMS11, KMS27, KMS34, KMM1 and RPMI8662), SW480 cells and HeLa were treated with 0.5–50 μM of each compound for 72 h and their viability was examined by means of MTS assay (Table 1). Q15 (Fig. 1A) showed the highest cytotoxicity among the compounds examined towards the cell lines tested here. Next, we compared Q15 with gefitinib in a KMS34 cell proliferation assay (Fig. 1B). The results indicated that Q15 is 6 times more potent than gefitinib.

In order to see whether the mechanism of action of Q15 differs from that of gefitinib, we examined inhibition of phosphorylation of ERK1/2, which is a key growth transducer of tumor cells, by Q15 and gefitinib. A549 cells were treated with 5 μM Q15 or gefitinib for 24 h in the absence or presence of EGF, then subjected to immunoblot analysis. Gefitinib inhibited the phosphorylation of ERK1/2, but Q15 did not (Fig. 1C). Furthermore, Q15 did not inhibit the phosphorylation stimulated by FGF2, HGF, VEGF or IL-6 (data not shown). Thus, these results indicate that the mechanism of action of Q15 is different from that of

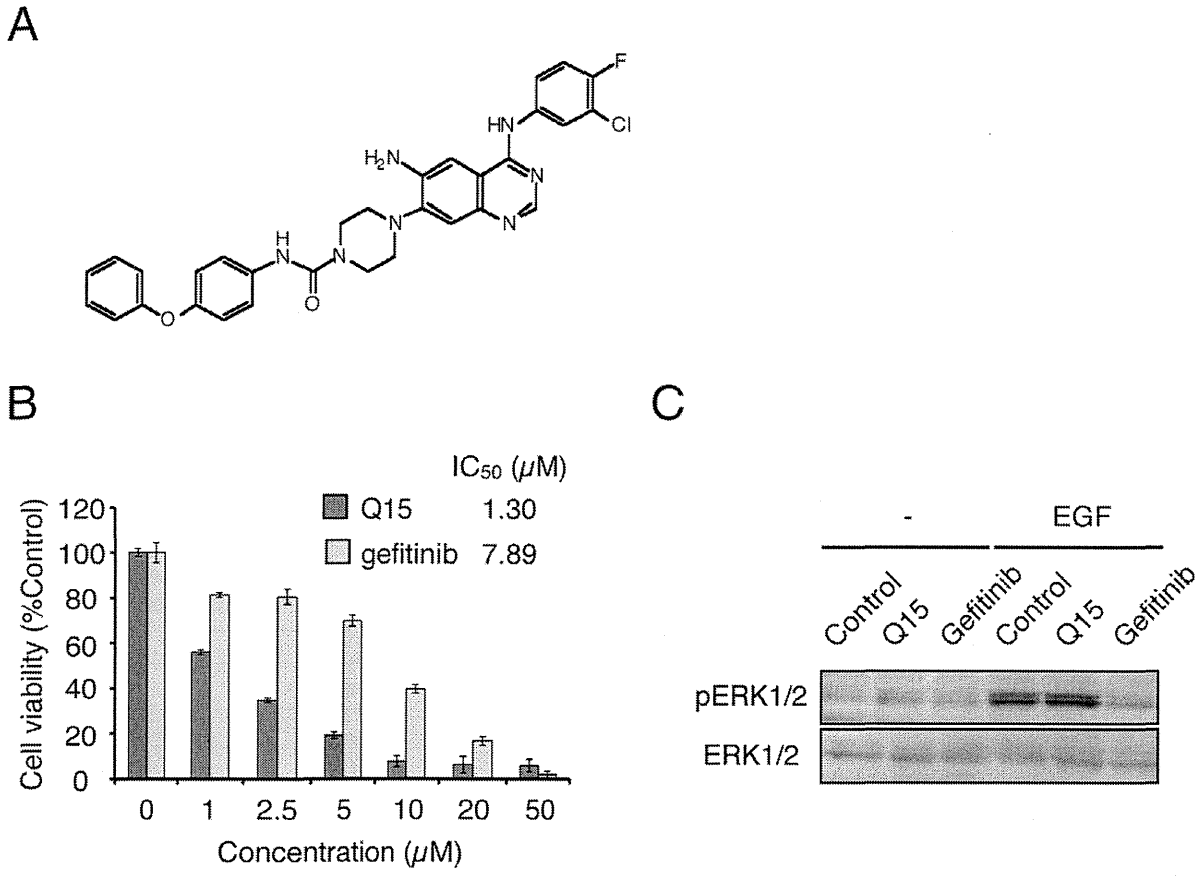

Figure 1. Q15 inhibits proliferation of tumor cells through a different mechanism from that of gefitinib. (A) Chemical structure of Q15. (B) KMS34 cells were incubated with 0–50 μM Q15 or gefitnib for 72 h. Then, cell viability was determined by means of MTS assay. (C) A549 cells were incubated with DMSO or 5 μM Q15 for 24 h in the absence or presence of 50 ng/mL EGF for 48 h. Whole cell lysates were analyzed by Western blotting with anti-pERK1/2 or anti-ERK1/2 antibody.
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Table 2. IC_{50} values (μM) of Q15 for inhibiting proliferation of different human tumor cell lines.

Cell Lines	IC_{50} (μM)	Cell Lines	IC_{50} (μM)
Breast cancer		Lung cancer	
HBC−4	2.2	NCI-H23	4.4
BSY-1	3.0	NCI-H226	3.1
HBC-5	3.1	NCI-H522	0.4
MCF-7	3.4	NCI-H460	1.7
MDA-MB-23	1.6	A549	1.3
Brain cancer		DMS273	1.3
U251	1.8	DMS114	1.7
SF-295	1.3	Kidney cancer	
SF-539	2.3	RXF-631L	1.8
SNB-75	1.6	Gastric cancer	
SNB-78	3.1	St-4	1.7
Colon cancer		MKN1	3.1
HCC2998	2.2	MKN7	1.6
HT-29	0.6	MKN28	2.7
HCT-15	1.7	MKN45	1.8
HCT-116	2.4	MKN74	1.4
Ovary cancer		Melanoma	
OVCAR-4	2.3	LOX-IMVI	0.4
SK-OV-3	2.9		

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gefitinib, despite the structural similarity between the two compounds.

We next tested the antitumor activity of Q15 against several human cancer cell lines, including breast, brain, colon, lung, ovary, kidney, gastric cancer and melanoma (Table 2). Q15 inhibited the growth of all the tumor cell lines examined, suggesting that it may have activity against a wide range of human cancers.

