A Master's Thesis

Myricetin as an anti-metastatic reagent against the irradiation-tolerant A549 lung cancer cells

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미리세틴의 방사선 내성 A549 폐암세포 전이능 억제 효과

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Abstract

Even though radio-therapy is extensively used for treating non-small cell lung cancer (NSCLC), many studies suggest that clinical usefulness of radio-therapy may be limited as it could increase the invasiveness of cancer cells. However, its underlying molecular mechanisms still need to be unveiled. Many studies revealed that phytochemicals potentially have abilities to reduce properties of malignancy. Thus, the objective of this study is to establish radiation-tolerant cancer cell line from A549 lung cancer cells treated with various conditions of irradiation, and compare the metastasis inhibitory effect of phytochemicals in radiation-tolerant cells. A549-IR cell line was established from 12 Gy-irradiated A549 cells. Compared to A549 cells, A549-IR cells have significantly enhanced migration ability and increased the level of Vimentin and Slug, representative markers of mesenchymal cells, but significantly decreased the level of E-cadherin, a representative marker of epithelial cells. Among the tested phytochemicals, myricetin showed highest ability to reduce the migration of A549-IR cells. Even though the recovery of E-cadherin and the suppression of Vimentin were not observed, myricetin effectively suppressed MMP-2, -9 expression. These results demonstrate that myricetin inhibits migration of A549-IR by suppressing MMP-2, -9 expression.

요약문

암 재발 및 생존율 감소의 주요 요인으로 방사선조사 후 암 세포의 전이능의 증가를 들 수 있다. 이전 연구를 통해 방사선 조사에 따른 전이능 증가에 대해서는 일부 보고된 바 있지만, 이에 대한 기작 규명과 이를 예방하기 위한 치료법에 대한 연구가 더 요구되고 있다. 이에, 본 연구에서는 다양한 선량의 방사선 조사 후에 살아남은 세포 중 이동능이 증가된 세포를 선발하여 세포주를 구축하고, 이 세포의 이동능을 억제할 수 있는 식물 유래의 생리활성물질을 탐색하였다. 12 Gy 를 조사 후에 살아남은 세포 중에서 A549 세포 대비해서 세포의 이동능이 특이적으로 증가되어 있고, mesenchymal cells 의 마커 단백질들인 Slug, Vimentin 의 발현은 증가한 반면, epithelial cells 의 마커 단백질인 E-cadherin 의 발현이 현저하게 감소되어 있는 세포주(A549-IR)를 구축하였다.–다양한 종류의 식물 유래의 생리활성물질을 대상으로 A549-IR 세포의 이동능 억제 효능을 분석한 결과, 미리세틴이 세포의 이동능을 현저하게 억제함을 확인하였다. 미르세틴은 세포 내 E-cadherin 의 발현을 회복시키거나 Vimentin 의 발현을 억제하지 못했지만, 세포외기질을 분해하여 침윤성을 높이는 matrix metalloproteinase-2, -9 (MMP-2, -9)의 발현을 현저히 억제하였다. 따라서, 본 연구의 결과에서 미리세틴은 방사선 조사 후 전이능이 증가된 폐암세포에서 MMP-2, -9 의 발현 억제를 통해 전이능을 감소시킬 수 있음을 나타내고 있다.

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1. Introduction

Cancer is a major public health problem worldwide. Lung cancer accounts for more than one-querter (27%) of all cancer deaths in United States (1). There are two major histological types of lung cancer: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC, i.e., adenocarcinoma, squamous cell carcinoma, and large cell carcinoma) (2, 3). NSCLC accounts for 85% of lung cancer cases, 40% of which are non-resectable (3, 4). The major treatments for locally advanced NSCLC are chemo- and radio-therapy (3). Especially, radio-therapy is considered as the standard treatment for locally advanced non-resectable lung cancer (3). Nevertheless, several clinical studies have suggested that radiotherapy could lead to cancer metastasis and secondary malignancies (5-7). Alternative therapeutic approaches to inhibit cancer recurrence and microenvironment-metastasis, as well as the resistance of the standard therapeutic approaches, are urgently needed for advanced NSCLC patients to enhance their chances of survival.

Epithelial-mesenchymal transition(EMT) and its reverse process, mesenchymal-epithelial transition(MET) are important embryonic processes. EMT, which is well known as an activated developmental program during cancer invasion and metastasis, often occurs at the invasive front of many metastatic cancers (8). EMT leads to the loss of cell-cell contact, repression of E-cadherin expression, induction of Vimentin expression, and, as a result, increased cancer cell mobility (9). As epithelial cancer cells undergo EMT, they gain mobility, invasiveness, and metastatic ability. EMT is regulated by a network of

transcription factors including Snail, Slug Twsit, and Zeb1 (10). The zinc-finger transcriptional factors Snail and Slug has been characterized as key EMT regulators and E-cadherin repressors (11).

The classic cadherins, namely, epidermal cadherin(E-cadherin), neuronal cadherin(N-cadherin), and placental cadherin (P-cadherin), are type I transmembrane glycoproteins (12). The epidermal specific cadherin, E-cadherin, has five extracellular domain repeats that are involved in cell biding mediated by E-cadherin homotypic interaction (13). The intracellular domain consists of a conserved sequence with β-, γ-, and p120-catenins. The interaction of β- or γ-catenin with α-catenin links E-cadherin to the cytoskeletal matrix for stabilizing the adherent junction mediated by the homotypic E-cadherin complex (14). The involvement of E-cadherin in cell-cell interaction is well established in embryonic development, organ morgphogenesis, tissue integrity, and wound healing (15). In fact, E-cadherin is considered to function as a metastasis suppressor, for it inhibits cancer cell migration and invasion (16).

Recently, some studies have suggested that ionizing radiation stimulates the secretion of various cytokines and matrix metalloproteinases acting via the PI3K/Akt or MAPK pathway (17-19), while other research suggested that transforming growth factor (TGF)-β can play a key role in determining the cancer cell motility as ionizing radiation response (20). Although a number of reports have revealed that ionizing radiation is able to stimulate EMT and invasion of cancer cells, the underlying mechanisms remain poorly understood.

