

RESEARCH ARTICLE

Andrographolide enhances the anti-metastatic effect of radiation in Ras-transformed cells via suppression of ERK-mediated MMP-2 activity

Chih-Chia Yu¹✉, Chien-An Chen²✉, Shu-Ling Fu³, Hon-Yi Lin^{1,4}, Moon-Sing Lee^{1,4}, Wen-Yen Chiou^{1,4}, Yu-Chieh Su^{5,6}, Shih-Kai Hung^{1,4*}

1 Department of Radiation Oncology, Dalin Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Chia-Yi, Taiwan, R.O.C, **2** Department of Radiation Oncology, Zhongxing Branch, Taipei City Hospital, Taipei, Taiwan, **3** Institute of Traditional Medicine, National Yang-Ming University, Taipei, Taiwan, **4** School of Medicine, Tzu Chi University, Hualian, Taiwan, R.O.C, **5** Division of Hematology and Oncology, Department of Internal Medicine, Kaohsiung Medical University Hospital, Taiwan, **6** Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

✉ These authors contributed equally to this work.

* oncology158@yahoo.com.tw



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Abstract

Background

Activation of Ras oncogene in human tumors is associated with radiation-associated metastatic potential. Although ionizing radiation is one important method of cancer treatments, it has been shown to enhance matrix metalloproteinases (MMPs) activity and facilitates a more aggressive cancer phenotype. Our previous studies showed that andrographolide with lower dose rates of radiation could inhibit RAS-transformed cancer metastasis *in vivo*; however, the molecular mechanisms are not yet clear. In this study, we aimed to explore the anti-metastatic effect of andrographolide combined with radiation on Ras-transformed cells.

Methods

RAS-transformed cells were treated with andrographolide in the presence or absence of irradiation (2–4 Gy) or angiotensin II to examine cell invasion. *In vivo* tumorigenesis assays were also performed. The MMP-2 activity was detected by using Gelatin zymography. Signal transduction of NF-κB subunit, p65 and phosphor-ERK 1/2, were examined by using Western blotting analysis.

Results

Treatment with andrographolide inhibited migration of Ras-transformed cells. Andrographolide treatment with radiation significantly inhibited cancer metastasis *in vivo*. We found that andrographolide exhibited anti-migration and anti-invasive ability against cancer metastasis via inhibition of MMP2 activity rather than affected MMP-9 and EMT. In addition, combined andrographolide with radiation appeared to be more effective in reducing MMP-2

expression, and this effect was accompanied by suppression of ERK activation that inhibits cancer cell migration and invasion.

Conclusions

These findings suggest that andrographolide enhances the anti-metastatic effect of radiation in Ras-transformed cells via suppression of ERK-mediated MMP-2 activity.

Introduction

Radiotherapy (RT) is a curative treatment option for some malignant tumors. However, local relapse and distant metastasis are two major causes of RT failure. Tumor metastasis is driven by accumulation of intrinsic and extrinsic alterations in malignant cells that increase motility and invasiveness of the cells, which can allow them to be resistant to cytotoxic therapies.

Matrix metalloproteinases (MMPs) have been considered to increase the metastatic potential of various cancers because they degrade basement membrane, and alter cell-cell and cell-extracellular matrix (ECM) interactions, migration, and angiogenesis [1,2]. MMP-2 (gelatinase A) and -9 (gelatinase B), both of which are cancer-associated, zinc-dependent endopeptidases, have been implicated in playing a significant proteolytic role in cancer invasion and metastasis [3]. Previous studies have indicated that the expression of MMP-2 and MMP-9 are enhanced by radiation in a number of tumor types, and their expressions have been associated with poor prognosis [4–6].

The mitogen-activated kinases (MAPKs) pathway has been previously related with MMPs expression and metastasis progression. In addition, NF- κ B has been reported to be involved in regulating MMP-2 and MMP-9 expression [7,8]. Upregulation of MMP-9 has been observed in hepatocellular carcinoma (HCC) exposed to ionizing radiation, and radiation-induced HCC invasiveness is enhanced through the PI3K/AKT/NF- κ B signal transduction pathway [6]. It has also been reported that MMP-9 inhibition downregulates radiation-induced NF- κ B activity, leading to apoptosis in breast tumors [9]. The activation of MMP-2 has been shown to be a crucial step in tumor invasiveness and is associated with more aggressive phenotypes [10]. MMP-2 siRNA combined with radiation was shown to significantly reduce migration, invasion, growth, and angiogenesis in glioblastoma cells [11].

Andrographolide, a bicyclic diterpenoid lactone, is the primary ingredient of the medicinal herb *Andrographis paniculata*, and it has been reported to have a wide range of biological activities including anti-inflammatory [12], anti-allergic [13], anti-oxidation [14], and anti-cancer effects [15,16]. In addition, it has been demonstrated to have anti-metastatic activity *in vitro* and *in vivo* on various tumors via inhibition of MMP-2 or MMP-9 activity [17–20]. Moreover, andrographolide has been shown to have potent radiosensitizing activity through increased autophagy [21] and apoptosis [22], as well as through an anti-invasion effect [23]. As a result, andrographolide sensitizes tumor cells to the cytotoxic effects of radiation.

Our previous studies have shown that andrographolide produces a radiation-induced cytotoxic effect in Ras-transformed cell *in vitro* and *in vivo* via attenuating NF- κ B activity [24]. We also found that andrographolide combined with lower dose-rate radiation synergistically enhances the anti-metastatic effects of Ras-transformed cells in a xenograft mouse model [25]. Although andrographolide may be a promising strategy for enhancing the anti-metastatic effect of radiation, the molecular mechanism in Ras-transformed cells remains to be

elucidated. In the present study, we extended our previous study to explore the anti-metastatic effects of andrographolide with RT, and the potential molecular mechanisms involved.

Materials and methods

Reagents and chemicals

Andrographolide (Merck Millipore, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (DMSO) as a concentrated stock solution, and stored at -20°C until further use.

Cell lines and cell culture

Ras-transformed cells were obtained from the laboratory of Shu-Ling Fu. The cell line derived from rat kidney (RK3E/tv-a) was infected with a retrovirus carrying the oncogene Ras as described in a previous study [26]. The cells were regularly maintained in DMEM containing 10% FBS, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 3 $\mu\text{g}/\text{ml}$ puromycin in 10 cm dishes at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

In vivo tumorigenesis assays

The Ras/Luc cell line, which is a Ras-transformed cell line constitutively expressing the luciferase gene, was used in the present study. Briefly, 10^6 Ras/Luc cells in 100 μl phosphate-buffered saline were injected into the back or tail vein of nude mice. Male 6–8 week-old athymic nude mice (BALB/cAnN.Cg-Foxn1nu/CrlNarl) were obtained from the National Laboratory Animal Center, Taiwan. Mice were maintained under 12h light/dark cycle at room temperature ($24\pm 1^{\circ}\text{C}$) and $60\pm 5\%$ humidity. Mice were divided into four groups according to the treatment administered: group 1 ($n = 12$), andrographolide (10 μM); group 2 ($n = 12$), radiation (2 Gy) and vehicle (100 μl DMSO); group 3 ($n = 12$), andrographolide (10 μM) plus radiation (2 Gy); and group 4 ($n = 12$), vehicle (100 μl DMSO). Andrographolide dosage was selected based on a previous *in vivo* study [24]. Oral administration of andrographolide was given 3 hours prior to radiation. An electron linear accelerator (Varian Medical Systems, Palo Alto, CA, USA) was used to apply radiation. At the end of the experiments, all remaining mice were sacrificed by cervical dislocation. All animal protocols were performed according to the instructions issued by the Institutional Animal Care and Use Committee of National Yang-Ming Cheng University (IACUC no.1010611).

