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Original Article

Resveratrol-mediated ADAM9 degradation decreases cancer progression and provides synergistic effects in combination with chemotherapy

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Abstract: Metastasis is a crucial hallmark of cancer progression and remains the primary cause of patient deaths. Metastasis-associated proteases contribute to cancer progression by disrupting the extracellular matrix interaction to facilitate the spreading of cancer cells to other organs. ADAM9, a type of metalloprotease, has been reported to promote tumor biology and is associated with clinicopathological features such as poor outcome, therapy resistance, and metastasis formation. Targeting ADAM9 might serve as a putative therapeutic application; however, this option is currently unavailable. Resveratrol, a polyphenol from plants, has been shown to be promising for cancer treatment due to its wide variety of biological effects with few side effects. In this study, we demonstrated that resveratrol inhibits cancer cell migration and viability in lung and esophageal cancer cells through the regulation of ADAM9. Mechanistically, resveratrol inhibits ADAM9 protein expression in cancer cells through the ubiquitin-proteasome pathway. Moreover, resveratrol provides synergistic anticancer effects when combined with clinical chemotherapeutics. Our data suggests that resveratrol may inhibit human lung cancer and ESCC progression by inhibiting ADAM9 expression, thus providing a potential mechanism for the anticancer action of resveratrol.

Keywords: Resveratrol, ADAM9, metastasis

Introduction

Metastasis is the main cause of death in cancer patients, including those with lung cancer or esophageal cancer. Lung cancer is one of the most common cancers diagnosed worldwide and accounts for the highest number of cancer-related deaths in the US in 2019 [1]. Esophageal cancer is also a fatal cancer that is asymptomatic in the early stages and has a poor prognosis [2]. Metastasis of cancer cells requires multiple processes for cancer cell dissemination, such as disrupting the cell-extracellular matrix (ECM) interaction and degrading the ECM. Proteases involved in the degradation of the ECM and components of the basement membrane play important roles in cancer progression. Thus, metastasis-associated proteases are considered putative targets for reducing cancer progression.

A disintegrin and a metalloproteinase (ADAM) 9, a type I transmembrane protein, belongs to the ADAM family, which has been linked to several biological processes, including neurogenesis, angiogenesis, cell-cell interactions, and migration [3]. The structure of ADAM9 contains a metalloproteinase domain for shedding and a disintegrin domain for adhesion [4]. ADAM9 is overexpressed in many types of cancers, including breast, lung, esophageal and liver cancers [5-8], and it correlates with cancer progression and metastasis by enhancing cancer cell growth and migration ability [9, 10]. We have demonstrated that ADAM9 can promote lung cancer metastases to the brain through enhancing activation of CUB-domain-containing protein 1 (CDCP1) via facilitating the plasminogen activator-based pathway [11]. Thus, ADAM9 contributes to cancer progression and can serve as a potential target.

Resveratrol (trans-3,4',5-trihydroxystilbene, RSV), a small polyphenol that is present in a large variety of plant species, has a wide variety of biological effects, including anti-inflammatory, anticancer, antioxidative stress, antiangiogenic, cardioprotective, and antidiabetic activities, with fewer side effects than other compounds [12]. It is considered a minor cytotoxic cancer chemopreventive compound [13, 14] and has anticancer properties, such as inhibition of lung, prostate, and skin cancer [15-17]. The anticancer effect of RSV can be attributed to its apoptosis induction and its antiangiogenic, antioxidant, and anti-inflammatory activities. For example, resveratrol can downregulate genes that are associated with cell cycle and proliferation in colorectal cancer (CRC) [18] and induce apoptosis in HT-29 and WiDr colon cancer cells via downregulating telomerase activity [19]. RSV can also influence cancer cell migration by suppressing the expression of the transcription factors NF-kB and AP-1, resulting in reduced expression of the ECM-degradation enzyme u-PA and MMPs [20]. However, the molecular mechanism underlying the effect of RSV on tumor metastasis is still elusive, and whether it can influence ADAM9-mediated migration is unclear. In the present study, we demonstrate the inhibitory effects of RSV on cell migration in lung cancer and esophageal squamous cell carcinoma (ESCC) cells and investigate the possible underlying mechanisms. We reveal that RSV increases ADAM9 protein degradation in cancer cells and provides synergistic anticancer effects in combination with clinical chemotherapeutics.