Myricetin (3, 3',4', 5, 5', 7- hexahydroxyflavone) is a naturally occurring flavonoid which is frequently found in fruits, vegetables, and medicinal herbs. Many studies have revealed that myricetin potentially has anti-oxidant, anti-inflammatory, and anti-cancer effects (21-25). In addition, it was also revealed that myricetin, quercetin and kaempferol (the congeners of myricetin) could suppress migratory ability of cancer cells (24, 26-29).

The reports, which have suggested that radiation could enhance metastatic ability of cancer cell, prompted us to develop new therapeutic approaches by establishing radiation-induced metastatic model cell line and by figuring out useful phytochemicals to inhibit radiation-induced metastasis. Thus, in this study, the radiation-induced metastatic cancer cell line was established from radiation-tolerant A549 cells, and metastasis inhibitory effect of myricetin in radiation-induced metastatic cancer cells was examined.

2. Materials and Methods

2.1. Reagents

Kaighn's modification of Ham's F-12 medium (F-12K), 100×penicillin/streptomycin solution and trypsin-EDTA were purchased from Invitrogen Inc. (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Corning Incorporated-Life Sciences (Oneonta, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from Amresco Inc. (Solon, OH, USA). Gelatin and Bradford reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Amicon® Ultra-4 Centrifugal filter Units was purchased from Merck Millipore Ltd. (Country Cork, Ireland). BS ECL plus kit was purchased from Biosesang Co. (Gyeonggi-do, Korea). Primary antibodied were purchased as followed : E-cadherin, Slug, Vimentin, MMP-2, p-ERK1/2, and p-JAK2 antibodies were purchased from Cell signaling Technology (Danvers, MA, USA). β-actin antibody was purchased from Sigma Chemical Co. MMP-9 antibody was purchased from BD Biosciences (Franklin Lake, NJ, USA).

2.2. Cell culture

The lung adenocarcinoma A549 cell line was kindly provided by Dr. Min Young Kim in Jeju National University. An irradiation-tolerant A549-8G1 cell line and A549-12G1 cell line were established as a followed description. Cells were cultured in F-12K supplemented

with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were incubated at 37 ℃ in a humidified incubator with 5% CO_2.

2.3. Establishment of irradiation-tolerant cells lines.

Irradiation-tolerant cell lines were established as following description, by modifying previously reported methods (18, 20, 30-33) (Fig. 1).

A549 cells were counted at a density of 6 x 10^4 cells/mL in 1 mL PBS as suspension and then were subjected to irradiation with a single fraction(4, 8, and 12 Gy) to generate irradiation-tolerant cell lines. The irradiation was performed by using a ^{60}CO Theratron-780 tele-therapy unit (Applied Radiological Science Institute, Jeju National University, Korea) at a dose rate of 1.43 Gy per minute. Cells were kept at room temperature for < 30 minutes during irradiation. After irradiated, cells immediately were seeded in a culture flask (60 mm-culture flask) and incubated at 37 ℃ in a humidified incubator with 5% CO_2. Irradiated cells were allowed to grow for 15 days with change to fresh medium every 3-4 days. At the end of the incubation period, the surviving two single clones from 8 Gy-irradiated cells and seven single clones from 12 Gy-irradiated cells were transferred to each well of a 96-well culture plate. Reaching to sub-confluence, each single clone was transferred to a larger scale culture flask, 48-well, 24-well, 12-well, and 6-well plates in sequence. Finally, only one surviving clone from each irradiated cells was established and designated as A549-8G1 cell line and A549-12G1(hereinafter A549-IR) cell line, respectively.

For fractionated radiation of 2 Gy, A549 cells were cultured on a 60-mm culture plate. When the cells reached 80 % confluence, cells were harvested and suspended in 1 mL PBS for irradiation. Cells were exposed to 2 Gy of γ-ray at a dose rate of 1.43 Gy per minute, using a ^{60}CO Theratron-780 tele-therapy unit. When the irradiated cells reached approximately 80 % confluence, the cells were again harvested and irradiated. These procedures were repeated 10 times, twice a week. Following the last irradiation, the irradiated cell population were cultured and passaged for about 30 times under same conditions without irradiation. The established cells were named as A549 2-10 and used for further experiments.

2.4. Cell viability

The viability of A549 or A549-IR cells on sample treatment were determined by performing MTT colorimetric assay. The cells were counted and seeded on 96-well culture plates at a density of 4 x 10^3 cell/well. After overnight incubation, cells were treated with concentrations of the samples for 48 h. At the end of treatment, 20 μL of 5 mg/mL MTT solution was added to each well, and the treated cells were incubated at 37℃ for 4 h. Then, the growth medium was carefully removed and DMSO was added to each well to dissolve the formazan crystals. The intensity of produced formazan was measured in 30 minutes at 570 nm using a microplate reader. The percentage of cell viability was calculated as the percentage reduction in absorbance.

2.5. Wound healing assay

Cells were seeded in a 12-well plate at 2×10^5 cells per well. After the confluent monolayer was formed, scratched wounds were created using a sterile tip of the 200-μL or 1000-μL micropipette. Cells were washed with PBS to remove detached cells and cell debris. Then, each well was supplied with growth medium, contained indicated samples. Images were captured by phase microscopy at 0 , 24 , and 48 hours post-wounding to assess the cell migration. Percentages of wound closure were calculated as, wound closure % = [(0 hr cell-free area) - (48 hr cell-free area)] / (0 hr cell-free area**)** x 100.

2.6. Gelatin zymography analysis

Cells were seeded at 3×10^5 cells in 100-mm plates and cultured overnight. After overnight, cells were treated with myricetin in serum-free media for 48 h. Conditioned media were concentrated by centrifuging at 7500xg for 25 min at 4℃, using Amicon® Ultra-4 Centrifugal filter Units(Merk Millipore). The concentrated media were stored at -70℃ until used. The protein concentration were determined using Bradford reagent. MMP-2 and MMP-9 enzymatic activity were measured by gelatin zymography following the methods described in (16). Briefly, electrophoresis was performed under non-reducing conditions on 1% gelatin containing poly-acrylamide gels. As electrophoresis completed, wash the gel twice with wahing buffer(2.5% Triton X-100, 0.02% NaN_3). Then wash the gel for 10 minute in incubation buffer(50 mM Tris-HCl (pH 8.0), 5 mM $CaCal_2$, 0.0% NaN_3). Place gel in sealable container with fresh incubation buffer and incubate at 37℃ for 48 hours.