Q15 Inhibits Cell Proliferation of Multiple Myeloma and Induces Apoptosis Both *in vitro* and *in vivo*

We next examined the ability of Q15 to induce apoptosis of tumor cells. KMS34 cells were treated with 20 μM Q15 for 0–24 h and then immunoblot analysis of the whole cell lysates was performed. Activation of caspase-3 and 9, leading to cleavage of PARP, was detected (Fig. 2A). These results suggest that Q15 induces caspase-dependent apoptosis of tumor cells.

To examine whether Q15 exhibits antitumor activity *in vivo*, we performed *in vivo* tumor proliferation assay. KMS34 tumor xenografts were treated with intraperitoneal injection of 20 mg/kg of Q15 twice, with a three-day interval, and then the time-course of tumor volume was followed for 16 days (Fig. 2B and C). On the 16th day, Q15 achieved statistically significant inhibition of tumor growth ($P<0.02$). No significant change of body weight was observed in Q15-treated mice, suggesting that systemic toxicity of Q15 is likely low. In order to examine whether Q15 induces apoptosis of tumor cells *in vivo*, we also performed histopathological examination of tumor tissue. In Q15-treated mice, a number of tumor cells exhibited aggregation of chromatin, as compared with the control (Fig. 2D). This result indicates that Q15 induces apoptosis of tumor cells *in vivo*, as well as *in vitro*.

Identification of Q15-binding Protein using mRNA Display

To elucidate the mechanism through which Q15 inhibits proliferation of tumor cells, we set out to identify Q15-binding proteins by means of mRNA display [5–7], as illustrated in Fig. 3A. We prepared a cDNA library derived from total RNA of human colon carcinoma SW480 cells, because, like other tumor cells, SW480 cells were sensitive to Q15. Proteins that bind to Q15-Bio (Fig. 3B) immobilized on beads were selected using mRNA display. From the library obtained after 5 rounds of selection, we analyzed the DNA sequences of 100 clones. Among them, we obtained six clones of a fragment of the Luzp5/NCAPG2 gene encoding hCAP-$G2_{262–476}$ containing the HEAT (Huntingtin, elongation factor 3, a subunit of protein phosphatase 2A, TOR lipid kinase) repeat domain (Fig. 4A). Although three other clones were obtained redundantly, they were confirmed to be false-positive clones by means of binding assay (data not shown). hCAP-G2 is a subunit of condensin II complex, which is regarded as a key player in mitotic chromosome condensation [9,10,11].