Materials and methods

Cell culture and reagents

Human lung cancer cell lines (A549, F4, and Bm7) were used as previously described [21]. The human ESCC cell lines CE48T, CE81T, and CE146T were provided by Taipei Veterans General Hospital and maintained in DMEM/F-12 (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (GIBCO, Carlsbad, CA, USA) at 37°C in a humidified incubator with 5% CO_2. All cell lines were free of Mycoplasma contamination.

Resveratrol and 5-FU were purchased from Sigma Aldrich (St. Louis, MO), and dasatinib (Sprycel) was purchased from Bristol-Myers Squibb (Princeton, NJ).

Time-lapse migration assay

Cell movement was assessed as previously described [11]. Briefly, after culturing overnight, the medium was changed to serum-free culture medium, and cell migration was detected by inverted microscopes (Axio Observer Z1, Zeiss) in 37°C environmental chambers for a total of 16 hours. The accumulated distance was determined by tracking each cell using the Track Point function of NIH ImageJ software.

Quantitative reverse transcription PCR

After cancer cells were treated with resveratrol for 72 hours, total RNA was isolated using TRIzol reagent (Invitrogen). The RNA expression levels of ADAM9 were determined by quantitative reverse transcription PCR (RT-qPCR) using a LightCycler® 480 Real-Time PCR System (Roche Applied Science, Indianapolis, IN) as previously described [21]. The following primers were used for ADAM9 mRNA detection: 5'-CCCCCAAATTGTGAGACTAAAG-3' (forward), 5'-TCCGTCCCTCAATGCAGTAT-3' (reverse). The relative expression level of ADAM9 mRNA was normalized to internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

MTT assay and synergy analysis

Cancer cells (2×10^4) were plated in 96-well plates. After treatment with various concentrations of single or combination of resveratrol and chemotherapeutics, 10 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) (5 mg/ml) was added. Two and half hours later, the culture medium was removed, and the formazan crystals were dissolved in 100 μl of DMSO. The absorbance values were determined using a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. The half maximal inhibitory concentration (IC50) value was estimated using CalcuSyn V2.0 Software.

The therapeutic effect of a two-drug combination was assessed by MTT assays, and the combination index (CI) was calculated with the CalcuSyn program (Biosoft, Cambridge, United Kingdom), as previously described [22]. A CI of

<1, 1, or >1 is indicative of synergistic, additive, or antagonistic effects, respectively.

Immunoblot analysis

Cancer cells were treated with RSV for 24 hours and then treated with 5 μM MG132 for 3 hours before collecting the cell lysate; the cells were lysed in RIPA lysis buffer (Sigma, St. Louis, MO). Total lysates were loaded on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio Rad Laboratories, Inc.). Blocking was performed with 5% nonfat milk in a 1X mixture of Tris-buffered saline and Tween 20 (TBST). After blocking, the membranes were then incubated overnight at 4°C with antibodies against the following targets: ADAM9 (#2099, Cell Signaling, Danvers, MA), CDCP1 (ab1377, Abcam, Cambridge, MA), pMEK1/2 (#9121, Cell Signaling), HA-tag (Thermo, WA), pSrc (#6943, Cell Signaling), pERK (9101, Cell Signaling, Danvers, MA), and EF1α (#05-235, Millipore, Billerica, MA). Blotted proteins were detected using an enhanced chemiluminescence (ECL) system (Millipore) with the BioSpectrum Imaging System (UVP, Upland, CA, USA).