Wound healing assay

A monolayer of cells was grown to confluence in 10 cm plates, and at experimental time zero a scratch was made in each well using a pipette tip. The cells were washed twice with PBS before their subsequent incubation with culture medium in the absence (control) or presence of 10 μM andrographolide. Photos were taken of the scratches at 0 h and 12 h with a digital camera.

Western blotting

After treatment, cells were harvested and lysed, and protein concentrations were measured by using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA). All samples were separated on SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). After blocking the membranes with 5% (w/v) non-fat dry milk in TBS containing 0.1% Tween 20 (TBS-T) for 1 hour at room temperature, they were immunoblotted with the following monoclonal primary antibodies: vimentin, E-cadherin, MMP-2, MMP-9, phospho-ERK1/2, total-ERK1/2, NF- κB subunit p65, β -actin, and GAPDH (all from Cell Signaling

Technology, Beverly, MA, USA). Appropriate horseradish peroxidase-conjugated secondary antibodies, including mouse IgG and rabbit IgG antibodies (Abcam, Cambridge, MA, USA), were applied and incubated for 1 h at room temperature. Specific signals were visualized using a chemiluminescence (ECL) detection kit (Millipore).

Cell invasion assay

The invasion assay was performed using Millicell Transwell Cell Culture Inserts (8 μm pores, Millipore, Billerica, MA, USA). The cells (2×10^4 cells/insert) were placed in the upper well of the chamber, and serum-free conditioned medium was placed in the lower well as a chemoattractant. The filter was a polyvinylpyrrolidone-free polycarbonate membrane with 8.0 μm pores. The bottom wells of the system were filled with complete medium, and incubated for 48 h at 37°C. The cells from inserts were fixed with 4% paraformaldehyde (PFA) and stained with 0.1% crystal violet. Invasion was quantified by reading the optical density (OD) at 590nm after crystal violet staining.

Gelatin zymography

Gelatin zymography was carried out by using 10% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were washed twice with 100 mL distilled water containing 2.5% Triton X-100 for 30 min at room temperature to remove the SDS. The gel was then incubated in 100 mL reaction buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5mM CaCl₂) overnight at 37°C. Gels were stained with 2.5% Coomassie Brilliant Blue, and enzymatic activity was detected by observing the lack of gelatin protein in sample lanes, represented by a clear band.

Statistical analysis

All data were presented as the mean \pm standard deviation and were compared by using Student's t-test or ANOVA test. *P* values of < 0.05 were considered as a statistical significance.

Results

Andrographolide decreased the Ras-transformed cell migration activity

We firstly performed a wound healing assay to assess the effects of andrographolide on cell migration of Ras-transformed cells. The untreated cells displayed significant wound closure and tended to protrude into the wound site. However, the cells treated with andrographolide exhibited decreased migration ability when compared to the control cells (Fig 1A and 1B). These results imply that andrographolide inhibited the migration of Ras-transformed cells.

Andrographolide with radiation inhibited Ras-transformed cell metastasis *in vivo*

To further determine whether andrographolide could reduce metastasis *in vivo*, xenograft tumor models were used in BALB/c nude mice. No significant alterations in body weight were detected following andrographolide and/or radiation treatment. Mice lung metastatic rates are presented in Table 1. The mice with tumors/mice injected ratio of vehicle, andrographolide (10 μM), radiation (2 Gy), and andrographolide (10 μM) plus radiation (2 Gy) was 75.0%, 58.3%, 50.0%, and 33.3%, respectively (Table 1). The results indicated that andrographolide combined with radiation demonstrated an inhibitive effect on cancer metastasis.

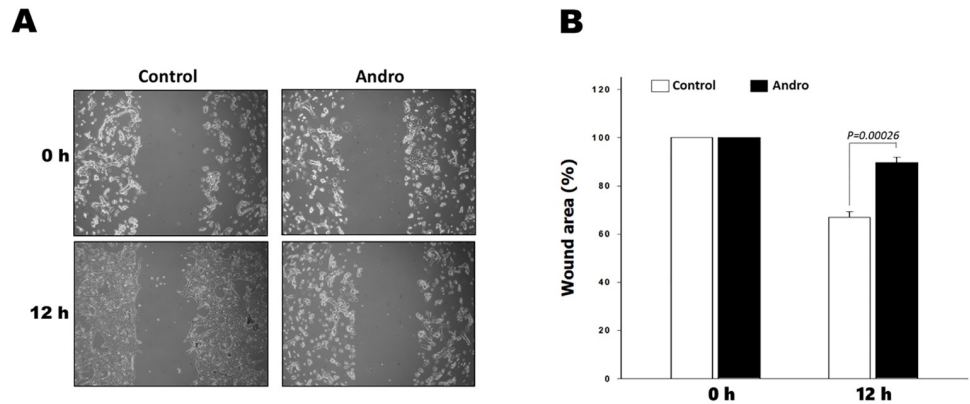


Fig 1. Andrographolide inhibits migration of RAS-transformed cells. Wound healing assay was evaluated after incubation with or without 10 μ M andrographolide (Andro). Migration distance was measured at 0 and 12 hours after cells were scratched. (A) Wound healing assay under microscope. (B) The graphical data represented the percentage of migration area. Data are presented as the means \pm SD of three experiments. *P* values indicate the statistical significance between control and andrographolide -treated cells for 12 hours.

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Andrographolide combined with radiation was associated with reducing MMP-2 activation

Epithelial-mesenchymal transition (EMT) is a developmental process, in which epithelial cells take on the characteristics of invasive mesenchymal cells and importantly determine the aggressiveness of cancer cells [27,28]. MMPs-dependent activation of the EMT program has been observed in a variety of cell types that involved in tumor development, cell proliferation, and metastatic dissemination [29,30]. Thus, we evaluated the effect of andrographolide with and without radiation on MMP-2, MMP-9, and EMT-related markers (i.e., E-cadherin, and vimentin protein) in Ras-transformed cells. Following treatment with andrographolide, the MMP-2 level was decreased significantly. Combination treatment of andrographolide and radiation more efficiently inhibited the expression of MMP-2 when compared with andrographolide alone. However, MMP-9, E-cadherin and vimentin protein levels were not altered by andrographolide alone or in combination with radiation (Fig 2A and 2B). To determine whether andrographolide regulates the expression of MMPs, we then measured MMP-2 and MMP-9 levels in the supernatant of *in vitro* cultured tumor cells by using gelatin zymography analysis. Andrographolide with radiation showed a significant reduction of MMP-2 enzyme activity, while MMP-9 activity was not significantly affected by the combined treatment (Fig 3). These data indicate that combined andrographolide and radiation synergistically inhibited MMP-2 activity.