After 48 hours, gel was stained with coomassie blue solution and detectable to confirm the activity of MMPs.

2.7. Western blot analysis

The treated or non-treated cells were collected and washed once with PBS. Then, the cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/mL aprotinin, and 25 μg/mL leupeptin) and incubated on ice for 30 min. The cell lysates were centrifuged for 30 min at 14,000 rpm in 4℃ condition, and the supernatants were collected. The collected supernatants were stored at –70℃ until used. The protein concentrations were measured using the BCA Protein Assay kit. For preparation of conditioned medium, treated cells were washed with PBS, and the medium was replaced with serum-free basal medium and incubated for 24 hours. The conditioned medium was further concentrated using Amicon® Ultra-4 Centrifugal filter Units following manufacturer's instructions. The protein oncentration of conditioned medium were measure using the Bradford reagent assay. Aliquots of the lysates were separated by 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membrane (Millipore Ltd., Billerica, MA, USA) using glycine transfer buffer (192 mM glycine, 25 mM Tris–HCl, pH 8.8, and 20% [v/v] methanol). The membranes were blocked with 5% skim-milk and incubated for 5 hours to overnight with primary antibodies in 4℃, followed by an additional 30 min incubation with secondary antibodies in Tris-buffered saline and

0.1% Tween 20. All primary antibodies were diluted at 1:1,000 for used. As the secondary antibody, horseradish peroxidase-conjugated anti-rabbit or -mouse IgG were used at a 1:5,000 dilution. To detect the protein bands, the membranes were exposed to X-ray film, using the BS ECL plus kit (Biosesang, Korea).

2.8. Quantitative real-time PCR analysis

Total cellular RNA was extracted from cells using the TRIzol^{TM} reagent (Invitrogen, USA). Reverse transcription of 1 μg of RNA was done using reverse transcription kit(Promega, Madison, WI, USA), following the manufacturer's instructions. Quantitative real-time PCR was performed by using TOPreal^{TM} qPCR preMIX (Enzynomics, Daejeon, Korea). PCR was initiated by incubating at 95℃ for 15 min, followed by 45 cycles of 10 s denaturation at 95℃, 15 s anealing at 59.5℃ (for E-cadherin) and 58.5℃ (for GAPDH), and 20 s elongation at 72℃. GAPDH was used as the reference gene. The relative levels of gene expression were represented as $\Delta Ct = Ct_{target} - Ct_{reference}$, and the fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer for amplification were as follows: E-cadherin, forward 5'- CGGGAATGCAGTTGAGGATC-3', reverse 5'-AGGATGGTGTAAGCGATGGC-3'; GAPDH, forward 5'-CACATGGCCTCCAAGGAGTAA-3', reverse 5'-TGAGGGTCTCTCTCTTCCTCTTGT-3'.

2.9. Flow cytometry

To detect cellular-tethered E-cadherin expression, flow cytometric measurement was performed as manufacturer's instruction. Briefly, Treated or non-treated cells were trypsinized, and centrifuged at 300xg for 10 minutes. Supernatant was removed completely, and pellet was resuspended in 100 μL buffer (phosphate-buffered saline (PBS), pH 7.2, 0.5% bovin serum albumin (BSA), and 2 mM EDTA). Then, 10 μL of the E-cadherin antibody (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) was added. The mixture was incubated for 10 minutes in the dark condition at 4 ℃. After that, cells were washed by 2 mL buffer by centrifuging at 300xg for 10 minutes. Supernatant was removed completely and cell pellet was resuspended in a suitable amount of buffer for analysis by flow cytometry (BD Accuri^{TM} C6, BD Bioscience, USA).

2.10. Statistical analysis

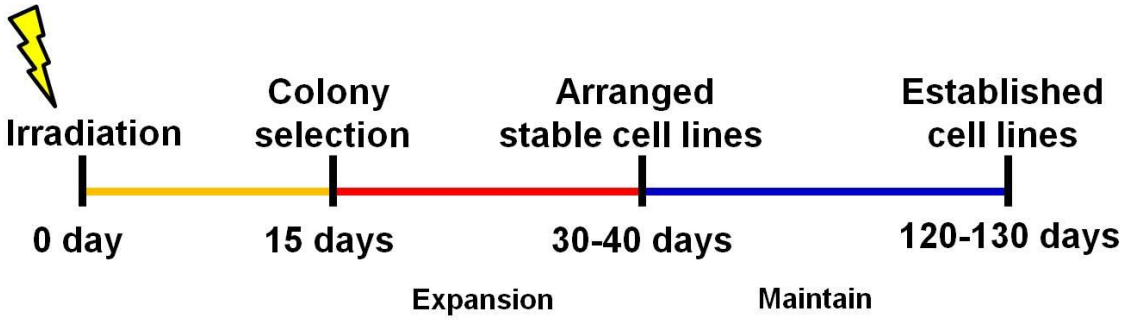
All results are shown as averages and standard deviations calculated by using Microsoft Excel. The data were subjected to one-way analysis using SPSS ver. 12.0 for Windows (Chicago, IL, USA). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3. Results

3.1. Irradiation-tolerant A549 cell lines present enhanced-invasive/migratory ability.

To determine whether established irradiation-tolerant A549 cell lines show increased invasive abilities, cell characteristics were first compared among A549, a clone from 8 Gy-irradiated A549 cells(8 Gy), a clone from 12 Gy-irradiated A549 cells(12 Gy), and 2 Gy-regularly-irradiated A549 cells(2 Gy X 10). By performing the wound healing assay, we quantified the migration ability of individual cells. A clone from 8 Gy-irradiated A549 cells (8 Gy) and a clone from 12 Gy-irradiated A549 cells (12 Gy) showed higher migration ability than that of A549 cells (Fig. 2A). Moreover, E-cadherin expression was decreased and Vimentin expression was increased in clones from 8 Gy-irradiated A549 cells (8 Gy) and 12 Gy-irradiated A549 cells (12 Gy), compared to A549 cells (Fig. 2B). Nonetheless, 2 Gy-regularly-irradiated A549 cells (2 Gy X 10) did not show enhanced-migration properties (Fig 2A, 2B). Further analysis of E-cadherin expression in cellular membrane showed that a clone from 12 Gy-irradiated A549 cells (12 Gy) has the least E-cadhein on its cellular membrane among 3 kinds of cell lines (Fig. 2C). Since E-cadherin is the hallmark of EMT process and migratory ability (34-37), a clone from 12 Gy-irradiated A549 cells (12 Gy) was selected for further experiments and named as A549-IR.