Immunoprecipitation

Cancer cells were transiently transfected with HA-Ub plasmid. The next day, cancer cells were treated with RSV for 24 h and then treated with 5 μM MG132 for 3 h before collecting the cell lysate. Cells were lysed with buffer containing 150 mM NaCl, 20 mM Tris, 5% NP40, 1 mM PMSF, 1 mM EDTA, 10 mM NaF, 2 mM sodium orthovanadate, and protease inhibitors (Bio-PINR-100, BioKit, Taiwan).

Cell lysates (1.5 mg) were mixed with anti-ADAM9 antibody (4 μl, Cell Signaling, Danvers, MA) for each reaction at 4°C overnight in a rotating wheel, and then 50 μL of protein A/G-coated beads was added and rotated 4°C for 1 hour. Immunoprecipitates were then washed with lysis buffer and eluted by boiling with 1% (wt/vol) SDS sample buffer for Western blotting with the indicated antibodies.

Statistical analysis

Student's t test was applied to calculate significance for at least three independent biological replicates, such as RT-QPCR analysis and cell migration analysis.

Results

Resveratrol reduces cell migration in ESCC

To investigate whether RSV can reduce the migration ability of ESCC and lung cancer cells, we treated cancer cells with RSV and measured the migration distance by tracking each cell's movement with time-lapse detection. We found that RSV significantly decreased the cumulative migration ability of CE48T, CE81T, CE146T ESCC, and Bm7, and A549 lung cancer cells (**Figure 1**). This finding suggests that RSV has an antimigratory effect on ESCC and lung cancer cells.

Cancer cells with a high level of ADAM9 expression are sensitive to RSV-mediated anticancer effects in ESCC and lung cancer cells

ADAM9 has been reported to promote tumor biology and is associated with clinicopathological features such as poor outcome, therapy resistance, and metastasis formation [23]. First, we investigate whether RSV provides anticancer effect in malignant cells when they contain high levels of ADAM9. To address the correlation of ADAM9 and the capable of RSV in suppressing cancer cell proliferation, we detected cell viability by MTT assays in control and ADAM9 knockdown ESCC and lung cancer cells treated with different doses of RSV. When we knocked down ADAM9 by viral transduction of shRNA to obtain a stable ADAM9 knockdown cancer cell population (shADAM9) and treated these cells with RSV, we found that the IC50 value for RSV was nearly 2-fold higher in ADAM9 knockdown CE-146T and KYSE170 ESCC cells than in control cells (**Figure 2A**, **2B**). Similar results were found that ADAM9 knockdown lung cancer cells have higher IC50 value for RSV (**Figure 2C**, **2D**). These findings suggest that cancer cells with ADAM9 expression are sensitive to RSV treatment and that ADAM9 may be the target for RSV-mediated anticancer effects.

Resveratrol promotes ADAM9 protein degradation via the ubiquitin-proteasome pathway

ADAM9 has been reported to promote lung cancer metastasis by increasing CDCP1 expres-