To confirm the interaction between hCAP-G2 and Q15, we performed an *in vitro* binding assay. Whole cell lysates were prepared from SW480 and KMS34 cells and incubated with Q15-immobilized beads for 1 h, followed by immunoblot analyses with specific antibodies (Fig. 4B and C). We found that hCAP-G2 in the cell lysates from both SW480 and KMS34 interacted specifically with Q15-immobilized beads; no interaction with mock beads was detected. Further, we found that SMC2, another subunit of condensin II complex, was also retained specifically on the Q15-immobilized beads. The interaction between hCAP-G2 and Q15 was further investigated by means of a competitive binding assay. Binding of hCAP-G2 to Q15 was inhibited in the presence of 100 μM free Q15, indicating that hCAP-G2 interacts not with the biotin linker, but with Q15 itself (Fig. 4D). We also confirmed that *in vitro* translated-hCAP-$G2_{262–476}$ binds directly to Q15 (data not shown). These results suggest that Q15 binds to the condensin II complex through direct interaction with hCAP-G2 in cell lysates prepared from both SW480 and KMS34.

Q15 Compromises Mitotic Chromosome Segregation and Eventually Induces Apoptosis

Condensins contribute to chromosome assembly and segregation in mitosis [9,12,13]. Therefore, we carried out an immunofluorescence analysis to examine the effects of Q15 on the behavior of chromosomes. For this purpose, we selected HeLa cells, since they have a large nucleus and intranuclear structures can be easily observed, whereas KMS34 and SW480 cells are too small for convenient observation of intracellular or intranuclear components. After 24 h treatment with Q15, HeLa cells were labeled with antibodies against hCAP-G and hCAP-H2 to visualize the distribution of condensin I and condensin II, respectively (Fig. 5A). In the Q15-treated cells, we observed about 80% of roundish and swollen chromosomes in which the otherwise distinct localizations of condensin I and condensin II were somewhat obscured (Fig. 5B). The defect in chromosome morphology observed here was reminiscent of, if not identical to, that reported previously in cells depleted of hCAP-G2 [9].

To examine whether or how Q15 may affect cell cycle progression, we next performed immunofluorescence labeling of cells with an antibody against α-tubulin and CREST, an autoimmune antiserum that recognizes the kinetochore/centromere region. When HeLa cells were treated with Q15 at a final concentration of 10 μM, atrophy of the cytoplasm was observed during interphase (Fig. 6A). Moreover, the frequency of cells with