1A



1B

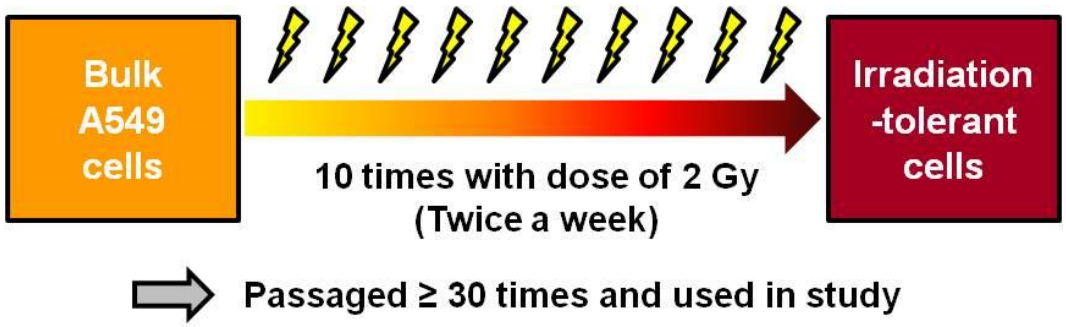
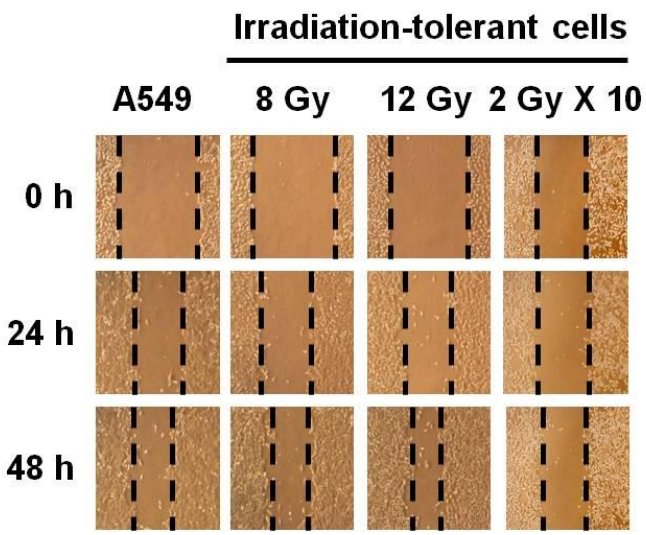


Figure 1. Schemes of establishment of irradiation-tolerant cells.

2A



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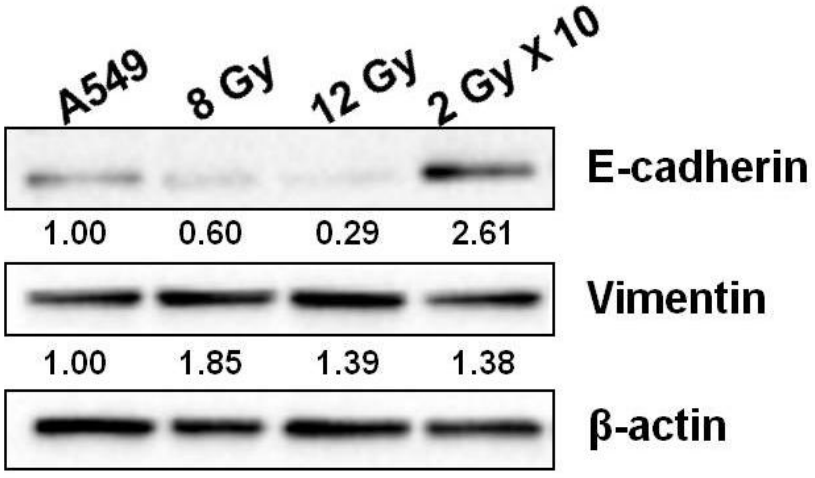


2C

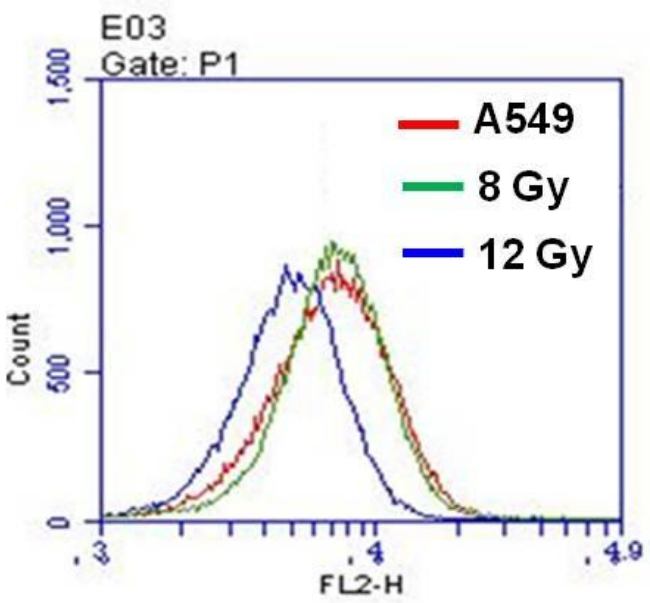


Figure 2. Migratory characteristic of established irradiation-tolerant cells. (A) Wound healing assay was performed to assess migration ability of established irradiation-tolerant cells. (B) E-cadherin and Vimentin expression in established irradiation-tolerant cells were examined by western blot analysis. (C) The level of cellular membrane-tethered E-cadherin was measured by flow cytometry.

