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Original Research Article

Anticancer effects of Mori radicis cortex, Mori ramulus, Angelica tenuissima, and Inonotus obliquus extract mixtures on B16F10 murine melanoma cells via up-regulation of p53 and down-regulation of Akt expression

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Abstract

Purpose: *To investigate the anticancer effects of the extract mixture of Mori radicis cortex, Mori ramulus, Angelica tenuissima, and Inonotus obliquus extract (MRME), a new herbal preparation from Korea, on B16F10 cells.*
Methods: *The MRME was extracted with water. Cell Counting Kit-8 (CCK-8) assays and 4',6-diamidino-2-phenylindole (DAPI) staining were used to investigate the effect of MRME on cell proliferation and apoptosis in vitro. The expression of p53, p-p53, and phosphorylated Akt (p-Akt) were analyzed by western blotting. Wound-healing assay was used to analyze cell migration.*
Results: *Based on CCK-8 and DAPI staining results, MRME inhibited the growth of B16F10 cells and induced apoptosis in B16F10 cells in a time- and dose-dependent manner ($p < 0.05$). MRME increased the expression of p53 and p-p53 and decreased the expression of p-Akt in a dose-dependent manner. MRME treatment inhibited cell migration and invasion ($p < 0.05$).*
Conclusion: *Mori Radicis Cortex Mixture Extract exerts anticancer effect by inducing the expression of p53 and p-p53 and decreasing the expression of p-Akt, which may benefit patients with malignant melanoma when used in combination with v-raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitors.*

Keywords: *Mori Cortex Radicis, Ramulus Mori, Angelica tenuissima, Inonotus obliquus, Melanoma, Anticancer, p53, Akt*

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INTRODUCTION

Malignant melanoma is the most aggressive form of skin cancer and is one of the leading causes of death [1]. The mortality rate worldwide is 0.7 % in men and 0.4 % in women [2]. Although surgical intervention for early diagnosis may treat malignant melanoma in most patients, a significant proportion of these cases are refractory, warranting research and the

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development of novel therapies against melanoma. Many studies have elucidated the roles of cellular signaling molecules, including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)-Akt, nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), Janus kinase (JAK)-signal transducer and activator of transcription, Wnt, Notch, and transforming growth factor β (TGF-β), which are involved in melanoma development. These compounds can potentially serve as treatment targets [3].

Among the signaling pathways involving these molecules, MAPK pathway alterations are frequently found in cutaneous melanoma; 40 – 50 % of melanomas carry v-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutations, 20 – 30 % harbor *NRAS* mutations, and 10 – 15 % have mutations involving *NF1* [4]. Recent data show that treatment with BRAF/MAPK kinase (MEK)-targeted therapy improves the overall five-year survival rate to 34 % [5]. However, a significant proportion of patients are refractory to MEK-targeted therapy and may experience severe side effects. Therefore, many Korean patients seek alternative treatments in the form of traditional Korean and Chinese medicinal herbs, such as *Mori Cortex Radicis, Ramulus Mori* (Sangzhi), *Angelica tenuissima* (gobon), and *Inonotus obliquus* (chaga), to avoid chemotherapy-induced toxicity and for their documented advantages in terms of anti-inflammatory and anticancer effects [6–9]. *Mori Cortex Radicis* is the bark of *Morus alba*, also known as white mulberry. *Mori Cortex Radicis* extract suppresses the expression of cytokines, c-Fos, activated p38-MAPK, and NF-κB [6]. *Ramulus Mori* (Sangzhi), which refers to the young twigs of *Morus alba*, exhibits potent tyrosinase inhibitory activity and robust melanogenesis inhibitory activity [7]. Previous studies have identified the antimelanogenic effects of *A. tenuissima* (gobon) root fermented by *Aspergillus oryzae*. Recently, anti-inflammatory activities of *Angelica tenuissima* have been identified [8]. *Inonotus obliquus* (*I. obliquus*) has significant inhibitory effects on nitric oxide production and NF-κB luciferase activity in RAW 264.7 cells, and exhibits cytotoxicity against various human carcinoma cells [9]. MRME is a commonly used ingredient in traditional Korean herbal medicines. In this study, we used a new preparation to extract a mixture of *Mori Radicis Cortex*, *Mori Ramulus*, *A. tenuissima*, and *I. obliquus* using water, explored the effects of the extract on melanoma cells, and investigated the underlying signaling pathway.