Table 1. Lung metastatic rate in mice.

Group	Tumorigenesis in lung area	
	Mice with tumors/mice injected	Ratio (%)
D0	9/12	75.0
A0	7/12	58.3
D2	6/12	50.0
A2	4/12	33.3

A2, andrographolide (10 μ M) plus radiation (2 Gy); A0, andrographolide (10 μ M); D2, radiation (2 Gy); D0, DMSO (100 μ l; control)

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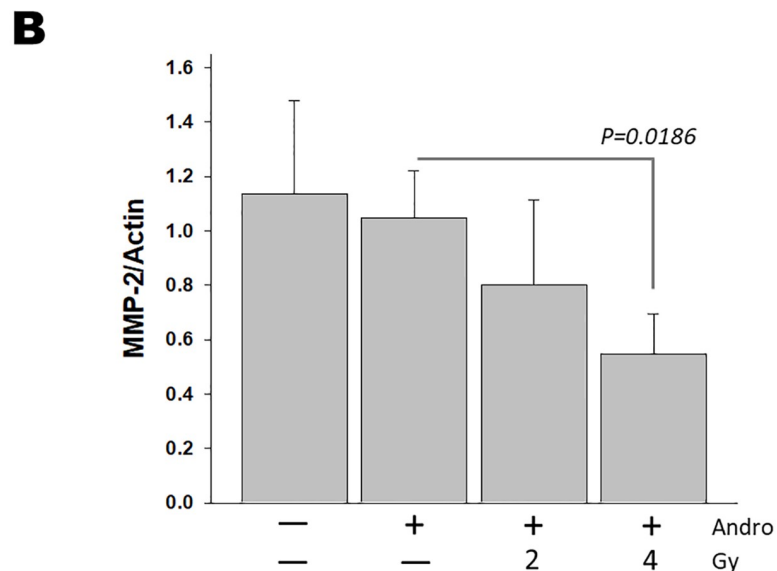
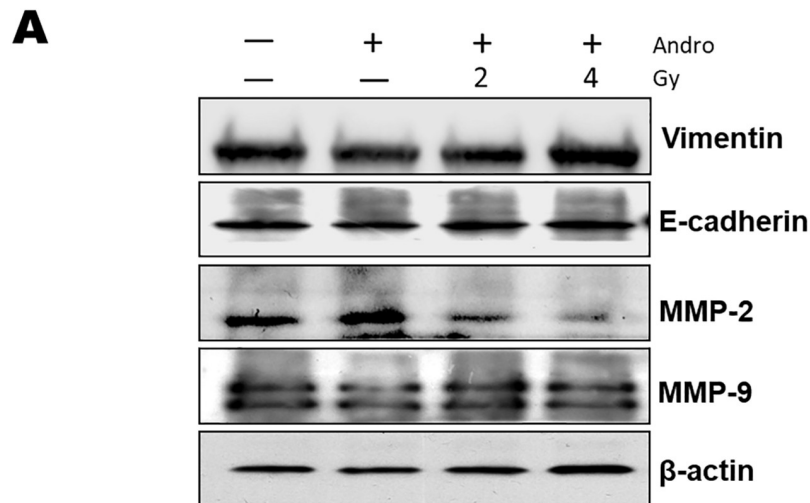


Fig 2. Effects of andrographolide and radiation on the expression EMT-related markers (E-cadherin, vimentin) and matrix metalloproteinases MMP-2 and -9. (A) RAS-transformed cells were treated with 10 uM andrographolide for 6 hours with and without 2 or 4 Gy radiation. After 24 hours, the cells were harvested for preparation of whole-cell protein lysates followed by Western blotting to detect the given proteins. (B) Quantitative analyses of MMP-2 expression evaluated by MMP-2/GAPDH ratio. The results are shown as mean \pm SD (n = 3). * $p < 0.05$ for andrographolide combined with 4 Gy vs. andrographolide alone.

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Andrographolide with radiation significantly inhibited MMP-2 activity through the suppression of ERK1/2 activation

MMP-2 is a critical factor that regulates cancer metastasis, and appears to affect cell migration and invasion by affecting ERK1/2 and NF- κ B signaling [31,32]. Our previous study found that

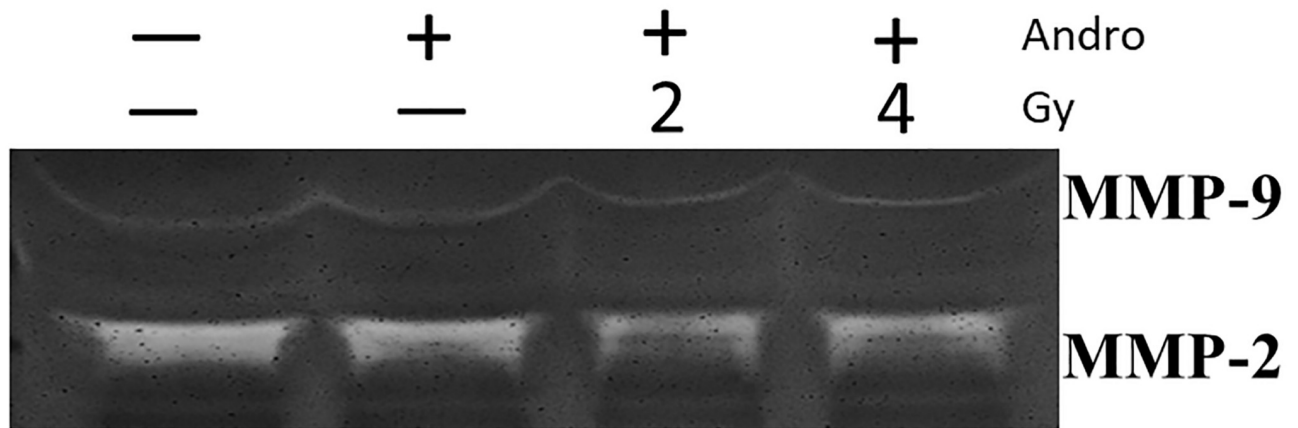


Fig 3. Andrographolide and radiation reduces MMP-2 expression. Gelatin zymography was performed using the conditioned media that were harvested after 48 hours in the presence or absence of 10 uM andrographolide, and then treated with/without radiation (2 or 4Gy) for 24 hours. The samples were applied without reduction to a 10% polyacrylamide gel containing gelatin, and proteolytic activity was demonstrated by digestion of the gelatin and clearing of the gel.