3.2. A549-IR cells show enhanced EMT-related protein level.

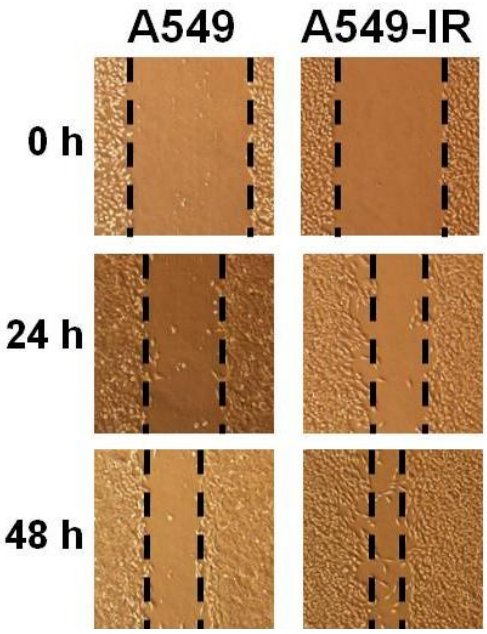
The enhanced-migratory ability of A549-IR cells, compared to A549 cells, was confirmed (Fig. 3A, B). E-cadherin expression was significantly decreased in A549-IR cells, whereas, Slug and Vimentin expression were increased (Fig. 3C). As a result of quantification real-time PCR, mRNA level of E-cadherin was typically decreased in A549-IR cells (Fig. 3D). These data indicate that EMT is induced in A549-IR cells and invasive/migration enhancement of A549-IR cells is related to EMT.

3.3. Soluble E-cadherin could play a crucial role in regulating MMPs expression in A549-IR cells.

The results show that E-cadherin expression was strongly decreased in A549-IR cells. Interestingly, the level of protein expression seems to have decreased more than that of mRNA transcription. Recent study also reported that existence of different forms of E-cadherin is one of the reasons why E-cadherin has multiple roles. E-cadherin is well known for existing in two forms, one as the membrane-tethered form and the other as the soluble form (38). Furthermore, other studies proved that soluble E-cadherin promotes cell survival and tumorigenesis (38-41). To confirm whether soluble E-cadherin was increased in A549-IR cells, conditioned media of A549 and A549-IR cells were carried for western blot analysis. Western blot analysis revealed that soluble E-cadherin level in conditioned media was increased in A549-IR cells, even though the full-length E-cadherin expression was

significantly decreased in A549-IR cells (Fig 4A). It is reported that soluble E-cadherin interacts and forms a complex with HER and IGF-1 receptor tyrosine kinases(41). To address whether soluble E-cadherin induces its effects through the HER/IGF-1R family of receptors, we examined the protein expression of downstream targets in A549 and A549-IR cells. Associated with the increase in soluble E-cadherin, phosphorylated Erk1/2 and phosphorylated JAK2 were increased in A549-IR cells (Fig. 4B). As matrix metalloproteinases, MMP-2 and MMP-9 are crucial executors in cancer invasion. MMP-2 and MMP-9 expression is well-controlled by MAPK and JAK-STAT3 pathways. As expected, the expression level of MMP-2 and MMP-9 was increased in A549-IR cells (Fig. 4C). Gelatin zymography analysis also proved that these increased (Fig 4D). Taken together, these results suggest that soluble E-cadherin can play a key role in enhanced-invasion in A549-IR cells via regulating MMP-2, -9 expression.

3A



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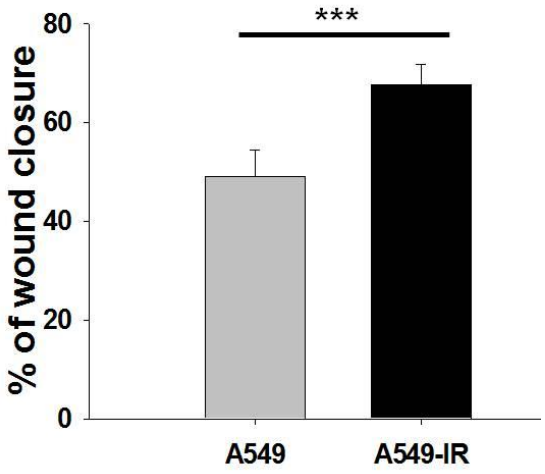

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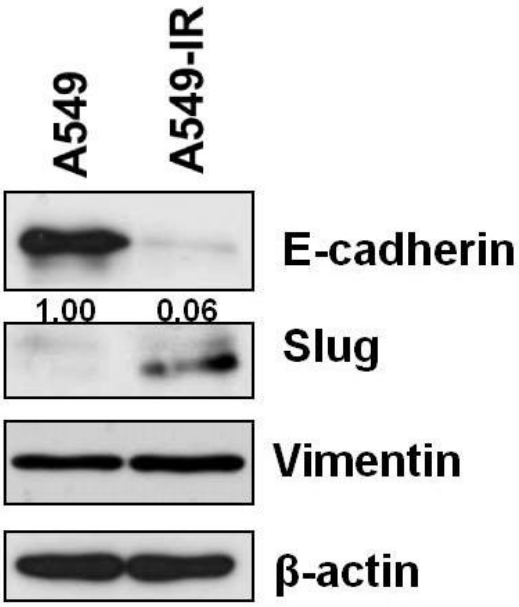