METHODS

Chemicals and cell culture

4′,6- diamidino-2-phenylindole (DAPI) and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). B16F10 murine melanoma cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle’s medium containing 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin at 37 °C under a 5 % CO_2 atmosphere.

Preparation of MRME

The herbal ingredients of MRME, including *Mori Radicis Cortex, Mori Ramulus*, *A. tenuissima*, and *I. obliquus*, were purchased from Dongyang Herb Co., Ltd. (Seoul, Republic of Korea). A mixture of *Mori Radicis Cortex* (52 g), *Mori Ramulus* (20 g), *A. tenuissima* (22 g), and *I. obliquus* (6 g) was extracted with 500 mL of distilled water at 90 °C for 8 h. The concentrated extract was dried by lyophilization and reconstituted in DMSO for *in vitro* studies.

Cell viability assays

The antiproliferative effects of MRME on B16F10 melanoma cells was determined using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer’s instructions. Cells (1×10^4 cells/well) were incubated in 96-well plates. Subsequently, the cells were treated with various concentrations of MRME (156, 312, 625, 1,250, 2,500, and 5,000 μg/mL) for 24 and 48 h. CCK-8 (10 μL) was added to each well and incubated for 3 h. Absorbance was measured at 450 nm using a microplate reader. The culture medium was used as a blank. Cytotoxicity in the MRME-treated culture was calculated using Eq 1.

Death (%) = (AM/AC)100 ……………. (1)

where AM and AC are the absorbance of MRME-treated and control cells, respectively.

Cell morphology studies

The nuclear morphology of MRME-treated B16F10 cells was evaluated using DAPI staining under a fluorescence microscope. B16F10 cells were seeded at a density of 1×10^4 cells/well in 6-well plates and subjected to MRME treatment at different concentrations (0, 250, 500, 1,000, 2,000, and 2,500 μg/mL) for 24 h. The cells were then treated with DAPI solution. Stained cells

were visualized under a fluorescence microscope (Olympus, Tokyo, Japan).

Western blot assay

B16F10 cells (2.5 × 10^5 cells/mL) were plated in 60-mm culture dishes prior to MRME treatment. After 24 h of cell attachment, the cells were treated with either growth medium alone, as a control, or with varying concentrations of MRME. At the end of the treatment, cells were lysed and protein concentrations were determined by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with albumin as the standard. The extracted protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The blotted membrane was blocked with Tris-buffered saline containing Tween-20 (2.7 M NaCl, 53.65 mM KCl, 1 M Tris-HCl, pH 7.4, and 0.1 % Tween-20) containing 5 % skim milk for 2 h and incubated with primary antibodies (p53, phospho-p53, Akt, phospho-Akt, p-B-Raf, B-Raf, and β-actin) for 24 h. Following this, the reaction with secondary antibodies was observed for 2 h at 25°C. Protein expression was confirmed using an enhanced chemiluminescence (ECL) solution. Bands were normalized using an anti-β-actin antibody.

Wound healing assay

B16F10 cells were plated at a density of 1 × 10^5 cells/mL in a 6-well plate. After culturing for 24 h, each well was scratched with a 200 μL sterile tip. Cell debris was removed three times with the medium. Thereafter, MRME was added to concentrations of 2,000 and 2,500 μg/mL. The plates were then incubated and wound widths were measured at the indicated times by microscopy (Olympus, Tokyo, Japan). The distance between cells was photographed every 6, 12, and 24 h.