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radiation induced NF-κB activity in Ras-transformed cells, and andrographolide co-treatment reduced radiation-induced NF-κB activation [24]. Andrographolide has also been shown to have anti-invasive activity against colon cancer cells via inhibition of MMP-2 [20]. In addition, we examined whether the anti-metastatic effect of andrographolide combined with radiation is related to attenuation of the ERK1/2 or NF-κB signaling pathway. Expression of phospho-ERK 1/2 and NF-κB p65 were slightly increased in irradiated cells when compared with control cells, although MMP-2 was unchanged after radiation treatment. MMP-2 activity was inhibited in the presence of andrographolide, which was accompanied by reduction of phospho-ERK 1/2 and NF-κB p65 (Fig 4). Moreover, andrographolide combining with radiation more effectively suppressed MMP-2 and phospho-ERK 1/2 activity, but only slightly decreased activation of NF-κB p65 when compared with andrographolide treatment alone (Fig 4). These results demonstrate that the enhanced anti-metastatic effect of andrographolide in combination with radiation comes from inhibition of MMP-2 through modulation of ERK1/2 activity.

Andrographolide with radiation inhibited cell invasion and this effect correlated with the down-regulation of MMP-2

MMP-2 has been demonstrated to play an important role in mediating tumor invasion in many types of cancer cells [33]. Angiotensin II (Ang II) is a multifunctional octapeptide with diverse effects, including activation of MMPs while cell migration and invasion [34,35]. To further examine the effects of andrographolide on MMP-2 production, we investigated the effect of Ang II on MMP-2 expression in RAS-transformed cells. Being consistent with the Western blotting data, ionizing radiation increased the level of MMP-2 activity when compared with untreated cells, whereas the addition of andrographolide decreased MMP-2 expression. While MMP-2 activity was increased in the presence of Ang II, pretreatment with andrographolide caused an obvious decrease in Ang II-induced MMP-2 activity. Co-treatment with andrographolide and radiation more effectively enhanced the attenuating effects of Ang II-induced MMP-2 expression (Fig 5A). MMP-2 was reported to be closely associated with invasion and metastasis in several cancers. Consequently, we investigated the anti-invasive activities of Ras-transformed cancer cells upon exposure to either radiation or andrographolide in the presence of Ang II using Transwell assays. Compared with the control group, Ang II markedly facilitated

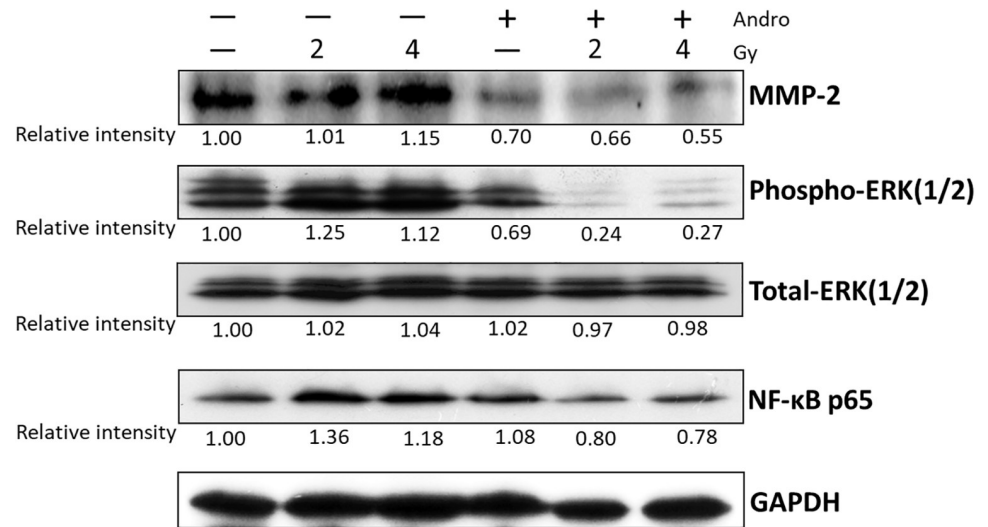


Fig 4. Andrographolide with radiation enhanced downregulation of radiation-induced MMP-2 leading to the suppression of ERK signaling. The cells were treated with 10 uM andrographolide with/without 2Gy or 4 Gy radiation for 24 hours. The figure displays typical data of MMP-2, phosphor-ERK1/2, total-ERK1/2 and NF-κB activity by Western blotting. Relative band intensities were analyzed by the Image J software. β-actin was used as an internal control for normalization. The numbers beneath the blots indicate the relative expression of each band when compared to the respective untreated control.

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invasion in Ras-transformed cells, whereas radiation treatment alone did not inhibit the Ang II-mediated response. In contrast, in the presence of andrographolide effectively inhibited Ang II-induced invasion. Notably, we observed that andrographolide combined with radiation more significantly suppressed cancer cell invasion when compared with treatment of radiation alone (Fig 5B). These results indicate that andrographolide effectively inhibits Ras-transformed cells invasion by suppressing MMP-2 activity, which might lead to the inhibition of metastasis.

Andrographolide-mediated inhibition of MMP-2 activity via suppression of ERK signaling led to significant decrease cell migration and invasion of irradiated cells

Previous studies demonstrated that ERK1/2 is an important upstream factor in MMP-2 activation [35]. To confirm the mechanism andrographolide and MMP-2 regulation of cell migration and invasion, we next assessed the expression of phospho-ERK 1/2 of Ras-transformed cells after administration of andrographolide and/or radiation in the presence or absence of Ang II. As the above results, andrographolide could significantly attenuate MMP-2 up-regulation when compared with radiation alone. In addition, this effect was accompanied by significantly reducing phospho-ERK-1/2 activity. Andrographolide in combination with radiation resulted in a significant decrease in phospho-ERK-1/2 activity and MMP-2 level (Fig 6). Taken together, these results suggest that andrographolide combined with radiation greatly reduce MMP-2 expression by decreasing the activation of ERK1/2. Accordingly, andrographolide was able to down-regulate MMP-2 levels through inhibition of ERK1/2, leading to enhance the anti-growth and anti-metastatic effects of radiation in Ras-transformed cells.

Discussion

RT is one of important treatments for cancer patients. Local recurrence and distance metastases, however, remain major obstacles to achieve a cure in patients with many solid tumors