3D

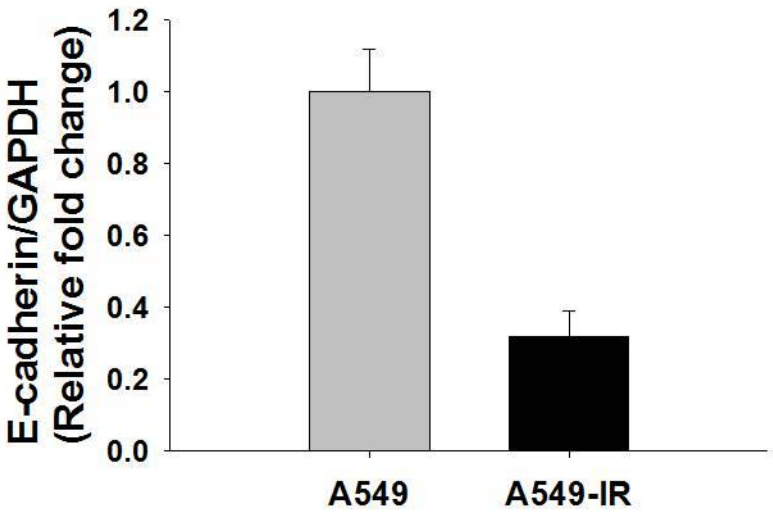
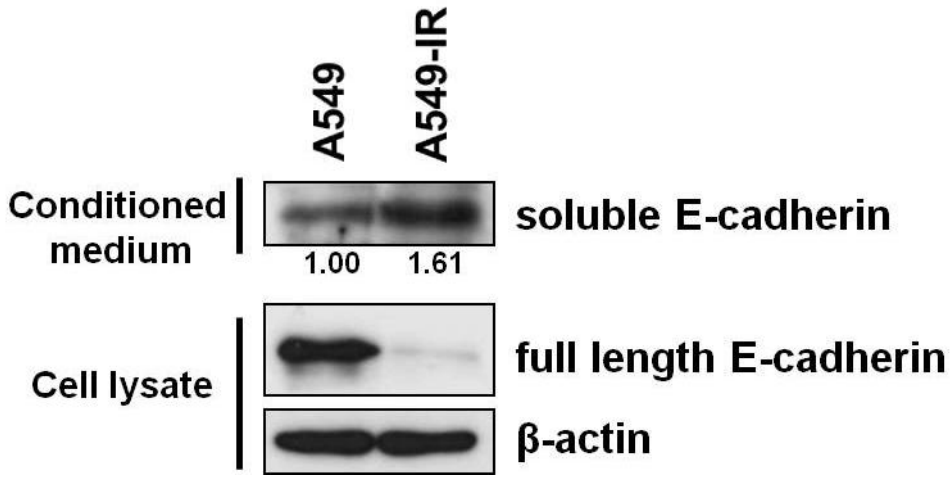
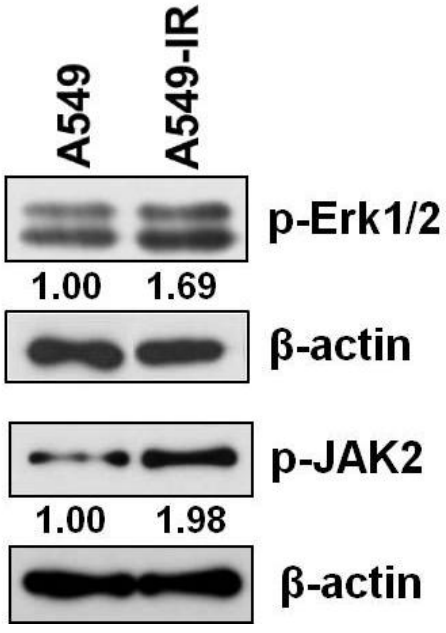


Figure 3. Characterization on EMT-related markers in A549-IR cells. (A) Wound healing assay was performed to assess migration ability of A549 and A549-IR cells. (B) The graph shows the percentage of wound closure, assessed from A. (C) EMT-relate proteins (E-cadherin, Slug, and Vimentin) expression in A549 and A549-IR cells were examined by western blot analysis. (D) E-cadherin gene transcriptional level in A549 and A549-IR cells were measured by performing quantitative real-time PCR analysis.

4A



4B



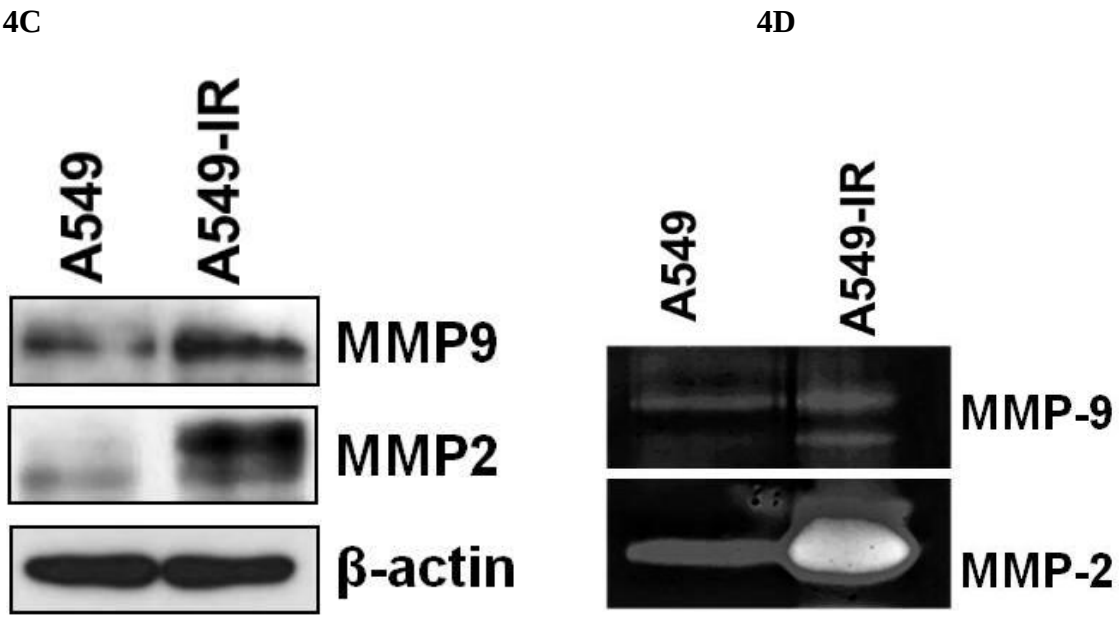


Figure 4. Soluble E-cadherin might play a crucial role in regulating MMPs expression in A549-IR cells. (A) The levels of soluble E-cadherin from conditioned medium of A549 and A549-IR cells and full-length E-cadherin expression in A549 and A549-IR cells were examined by western blot analysis. (B) Phosphorylated Erk1/2 and phosphorylated JAK2 expression in A549 and A549-IR cells were examined by western blot analysis. (C) MMP-2 and MMP-9 expression in A549 and A549-IR cells were examined by western blot analysis. (D) Gelatin zymography analysis was performed to assess the activity of MMP-2 and MMP-9 in A549 and A549-IR cells.

3.4. Myricetin suppresses migration in A549-IR cells.

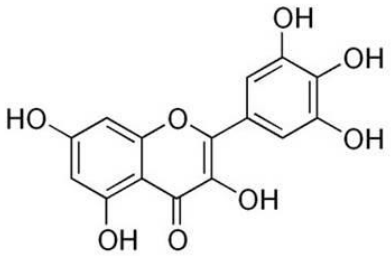
Next, we examined to figure out which phytochemicals could inhibit the radiation-induced invasion enhancement in A549-IR cells. First, to evaluate the anti-proliferative effects of phytochemicals, we tested the cyto-toxicity of phytochemicals on A549-IR cells. A549-IR cells were treated with various kinds of phytochemicals for 48 h, and their cell viability was measured. Following this data, we determined IC_{90} values of each phytochemical, which had no influence on cell proliferation (Table 1). According to this result, A549-IR cells were treated with selected dosed of each phytochemical, for further analysis.