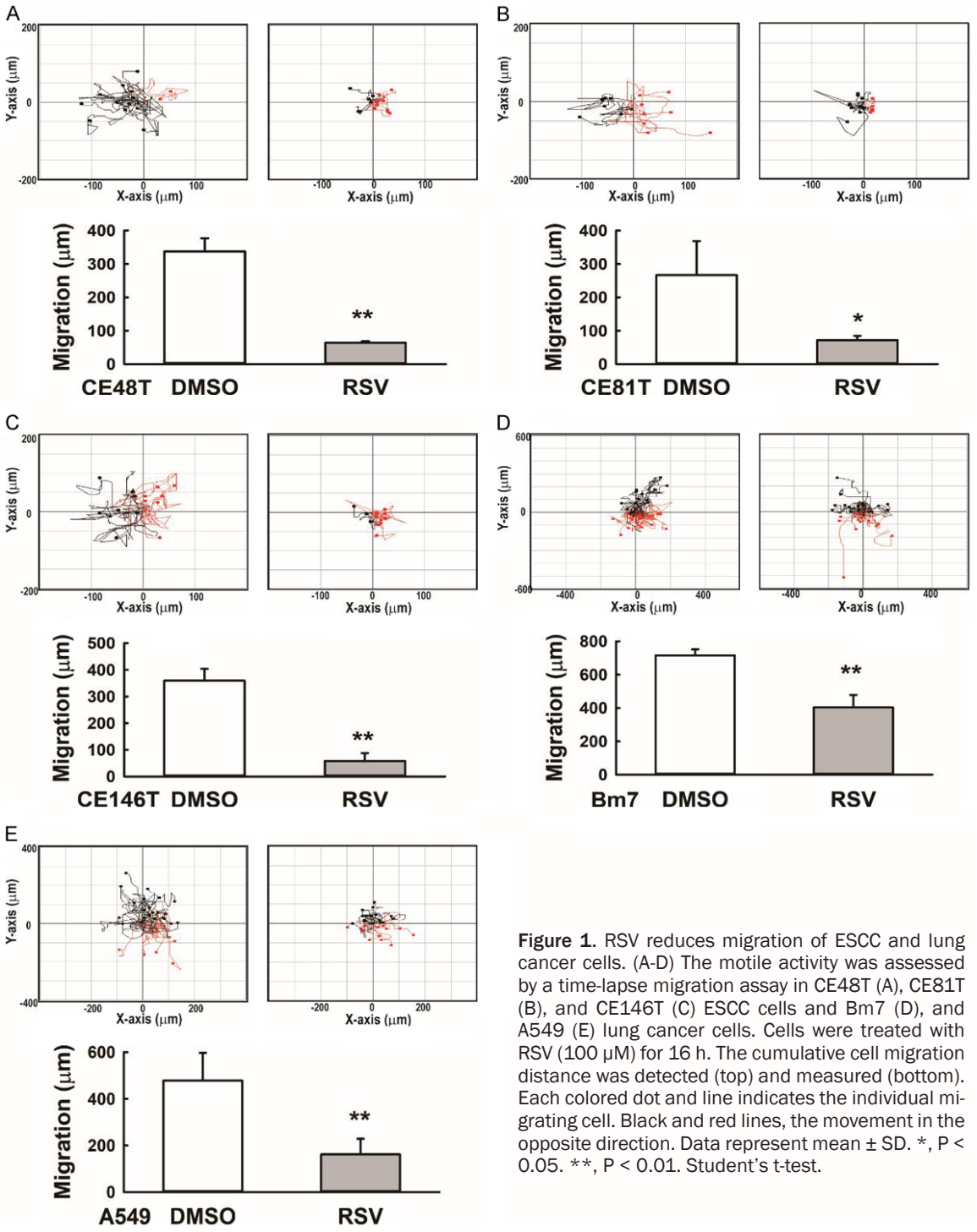

Figure 1. RSV reduces migration of ESCC and lung cancer cells. (A-D) The motile activity was assessed by a time-lapse migration assay in CE48T (A), CE81T (B), and CE146T (C) ESCC cells and Bm7 (D), and A549 (E) lung cancer cells. Cells were treated with RSV (100 μM) for 16 h. The cumulative cell migration distance was detected (top) and measured (bottom). Each colored dot and line indicates the individual migrating cell. Black and red lines, the movement in the opposite direction. Data represent mean ± SD. *, $P < 0.05$. **, $P < 0.01$. Student's t-test.