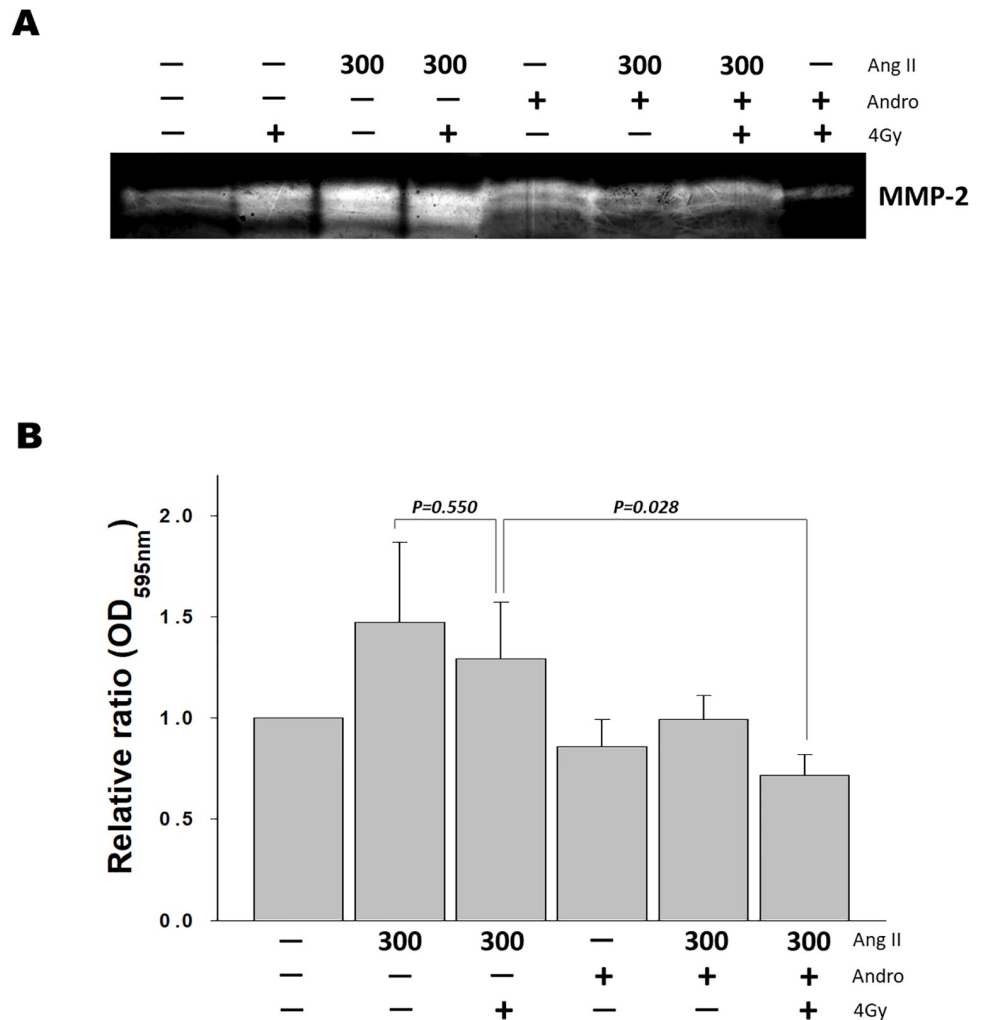


Fig 5. Andrographolide plus radiation reduced angiotensin II-induced MMP-2 activation and invasion. The Ras-transformed cells were incubated with/without 300 nM angiotensin II (Ang II) for 1 hour, and then treated with andrographolide, or radiation, or both for 24 hours. (A) The typical data show the gelatin zymography analysis band of the MMP-2 in conditioned media. (B) Invasion assays were performed using Transwell inserts of 8-micron pore size membrane and matrigel. After treatment, the cells were seeded in Transwell plates for 72 hours. The invaded cells were stained and quantified at an optical density of 560 nm. The experiments have been repeated three times; representative results of three independent experiments were shown. Quantification of cell invasion expressed as the percentage of control; one-way ANOVA was used for statistical analysis.

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treated with RT. Therefore, investigating strategies to improve the sensitivity of cells to the cytotoxic effects of RT is required.

The RAS gene is known to be overstimulated in malignancies, and it is associated with an increased propensity for metastasis. Transfected Ras oncogenes have been shown to induce metastatic properties in some cells [36]. Additionally, overexpression of the Ras oncogene has been implicated in the development of radioresistance of various tumors [37,38], which can increase the risk of local recurrence after radiotherapy. Accordingly, in this study we used a Ras-transformed cell model to characterize the metastatic process and to explore the possible mechanism for the synergistic anti-metastatic effect of andrographolide in combination with RT.

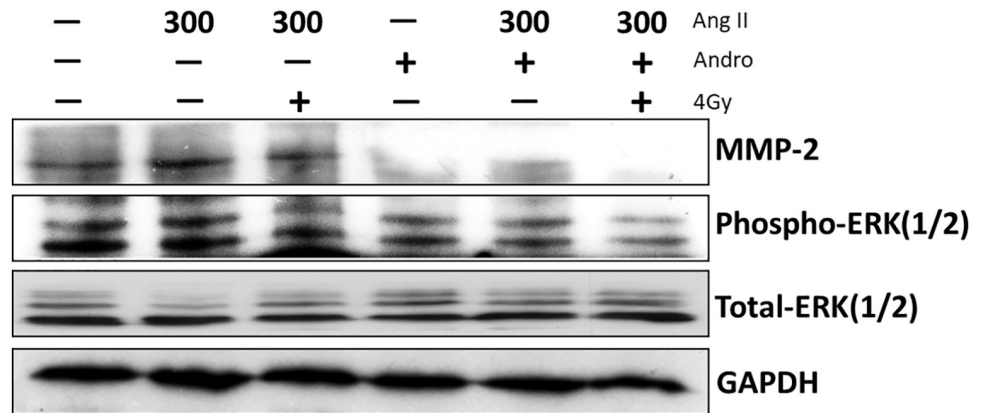


Fig 6. Andrographolide with radiation suppressed angiotensin II-induced MMP-2 expression through inhibition of ERK1/2 signaling. Cells were incubated with 300 nM Ang II for 1 hour and then treated with andrographolide, irradiation, or both for 24 hours. Protein level was determined by Western blotting.

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Andrographolide and its derivatives have been shown to have anti-cancer activities in both *in vitro* and *in vivo* experimental models of cancer [39–41]. Recent studies demonstrated that andrographolide exerts inhibitory effects on malignant progression by repression of migration, matrix degradation, and invasion [42,43]. On the other hand, andrographolide also has been validated as a candidate for a radiosensitizing approach [24]. Studies have shown that andrographolide combined with RT have a synergistic anti-tumor effect in Ras-activated cancer cells [24,25]. We have further found that andrographolide enhanced the effectiveness of RT against Ras-dependent tumorigenesis. However, little is known about the mechanisms by which andrographolide enhances the sensitivity of cells to radiation. We found that andrographolide suppressed the migration and invasion of Ras-transformed cells. As a result, andrographolide may prevent cancer progression, such as metastasis. These results confirm our previous observations that andrographolide exerted marked inhibition of the metastasis of Ras-transformed cell *in vivo*.

MMP-2 and MMP-9 are major proteolytic enzymes contributed to ECM degradation to promote tumor cell migration and invasion [44–46]. Previous reports showed that radiation enhanced MMP-2 and MMP-9 secretion and activity, which enhanced tumor aggressiveness and promoted tumor metastasis [45,47]. We observed that ionizing radiation induced an increase in MMP-2 production but did not affect activation of MMP-9. MMP-2 is involved in tumor-induced and radiation-enhanced migration and invasion, and pre-treatment with andrographolide significantly decreased MMP-2 expression in irradiated cells, which reduced migration and invasion. On the other hand, EMT has been invoked as a critical component of the metastatic process, which endowed tumor cells with a more aggressive behavior [28,30,48]. In this study, we examined the levels of E-cadherin and vimentin (a mesenchymal marker) after treatment with either andrographolide alone or in combination with radiation. The results showed that E-cadherin and vimentin levels were not significantly different between the treatment and control groups.