To determine the migration-inhibitory effect of phytochemicals on A549-IR cells, we performed wound healing assay. Most of them were not effective in reducing migration of A549-IR cells (data not shown), but, myricetin showed significant inhibitory effect against migration of A549-IR cells (Fig 5C). Therefore, we speculated that the enhanced-migration/invasion ability of A549-IR cells may be inhibited by myricetin treatment.

Compound	IC_{90}
Gallic acid	≤ 20 μM
Trihydroxybenzaldehyde	≤ 200 μM
Ditertbutylphenol	≤ 20 μM
Quercetin	≤ 20 μM
Kaempferol	≤ 55 μM
Myricetin	≤ 100 μM
Dihydrokaemferol (Aromadendrin)	≤ 200 μM
Luteolin	≤ 10 μM
Baicalein	≤ 70 μM
Nobiletin	≤ 20 μM
Tangeretin	≤ 20 μM
Hesperidin	≤ 50 μM
Gliotoxin	≤ 50 μM
Resveratrol	≤ 30 μM
Auraptene	≤ 25 μM
Phytol	≤ 90 μM
Limonene	≤ 400 μM

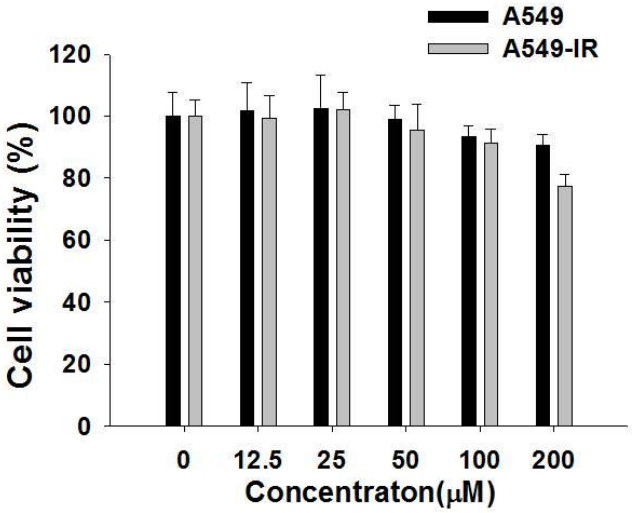
Table 1. IC_{90} value of phytochemicals on inhibition of proliferation in A549-IR cell.

5A

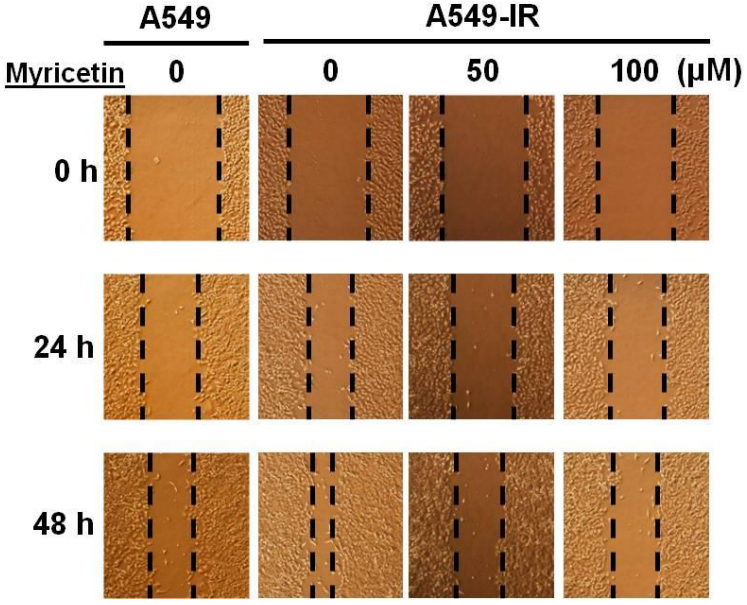


Myricetin

5B



5C



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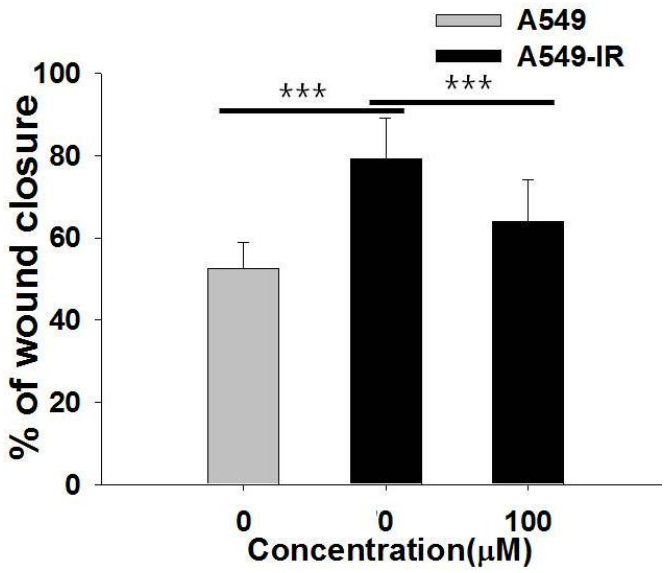
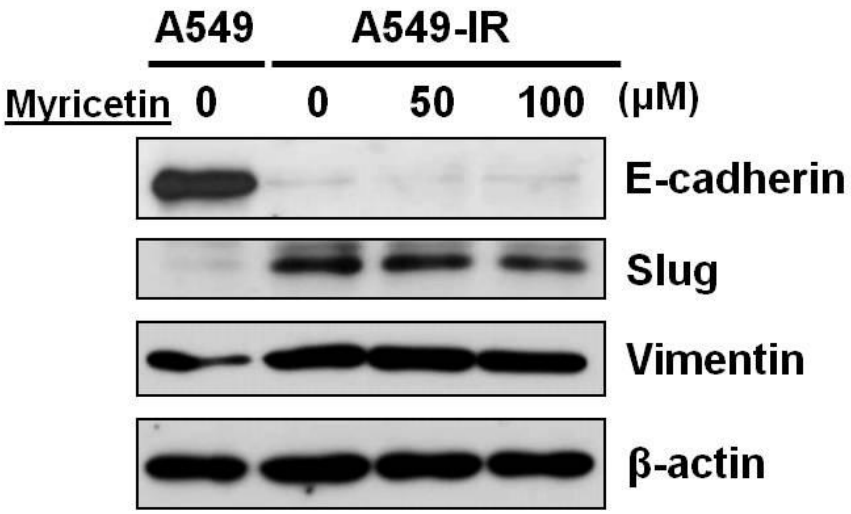