sion (full length of CDCP1) and CDCP1 activation (cleaved form of CDCP1) [24]. Based on the result showing that cancer cells with ADAM9 expression are sensitive to RSV treatment, we wanted to investigate whether RSV can regulate ADAM9 expression to support RSV-induced inhibition of migration. After RSV treatment, we found that the levels of ADAM9 were significantly decreased in A549 and Bm7 lung cancer cells (**Figure 3A**) and CE81T and CE146T ESCC cells (**Figure 3B**). Moreover, ADAM9-affected downstream signaling proteins, including CDCP1, p-Src, and p-MEK, which were strongly decreased in Bm7 lung cancer cells and two

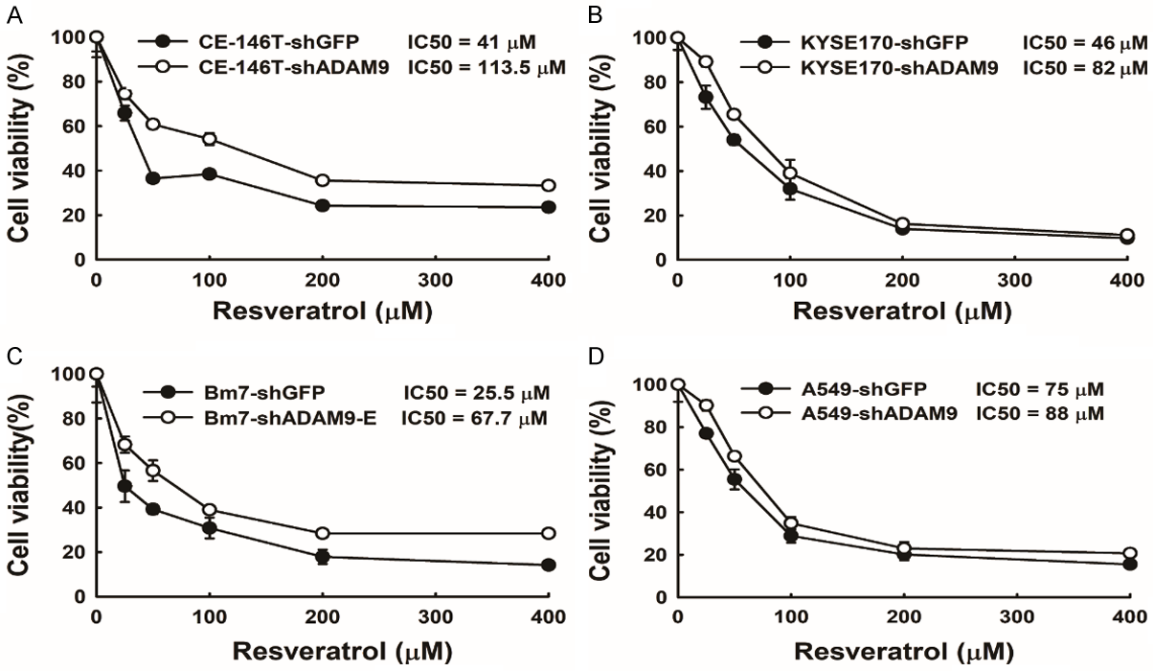

Figure 2. RSV decreases proliferation of ESCC cells. Cell viability was determined by MTT assay in control (shGFP) or ADAM9 knockdown (shADAM9) CE-146T cells (A), KYSE170 (B), Bm7 (C), and A549 (D) with resveratrol treatment for 72 h. Values are means ± SDs for triplicate at each concentration. The IC50 concentrations of RSV in cancer treatment were shown.

ESCC cells, although CDCP1 and p-MEK were not reduced in A549 lung cancer cells treated with RSV. Notably, RSV had a minor effect on the regulation of ADAM9 mRNA in ESCC and lung cancer cells, despite a moderately reduced ADAM9 RNA in Bm7 cells treated with RSV (P=0.06) (**Figure 3C**), suggesting that RSV reduces ADAM9 protein expression mainly through protein regulation but not RNA regulation. By using MG132, a proteasome inhibitor, to reduce protein degradation, we found that MG132 strongly rescued the ADAM9 protein levels in RSV-treated Bm7, CE81T, and CE146T cells (**Figure 3D**). This suggested that RSV treatment in cancer cells leads to ADAM9 protein reduction through proteasomal degradation.