Multiple signaling pathways, including ERK1/2 and NF-κB, have been associated with the pharmacological activity of andrographolide [13,49]. In addition, elevated basal NF-κB and ERK1/2 activity in certain cancers have been linked with tumor resistance to radiation, suggesting that radiation-induced activation of these kinase cascades may enhance the survival of irradiated cells [50,51]. Our prior study and other published reports have indicated that andrographolide significantly attenuates NF-κB activity, thus increasing radiation-induced

cytotoxicity to tumor cells. In this study, we observed that radiation treatment alone induced NF- κ B activity in Ras-transformed cells, and it might contribute to aggressive tumor growth. However, in the presence of andrographolide, radiation-induced NF- κ B-dependent protein expression is inhibited.

ERK1/2 is a key molecule involved in the process of MAPKs pathway, which plays a crucial role in the progression of tumor cell proliferation and enhances cancer survival after irradiation. Additionally, ERK1/2 has been shown to play a critical role in the regulation of cancer cell invasiveness by inducing MMPs-mediated extracellular matrix degradation [52,53]. Several lines of evidence also revealed that inhibition of ERK1/2 is associated with MMP-2 downregulation to suppress cancer progression [20,54]. Furthermore, several studies showed that andrographolide reduces cancer invasion and metastatic abilities by inhibiting the activity of matrix metalloproteinases (MMPs) [20,42]. It showed that andrographolide suppresses the invasion ability of colon cancer cells via inhibition of MMP-2 activity and attenuation of the ERK signaling pathway [20]. Being consistent with these findings, our results indicated that andrographolide decreased MMP-2 activity, which was accompanied by a decrease of ERK1/2 phosphorylation, in Ras-transformed cells.

Further investigation showed that ERK1/2 is essential for MMPs expression mediated through activation of transcriptional factor NF- κ B [55]. In this study, andrographolide treatment resulted in significant inhibition of MMP-2 expression, which was associated with slightly decreased NF- κ B activation. More significantly, we observed that andrographolide inhibited radiation-induced MMP-2 expression, and was accompanied by the suppression of ERK1/2 activation. Combined treatment with andrographolide and radiation synergistically inhibited MMP-2 expression through the suppression of ERK1/2 signaling pathway in Ras-transformed cells could play an important role against the repression of metastasis. It has been demonstrated that Ang II induces expression of MMP-2 gene via ERK1/2 signaling pathway in different cell types [35]. In order to confirm our results, we further examined the effect of andrographolide on Ang II-induced MMP-2 in Ras-transformed cells. Andrographolide significantly attenuated Ang II-induced MMP-2 and related to diminished ERK1/2 activity in irradiated cells. Targeting and inhibiting both tumorigenesis and metastasis are the main strategies to increase the effectiveness of cancer treatments. Although targeted drugs are still developed in conjunction with RT to improve therapeutic efficacy in cancer patients, these agents are less effective for the prevention of metastasis. We have previously shown that andrographolide was able to enhance tumor regression in conjunction with ionizing radiation in the Ras-transformed model *in vitro* and *in vivo* [24]. Moreover, andrographolide combined with RT has been shown to have a synergistic effect that significantly inhibits cancer metastasis *in vivo*. Our findings indicate that andrographolide with radiation attenuates oncogenic RAS-expressing cell migration and invasion, which is mediated downregulation of activated MMP2 with the suppression of ERK1/2 signaling. The results suggest that andrographolide may be an effective strategy for overcoming radiation-induced cancer progression and metastasis.

Author Contributions

Data curation: Chih-Chia Yu, Chien-An Chen, Hon-Yi Lin, Moon-Sing Lee, Shih-Kai Hung.

Formal analysis: Chih-Chia Yu, Moon-Sing Lee, Wen-Yen Chiou, Yu-Chieh Su.

Investigation: Shu-Ling Fu, Hon-Yi Lin, Wen-Yen Chiou, Yu-Chieh Su.

Methodology: Shu-Ling Fu.

Validation: Shih-Kai Hung.

Writing – original draft: Chih-Chia Yu.

Writing – review & editing: Chien-An Chen, Shih-Kai Hung.

References

1. Gialeli C, Theocharis AD, Karamanos NK (2011) Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. *FEBS J* 278: 16–27. <https://doi.org/10.1111/j.1742-4658.2010.07919.x> PMID: 21087457
2. Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S (1980) Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* 284: 67–68. PMID: 6243750
3. Kumar A, Collins HM, Scholefield JH, Watson SA (2000) Increased type-IV collagenase (MMP-2 and MMP-9) activity following preoperative radiotherapy in rectal cancer. *Br J Cancer* 82: 960–965. <https://doi.org/10.1054/bjoc.1999.1025> PMID: 10732772
4. Heo W, Lee YS, Son CH, Yang K, Park YS, Bae J (2015) Radiation-induced matrix metalloproteinases limit natural killer cell-mediated anticancer immunity in NCI-H23 lung cancer cells. *Mol Med Rep* 11: 1800–1806. <https://doi.org/10.3892/mmr.2014.2918> PMID: 25385045
5. Liu WW, Zeng ZY, Wu QL, Hou JH, Chen YY (2005) Overexpression of MMP-2 in laryngeal squamous cell carcinoma: a potential indicator for poor prognosis. *Otolaryngol Head Neck Surg* 132: 395–400. <https://doi.org/10.1016/j.otohns.2004.09.050> PMID: 15746850
6. Cheng JC, Chou CH, Kuo ML, Hsieh CY (2006) Radiation-enhanced hepatocellular carcinoma cell invasion with MMP-9 expression through PI3K/Akt/NF-kappaB signal transduction pathway. *Oncogene* 25: 7009–7018. <https://doi.org/10.1038/sj.onc.1209706> PMID: 16732316
7. Lin KL, Tsai PC, Hsieh CY, Chang LS, Lin SR (2011) Antimetastatic effect and mechanism of ovatodiolide in MDA-MB-231 human breast cancer cells. *Chem Biol Interact* 194: 148–158. <https://doi.org/10.1016/j.cbi.2011.10.002> PMID: 22033475
8. Hsieh CY, Tsai PC, Chu CL, Chang FR, Chang LS, Wu YC et al. (2013) Brazilein suppresses migration and invasion of MDA-MB-231 breast cancer cells. *Chem Biol Interact* 204: 105–115. <https://doi.org/10.1016/j.cbi.2013.05.005> PMID: 23707804
9. Wei LH, Lai KP, Chen CA, Cheng CH, Huang YJ, Chou CH et al. (2005) Arsenic trioxide prevents radiation-enhanced tumor invasiveness and inhibits matrix metalloproteinase-9 through downregulation of nuclear factor kappaB. *Oncogene* 24: 390–398. <https://doi.org/10.1038/sj.onc.1208192> PMID: 15531921
10. Yu AE, Hewitt RE, Kleiner DE, Stetler-Stevenson WG (1996) Molecular regulation of cellular invasion—role of gelatinase A and TIMP-2. *Biochem Cell Biol* 74: 823–831. PMID: 9164651
11. Badiga AV, Chetty C, Kesanakurti D, Are D, Gujrati M, Klopfenstein JD et al. (2011) MMP-2 siRNA inhibits radiation-enhanced invasiveness in glioma cells. *PLoS One* 6: e20614. <https://doi.org/10.1371/journal.pone.0020614> PMID: 21698233
12. Shen YC, Chen CF, Chiou WF (2002) Andrographolide prevents oxygen radical production by human neutrophils: possible mechanism(s) involved in its anti-inflammatory effect. *Br J Pharmacol* 135: 399–406. <https://doi.org/10.1038/sj.bjp.0704493> PMID: 11815375
13. Xia YF, Ye BQ, Li YD, Wang JG, He XJ, Lin X et al. (2004) Andrographolide attenuates inflammation by inhibition of NF-kappa B activation through covalent modification of reduced cysteine 62 of p50. *J Immunol* 173: 4207–4217. PMID: 15356172
14. Batkhuu J, Hattori K, Takano F, Fushiya S, Oshiman K, Fujimiya Y (2002) Suppression of NO production in activated macrophages in vitro and ex vivo by neoandrographolide isolated from *Andrographis paniculata*. *Biol Pharm Bull* 25: 1169–1174. PMID: 12230111
15. Dai L, Wang G, Pan W (2017) Andrographolide Inhibits Proliferation and Metastasis of SGC7901 Gastric Cancer Cells. *Biomed Res Int* 2017: 6242103. <https://doi.org/10.1155/2017/6242103> PMID: 28194420
16. Banerjee M, Chattopadhyay S, Choudhuri T, Bera R, Kumar S, Chakraborty B et al. (2016) Cytotoxicity and cell cycle arrest induced by andrographolide lead to programmed cell death of MDA-MB-231 breast cancer cell line. *J Biomed Sci* 23: 40. <https://doi.org/10.1186/s12929-016-0257-0> PMID: 27084510
17. Zhai Z, Qu X, Li H, Ouyang Z, Yan W, Liu G et al. (2015) Inhibition of MDA-MB-231 breast cancer cell migration and invasion activity by andrographolide via suppression of nuclear factor-kappaB-dependent matrix metalloproteinase-9 expression. *Mol Med Rep* 11: 1139–1145. <https://doi.org/10.3892/mmr.2014.2872> PMID: 25374279