Statistical analysis

All statistical analyses were performed using SPSS v.21.0 (SPSS, Inc., Chicago, IL, USA) and data are presented as means ± standard deviation (SD). Student's *t*-test was used to analyze differences, and statistical significance was set at $p < 0.05$.

RESULTS

Anticancer effects of MRME

The effects of various concentrations of MRME on the growth of mouse-derived B16F10 melanoma cell was tested *in vitro* for 24 and 48 h. As shown in Figure 1, after treatment for 24 and 48 h, MRME induced a dose-dependent decrease in the viability of B16F10 cells, as analyzed using the CCK-8 assay.

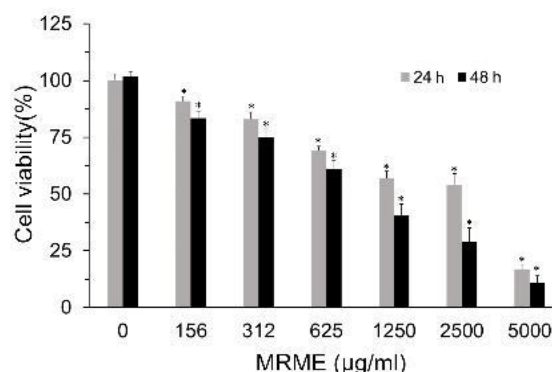

Figure 1: Effects of MRME on viability of B16F10 murine melanoma cells. Cells were treated with different concentrations of MRME (156, 312.5, 625, 1,250, 2,500, and 5,000 μg/mL) for 24 or 48 h. Cell viability was measured using CCK-8 assays. The data were normalized to those of control cells. Data are expressed as means ± SD (n = 5). $^*P < 0.05$, compared to the untreated control

In addition, at 48 h, B16F10 cell viability was generally more decreased in a time-dependent manner than that at 24 h. Overall, cell viability decreased in a dose-dependent manner. IC_{50} values at 24 and 48 h ranged from 625 – 1,250 and 2,500 – 5,000 μg/mL, respectively.

Apoptosis of B16F10 cells treated with MRME

MRME displayed high potential for anti-proliferative effects by stimulating apoptosis in B6F10 cells, as indicated by fluorescence investigations of DAPI-stained B6F10 cells, which exhibited more pronounced chromatin condensation as MRME concentration increased (Figure 2).

MRME down-regulates Akt phosphorylation and up-regulates p53 phosphorylation

Preliminary *in vitro* experiments confirmed that MRME induced apoptosis in B16F10 cells. Western blotting was performed to determine the expression levels of p53 and Akt, which are the major factors involved in the apoptosis signaling pathway. As shown in Figure 3, the expression of p53 and p-p53 increased, and that of p-Akt decreased in a dose-dependent manner. p-B-Raf and B-Raf levels did not exhibit any significant changes.

These findings revealed that MRME increased the expression of p53 and p-p53 and inhibited the expression of phosphorylated Akt (p-Akt),

highlighting the mechanisms by which MRME inhibits cell proliferation and induces apoptosis in B16F10 cells.

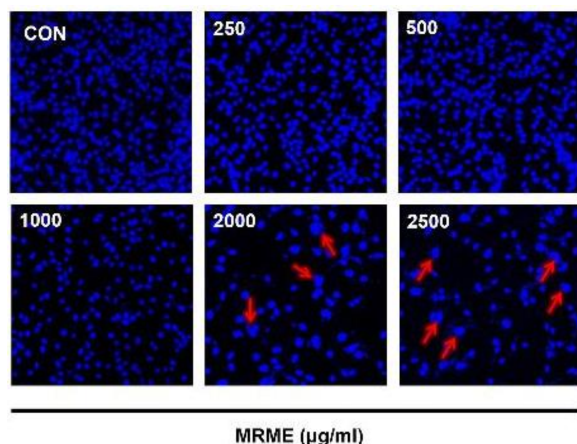

Figure 2: Effects of MRME on apoptosis induction in B16F10 murine melanoma cells. Cells were treated with different concentrations of MRME (0–2,500 μg/mL) for 48 h. Apoptotic cells were stained with DAPI. The arrows point at changes in the morphology of B16F10 cells: membrane and nuclear fragmentation, indicating apoptosis when compared to the untreated control group. MRME dose-dependently inhibited the growth of B16F10 cells