Next, to further examine whether RSV promotes ADAM9 protein degradation by ubiquitination in cancer cells, we transiently transfected HA-tagged ubiquitin into cancer cells and measured ubiquitin conjugation to ADAM9 proteins. When F4 cancer cells were treated with RSV, ADAM9 protein levels were decreased and the polyubiquitin conjugation level increased compared to that in the untreated ADAM9 immunoprecipitation fraction (**Figure 4A**). Similar results were detected in Bm7 cancer cells (**Figure 4B**). Taken together, RSV contributes to ADAM9 degradation through ubiquitin-proteasome degradation pathways.

Resveratrol provides synergistic anticancer effects with dasatinib or 5-FU

Dasatinib, a Src/Abl kinase inhibitor, is a targeted therapy drug used to treat certain types of chronic myeloid leukemia and acute lymphoblastic leukemia [25]. Since we have previously demonstrated that ADAM9 knockdown can reduce the levels of pSrc in lung cancer cells [11], we wanted to investigate whether the combination of RSV and dasatinib could enhance the suppression of pSrc even more strongly and synergistically inhibit cancer cell growth; this was done by measuring the combination index (CI), an indicator for evaluating the therapeutic effect of two-drug treatment. The results showed that the two-drug treatment combination acted synergistically (CI value <1 indicates synergism); the combination of RSV and dasatinib suppressed cancer cell growth more potently than either single drug treatment (**Figure 5A**). A similar result was obtained in