18. Luo W, Liu Y, Zhang J, Luo X, Lin C, Guo J (2013) Andrographolide inhibits the activation of NF-kappaB and MMP-9 activity in H3255 lung cancer cells. *Exp Ther Med* 6: 743–746. <https://doi.org/10.3892/etm.2013.1196> PMID: 24137258
19. Pratheeshkumar P, Kuttan G (2011) Andrographolide inhibits human umbilical vein endothelial cell invasion and migration by regulating MMP-2 and MMP-9 during angiogenesis. *J Environ Pathol Toxicol Oncol* 30: 33–41. PMID: 21609314
20. Chao HP, Kuo CD, Chiu JH, Fu SL (2010) Andrographolide exhibits anti-invasive activity against colon cancer cells via inhibition of MMP2 activity. *Planta Med* 76: 1827–1833. <https://doi.org/10.1055/s-0030-1250039> PMID: 20539971
21. Zhang C, Qiu X (2015) Andrographolide radiosensitizes human ovarian cancer SKOV3 xenografts due to an enhanced apoptosis and autophagy. *Tumour Biol* 36: 8359–8365. <https://doi.org/10.1007/s13277-015-3578-9> PMID: 26014516
22. Wang ZM, Kang YH, Yang X, Wang JF, Zhang Q, Yang BX et al. (2016) Andrographolide radiosensitizes human esophageal cancer cell line ECA109 to radiation in vitro. *Dis Esophagus* 29: 54–61. <https://doi.org/10.1111/dote.12255> PMID: 25059546
23. Nantajit D, Jetawattana S, Suriyo T, Grdina DJ, Satayavivad J (2017) Andrographis paniculata Diterpenoids Protect against Radiation-Induced Transformation in BALB/3T3 Cells. *Radiat Res* 188: 66–74. <https://doi.org/10.1667/RR14698.1> PMID: 28492344
24. Hung SK, Hung LC, Kuo CD, Lee KY, Lee MS, Lin HY et al. (2010) Andrographolide sensitizes Ras-transformed cells to radiation in vitro and in vivo. *Int J Radiat Oncol Biol Phys* 77: 1232–1239. <https://doi.org/10.1016/j.ijrobp.2010.01.007> PMID: 20610044
25. Fu SL, Chen CA, Hung LC, Lee MS, Chiou WY, Lin HY et al. (2016) Preliminary results of a non-invasive method to measure tumor size and distribution in vivo. *Exp Ther Med* 12: 3614–3620. <https://doi.org/10.3892/etm.2016.3819> PMID: 28105095
26. Fu SL, Huang YJ, Liang FP, Huang YF, Chuang CF, Wang SW et al. (2005) Malignant transformation of an epithelial cell by v-Src via tv-a-mediated retroviral infection: a new cell model for studying carcinogenesis. *Biochem Biophys Res Commun* 338: 830–838. <https://doi.org/10.1016/j.bbrc.2005.10.045> PMID: 16256070
27. Ponnusamy MP, Seshacharyulu P, Lakshmanan I, Vaz AP, Chugh S, Batra SK (2013) Emerging role of mucins in epithelial to mesenchymal transition. *Curr Cancer Drug Targets* 13: 945–956. PMID: 24168188
28. He B, Gao SQ, Huang LD, Huang YH, Zhang QY, Zhou MT et al. (2015) MicroRNA-155 promotes the proliferation and invasion abilities of colon cancer cells by targeting quaking. *Mol Med Rep* 11: 2355–2359. <https://doi.org/10.3892/mmr.2014.2994> PMID: 25420938
29. Radisky ES, Radisky DC (2010) Matrix metalloproteinase-induced epithelial-mesenchymal transition in breast cancer. *J Mammary Gland Biol Neoplasia* 15: 201–212. <https://doi.org/10.1007/s10911-010-9177-x> PMID: 20440544
30. Wang W, Zhang X, Qin JJ, Voruganti S, Nag SA, Wang MH et al. (2012) Natural product ginsenoside 25-OCH3-PPD inhibits breast cancer growth and metastasis through down-regulating MDM2. *PLoS One* 7: e41586. <https://doi.org/10.1371/journal.pone.0041586> PMID: 22911819
31. Lin ML, Lu YC, Chung JG, Wang SG, Lin HT, Kang SE et al. (2010) Down-regulation of MMP-2 through the p38 MAPK-NF-kappaB-dependent pathway by aloe-emodin leads to inhibition of nasopharyngeal carcinoma cell invasion. *Mol Carcinog* 49: 783–797. <https://doi.org/10.1002/mc.20652> PMID: 20607722
32. Poudel B, Lee YM, Kim DK (2015) DDR2 inhibition reduces migration and invasion of murine metastatic melanoma cells by suppressing MMP2/9 expression through ERK/NF-kappaB pathway. *Acta Biochim Biophys Sin (Shanghai)* 47: 292–298.
33. Furukawa K, Kumon Y, Harada H, Kohno S, Nagato S, Teraoka M et al. (2006) PTEN gene transfer suppresses the invasive potential of human malignant gliomas by regulating cell invasion-related molecules. *Int J Oncol* 29: 73–81. PMID: 16773187
34. Touyz RM (2005) Intracellular mechanisms involved in vascular remodelling of resistance arteries in hypertension: role of angiotensin II. *Exp Physiol* 90: 449–455. <https://doi.org/10.1113/expphysiol.2005.030080> PMID: 15890798
35. Wang C, Qian X, Sun X, Chang Q (2015) Angiotensin II increases matrix metalloproteinase 2 expression in human aortic smooth muscle cells via AT1R and ERK1/2. *Exp Biol Med (Maywood)* 240: 1564–1571.
36. Chambers AF, Tuck AB (1993) Ras-responsive genes and tumor metastasis. *Crit Rev Oncog* 4: 95–114. PMID: 8420573