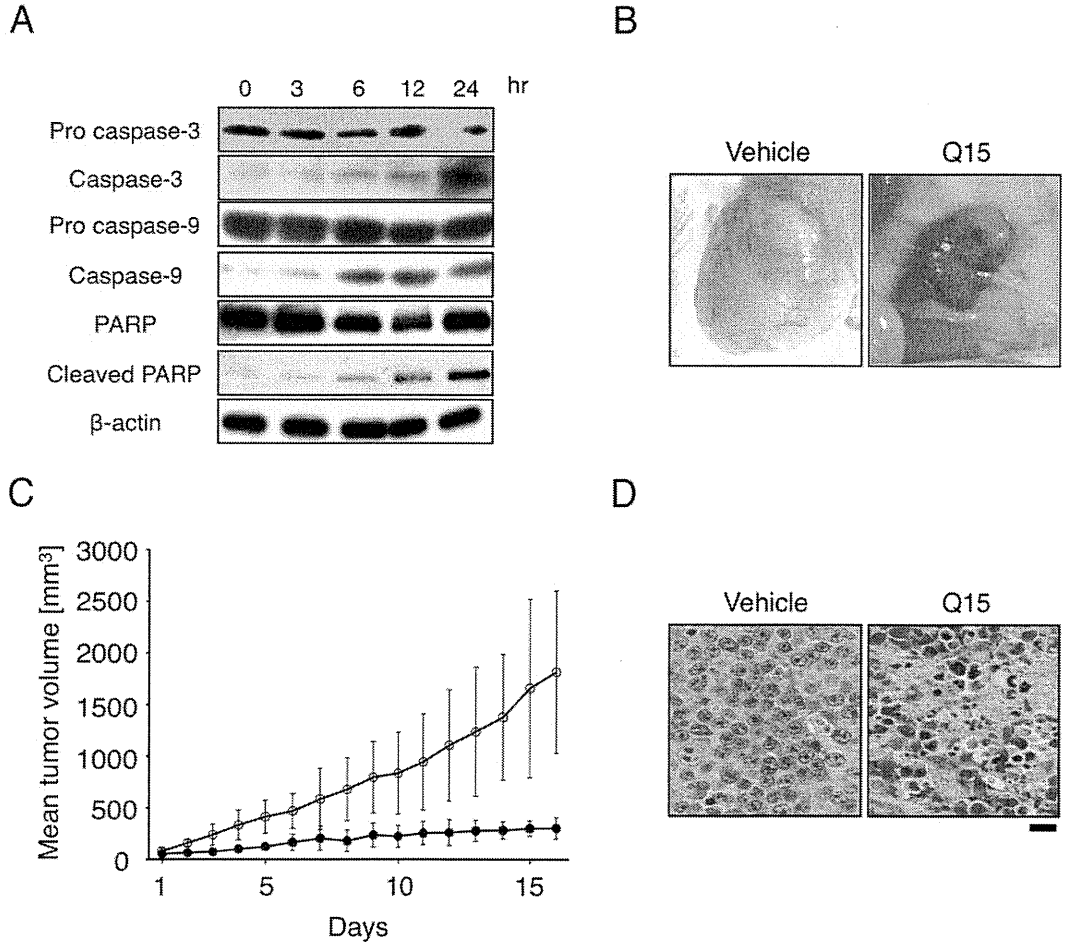

Figure 2. Q15 suppresses tumor growth and induces apoptosis of multiple myeloma cells *in vitro* and *in vivo*. (A) KMS34 cells were incubated with 20 μM Q15 for 0–24 h. The whole cell lysates were analyzed by immunoblot with anti-caspase-3, caspase-9, PARP or β-actin antibody, respectively. (B) KMS34 cells (3×10^7 cells) were inoculated subcutaneously into Icr/scid mice. Then, 20 mg/kg Q15 was injected intraperitoneally twice every 3 days. Representative tumor from each group. (C) The width and length of the plasmacytoma were measured and tumor volume was calculated. (D) Sections were stained with hematoxylin and eosin.
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defects in metaphase and anaphase was increased as compared with the control (Fig. 6B). In the metaphase population, more than 50% of mitotic cells showed defects in chromosome alignment (Fig. 6B, metaphase Incomplete). In the ana/telophase population, the frequency of cells with chromosomal bridging and lagging chromosomes among Q15-treated cells was about twice that in untreated cells. As shown in Fig. 6C, the most obvious defect was incompletely aligned chromosomes in metaphase. In these cells, poorly organized chromosomes were scattered and failed to be aligned properly on the metaphase plate. Again, this mitotic phenotype was reminiscent of that previously observed in cells depleted of condensin II [13]. Thus, these results indicate that Q15 compromises proper assembly and segregation of chromosomes, possibly by interfering with the function of condensin II.

We finally examined whether abnormal cell division induced by Q15 affects the nuclear structure of cells. KMS34 cells were treated with 5 μM Q15 for 24 h and observed with an electron microscope (Fig. 7). The Q15-treated cells showed segmented nuclei, while control cells had a single nucleus. These results suggest that interaction of Q15 with hCAP-G2 induces abnormal mitosis, resulting in multinucleated cells.

Discussion

In this study, we identified a novel anilinoquinazoline derivative Q15 as a potent inhibitor of proliferation of cancer cell lines derived from a variety of tissues. Our results also indicated that Q15 has a more potent antitumor activity than gefitinib, an anilinoquinazoline derivative that has a well-established antitumor effect on recurrent non-small-cell lung tumor [14]. However, unlike gefitnib, Q15 did not inhibit intracellular signaling or the phosphorylation status of ERK1/2, indicating that the mechanism of its antitumor effect is different from that of gefitinib.

We have developed mRNA display using IVV [7,15,16] as a simple and totally *in vitro* screening tool for protein-protein [15], protein-peptide [17–19], antigen-antibody [20,21], protein-DNA [22], protein-RNA [23] and protein-drug [24] interactions. In this mRNA display methodology, molecules that interact with target proteins are amplified by RT-PCR, and the amplified sequences are identified by DNA sequencing. Functional domains are easily extracted based on the identified sequences obtained from a randomly primed prey library as a non-biased representation [16,25]. Bait mRNA templates were prepared using an *in vitro* procedure that makes the previously employed *in vivo* IVV cloning steps unnecessary [7]. Because mRNA display using IVV is an