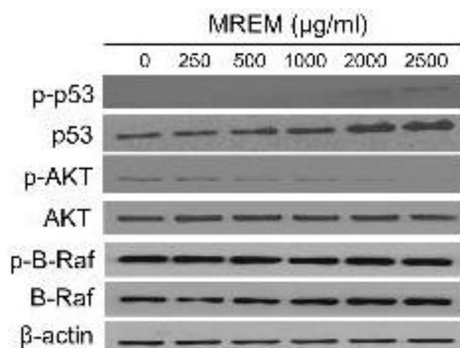

Figure 3: Effect of MRME on cell signaling molecules in B16F10 murine melanoma cells. B16F10 cells were treated with 250, 500, 1,000, 2,000, and 2,500 μg/mL of MRME for 24 h. Western blot analysis was performed using antibodies against p53, p-p53, Akt, p-Akt, B-Raf, p-B-Raf, and β-actin

Cell migration analysis

Wound-healing assays were performed to analyze whether MRME inhibited B16F10 cell migration. B16F10 cells were treated with 0, 2,000, or 2,500 μg/mL MRME for 6, 12, or 24 h. The results showed that MRME treatment inhibited cell migration, with the effect increasing with increasing concentration after 6, 12, and 24 h of incubation (Figure 4), suggesting that MRME inhibited the migration and invasion of B16F10 cells in a dose-dependent manner.

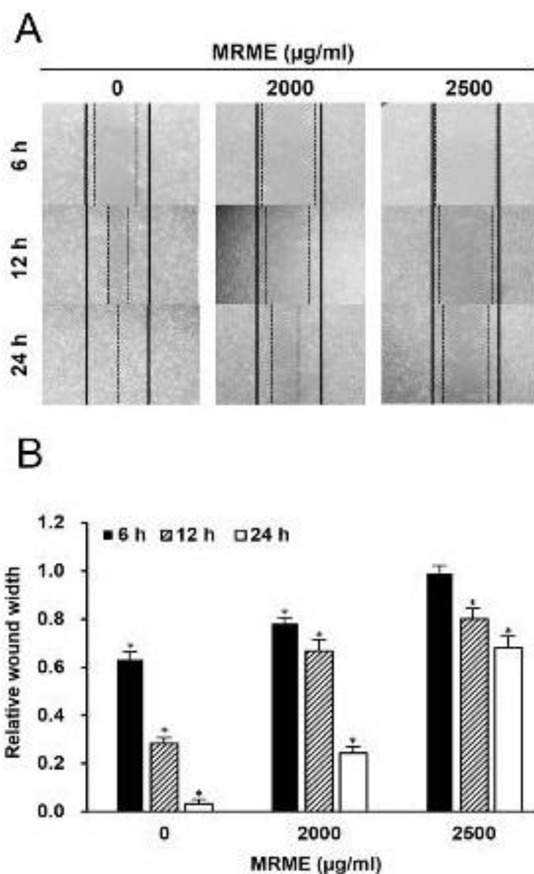

Figure 4: Effects of MRME on cell migration by B16F10 murine melanoma cells. Cells were treated with 0, 2,000, and 2,500 μg/mL of MRME and monitored for 6, 12, and 24 h. (A) Images of wound healing assay results after 6, 12, and 24 h. (B) Quantitative analysis of wound width, an indicator of cell migration, in the wound healing assay. $^*P < 0.05$ when compared with the untreated control at 0 h

DISCUSSION

This study has demonstrated that MRME inhibits the tumorigenesis and metastasis of malignant melanoma cells *in vitro*. The results revealed that MRME inhibited melanoma cell growth and proliferation by inducing apoptosis in a dose- and time-dependent manner. Furthermore, there was an increase in the inhibition of migration and invasion of B16F10 cells in a dose-dependent manner, suggesting that MRME prevents further tumor invasion and metastasis.