37. Gupta AK, Bakanauskas VJ, Cerniglia GJ, Cheng Y, Bernhard EJ, Muschel RJ et al. (2001) The Ras radiation resistance pathway. *Cancer Res* 61: 4278–4282. PMID: [11358856](#)
38. Samid D, Miller AC, Rimoldi D, Gafner J, Clark EP (1991) Increased radiation resistance in transformed and nontransformed cells with elevated ras proto-oncogene expression. *Radiat Res* 126: 244–250. PMID: [2023995](#)
39. Reabroi S, Saeeng R, Boonmuen N, Kasemsuk T, Saengsawang W, Suksen K et al. (2018) The anti-cancer activity of an andrographolide analogue functions through a GSK-3beta-independent Wnt/beta-catenin signaling pathway in colorectal cancer cells. *Sci Rep* 8: 7924. <https://doi.org/10.1038/s41598-018-26278-8> PMID: [29784906](#)
40. Jada SR, Subur GS, Matthews C, Hamzah AS, Lajis NH, Saad MS et al. (2007) Semisynthesis and in vitro anticancer activities of andrographolide analogues. *Phytochemistry* 68: 904–912. <https://doi.org/10.1016/j.phytochem.2006.11.031> PMID: [17234223](#)
41. Satyanarayana C, Deevi DS, Rajagopalan R, Srinivas N, Rajagopal S (2004) DRF 3188 a novel semi-synthetic analog of andrographolide: cellular response to MCF 7 breast cancer cells. *BMC Cancer* 4: 26. <https://doi.org/10.1186/1471-2407-4-26> PMID: [15207007](#)
42. Shi MD, Lin HH, Chiang TA, Tsai LY, Tsai SM, Lee YC et al. (2009) Andrographolide could inhibit human colorectal carcinoma Lovo cells migration and invasion via down-regulation of MMP-7 expression. *Chem Biol Interact* 180: 344–352. <https://doi.org/10.1016/j.cbi.2009.04.011> PMID: [19426720](#)
43. Yang W, Zhao J, Wang Y, Xu H, Wu Z, Hu Y et al. (2017) In vivo inhibitory activity of andrographolide derivative ADN-9 against liver cancer and its mechanisms involved in inhibition of tumor angiogenesis. *Toxicol Appl Pharmacol* 327: 1–12. <https://doi.org/10.1016/j.taap.2017.04.022> PMID: [28438631](#)
44. Kessenbrock K, Plaks V, Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 141: 52–67. <https://doi.org/10.1016/j.cell.2010.03.015> PMID: [20371345](#)
45. Fujita M, Yamada S, Imai T (2015) Irradiation induces diverse changes in invasive potential in cancer cell lines. *Semin Cancer Biol* 35: 45–52. <https://doi.org/10.1016/j.semcancer.2015.09.003> PMID: [26410035](#)
46. Bauvois B (2012) New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: outside-in signaling and relationship to tumor progression. *Biochim Biophys Acta* 1825: 29–36. <https://doi.org/10.1016/j.bbcan.2011.10.001> PMID: [22020293](#)
47. Moncharmont C, Levy A, Guy JB, Falk AT, Guilbert M, Trone JC et al. (2014) Radiation-enhanced cell migration/invasion process: a review. *Crit Rev Oncol Hematol* 92: 133–142. <https://doi.org/10.1016/j.critrevonc.2014.05.006> PMID: [24908570](#)
48. Lambert AW, Pattabiraman DR, Weinberg RA (2017) Emerging Biological Principles of Metastasis. *Cell* 168: 670–691. <https://doi.org/10.1016/j.cell.2016.11.037> PMID: [28187288](#)
49. Tsai HR, Yang LM, Tsai WJ, Chiou WF (2004) Andrographolide acts through inhibition of ERK1/2 and Akt phosphorylation to suppress chemotactic migration. *Eur J Pharmacol* 498: 45–52. <https://doi.org/10.1016/j.ejphar.2004.07.077> PMID: [15363974](#)
50. Ahmed KM, Li JJ (2008) NF-kappa B-mediated adaptive resistance to ionizing radiation. *Free Radic Biol Med* 44: 1–13. <https://doi.org/10.1016/j.freeradbiomed.2007.09.022> PMID: [17967430](#)
51. Munshi A, Ramesh R (2013) Mitogen-activated protein kinases and their role in radiation response. *Genes Cancer* 4: 401–408. <https://doi.org/10.1177/1947601913485414> PMID: [24349638](#)
52. Bozzuto G, Ruggieri P, Molinari A (2010) Molecular aspects of tumor cell migration and invasion. *Ann Ist Super Sanita* 46: 66–80. https://doi.org/10.4415/ANN_10_01_09 PMID: [20348621](#)
53. Yang M, Huang CZ (2015) Mitogen-activated protein kinase signaling pathway and invasion and metastasis of gastric cancer. *World J Gastroenterol* 21: 11673–11679. <https://doi.org/10.3748/wjg.v21.i41.11673> PMID: [26556994](#)
54. Xu HY, Qian AR, Shang P, Xu J, Kong LM, Bian HJ et al. (2007) siRNA targeted against HAb18G/CD147 inhibits MMP-2 secretion, actin and FAK expression in hepatocellular carcinoma cell line via ERK1/2 pathway. *Cancer Lett* 247: 336–344. <https://doi.org/10.1016/j.canlet.2006.05.017> PMID: [16815629](#)
55. Dhawan P, Richmond A (2002) A novel NF-kappa B-inducing kinase-MAPK signaling pathway up-regulates NF-kappa B activity in melanoma cells. *J Biol Chem* 277: 7920–7928. <https://doi.org/10.1074/jbc.M112210200> PMID: [11773061](#)