Figure 5. Migration inhibitory effects of myricetin in A549-IR cells. (A) The structure of myricetin. (B) The cytotoxicity of myricetin on A549 and A549-IR cells was measured by performing MTT assay. Cells were treated with indicated doses of myricetinn for 48 hours. (C) Wound healing assay was performed to assess migratory inhibition effect of myricetin on A549 and A549-IR cells. (D) The graph showed percentage of wound closure assessed from C.

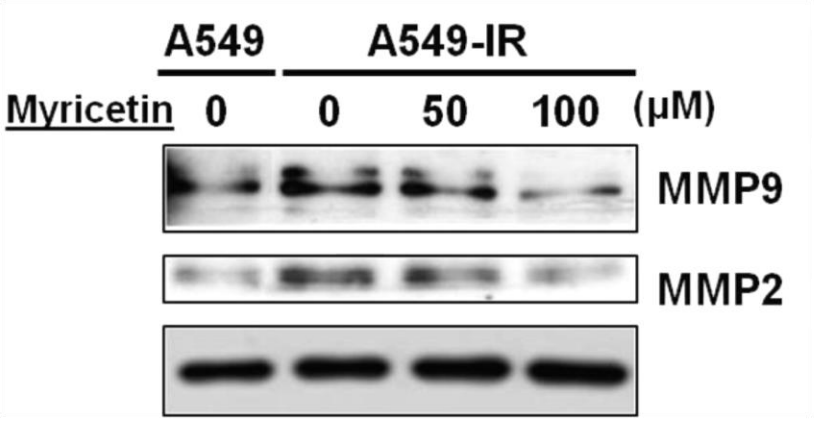
3.5. Myricetin inhibited migratory ability via decreasing MMP-2, -9 expression in A549-IR cells.

To examined whether myricetin induces MET in A549-IR cells, expression of EMT-related protein was evaluated in A549-IR cells after 48 hours myricetin treatment. However, elevation of E-cadherin expression and abrogation of Vimentin expression in A549-IR cells were not observed upon myricetin treatment (Fig. 6A). On the other hand, expression of MMP-2 and MMP-9 were remarkably decreased following myricetin treatment in A549-IR cells (Fig. 6B, C). These results indicate that myricetin hardly induces MET in A549-IR cells, but suppresses migration of A549-IR cells through reducing MMP-2 and MMP-9 expression.

6A



6B



6C

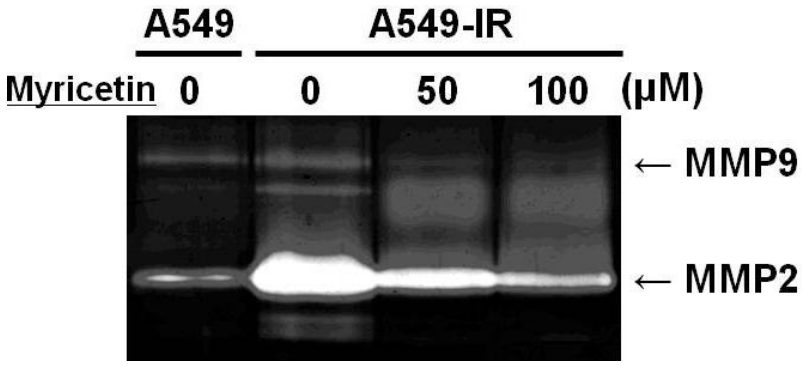


Figure 6. Myricetin failed to induce Mesenchymal-epithelial transition, but, suppresses MMP-2, MMP-9 expression in A549-IR cells. (A) EMT-relate proteins (E-cadherin, Slug, and Vimentin) expression in A549 and A549-IR cells, after myricetin treatment for 48 hours, were examined by western blot analysis. (B) MMP-9 and MMP-2 expression in A549 and A549-IR cells after myricetin treatment for 48 hours. (C) Gelatin zymography analysis was performed to assess the activity of MMP-2 and MMP-9 in A549 and A549-IR cells, after myricetin treatment for 48 hours.

4. Conclusion

Our results indicate that A549-IR cells, which is established from 12 Gy-irradiation-tolerant A549 cells, show enhanced migration/invasion ability. As the marker of EMT, E-cadherin expression was significantly decreased in A549-IR cells, compared to that of A549 cells. Concomitant with decreased E-cadherin expression, A549-IR cells showed higher expression of Slug and Vimentin than A549 cells expressed. As the marker of invasiveness, MMP-2 and MMP-9 expression were increased in A549-IR cells, compared to A549 cells. Remarkably, soluble E-cadherin, which is an extracellular shedding domain of E-cadherin and a contributor to cancer cell proliferation, was significantly increased in conditioned media of A549-IR cells. Taken together, these results indicate that migration enhancement of A549-IR cells is the result of soluble E-cadherin-mediated increasing MMP-2 and MMP-9 expression, via activation of HER/IGF-1R signaling pathway.

Myricetin showed the ability to reduce the migration of A549-IR cells. Even though elevation of E-cadherin was not observed, myricetin effectively suppressed MMP-2 and MMP-9 expression. It was suggested that inhibitory effect of myricetin against migratory activity of A549-IR cells was mediated by down-regulation of MMP-2 and MMP-9 expression.

In conclusion, these results have provided a possible model for irradiation-induced metastatic lung cancer cells, and suggested myricetin as a potential anti-metastatic reagent against the irradiation-induced metastatic lung cancer cells, via down-regulation of MMP-2

and MMP-9 expression.

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진심으로 감사드립니다.