Western blotting was performed to elucidate the involvement of MRME in major apoptotic signaling pathways. The results revealed a dose-dependent increase in p53 and p-p53 expression, and a decrease in Akt expression. However, p-B-Raf and B-Raf expression levels remained unchanged. These results indicate that MRME selectively modulates the PI3K-Akt pathway through Akt phosphorylation and may increase the expression of p53-up-regulated

modulator of apoptosis (PUMA) and NOXA by increasing p53 expression [10]. Two proteins,

PUMA and p-Akt, have been implicated in poor survival of melanoma patients [11]. The p53 directly targets *PUMA* transcription and increases its expression in response to DNA damage [12]. PUMA binds to and neutralizes pro-survival members of the Bcl-2 family to promote apoptosis [12]. In contrast to most BH3-only proteins, which target only a subset of pro-survival Bcl-2 proteins, PUMA interacts with all pro-survival Bcl-2-like proteins (BCL-2, BCL-W, BCL-XL, BFL-1/A1, and MCL-1), suggesting that PUMA is the chief component of apoptosis [13]. Along with pro-apoptotic effector inactivation, excessive activation of proliferation signaling pathways contributes to the aggressive characteristics of melanoma. In 43 – 67 % of melanomas, serine/threonine kinase Akt (protein kinase B) is constitutively activated [14]. Upon activation, Akt induces transcription of various cell survival and proliferation genes. In melanoma, however, the PI3K-Akt pathway is activated in multiple ways, including activating mutations in the oncogene *NRAS* (15 – 20 %) or the loss of expression or function of the tumor suppressor *PTEN* (20 – 30 %) [15].

Recently, targeted small-molecule inhibitors and immunotherapy have been developed to improve the overall survival of patients with melanoma. Therapies targeting BRAF and MEK to block the MAPK pathway were the first to show unprecedented clinical responses regarding overall survival (34 % at five years) and progression-free survival rates (19 % at five years) [5,16]. Most patients have intrinsic resistance or rapidly acquire resistance to the MAPK pathway and immune checkpoint inhibitors [17]. One of the major causes of resistance to BRAF inhibitors (BRAFi) is reactivation of the MAPK pathway (80 %) [17]. Other mechanisms of BRAFi resistance include activation of the PI3K/AKT/mTOR signaling pathway [18]. Owing to the multiplicity of mechanisms, future research goals would be to develop combination targeted therapies.

Limitations of the study

This study has some limitations. The effects of individual components of the MRME mixture were not tested. The effects of MRME on the cell cycle were not distinctly identified by flow cytometry. Animal experiments are required to determine the effects of MRME *in vivo*, which requires further research. However, this study suggests the potential of the combined use of MRME and BRAFi-based therapies.

CONCLUSION

The findings of this study confirm the *in vitro* anticancer effects of MRME which are mediated by modulation of PI3K-Akt pathway through Akt phosphorylation and increased p53 expression. Thus, MRME is a potential therapeutic agent when combined with BRAFi-based therapies.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this study was conducted by the authors of this manuscript. All liabilities regarding claims relating to the content of this article will be borne by the authors. Kangduk Choi conceived and designed the study. Kyeng Min Kim, Eunhee Choi, Jung-Hyun Lee, Kukdong Kim, Prabhat Kumar Mandal, and Kangduk Choi collected and analyzed the data and wrote the manuscript. All authors read and approved the manuscript for publication.

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