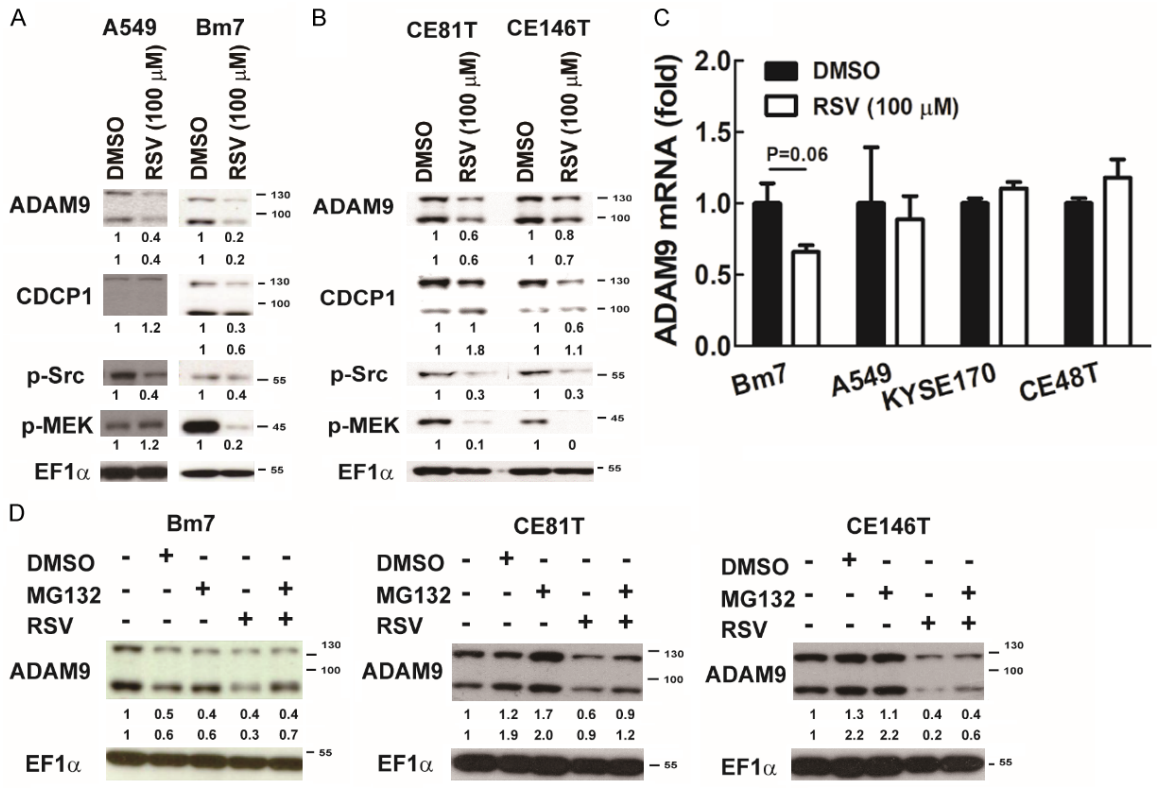

Figure 3. RSV promotes ADAM9 protein degradation. (A, B) Western blot analysis of the indicated proteins in lung cancer cells (A) and ESCC cells (B) after RSV treatment for 24 hours. EF1α served as the internal control. (C) RT-qPCR analysis of ADAM9 mRNA expression in cancer cells after RSV treatment for 24 hours. Data represent mean ± SD. (D) Western blot analysis of the indicated proteins in cancer cells after RSV (100 μM) and MG132 (5 μM) treatment. The numbers below the blots indicate the relative protein expression.

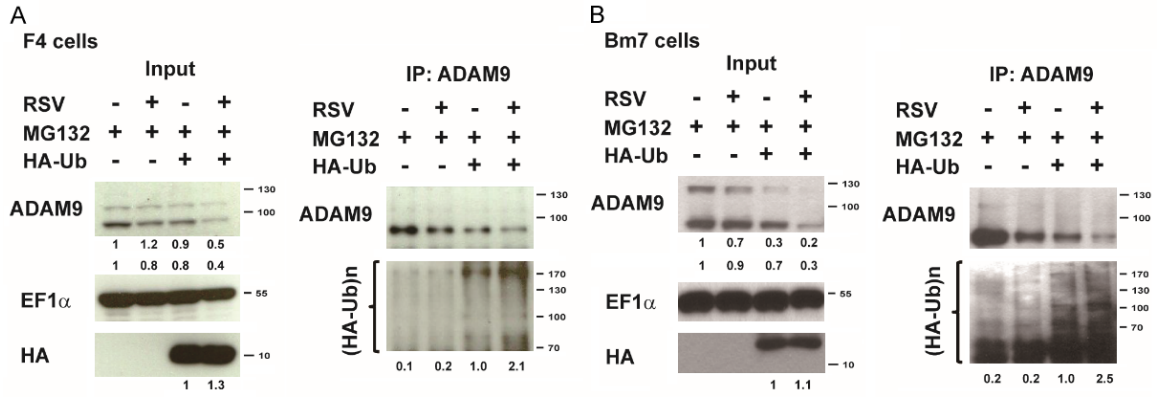

Figure 4. RSV-induced ADAM9 protein degradation is mediated by the ubiquitin-proteasome pathway. (A, B) HA-Ub was overexpressed in F4 (A) and Bm7 (B) cells treated with RSV (100 μM) and MG132 (5 μM) for 24 hours. Input (left), 10% of the total protein lysate for immunoprecipitation (IP). IP was performed with anti-ADAM9 antibodies, followed by Western blotting with the anti-HA antibody. EF1α, loading control. The numbers below the blots indicate the relative protein expression.

Bm7 lung cancer cells treated with RSV and dasatinib (**Figure 5B**). Inhibition of ADAM9 expression has been reported to sensitize human prostate cancer to chemotherapy [26]. To further investigate whether RSV could provide synergistic anticancer effects in combination with clinical chemotherapeutics, we evaluated the therapeutic effect of RSV and 5-FU, an anticancer drug with that disrupts dNTP synthesis, in ESCC cells. This combination showed a

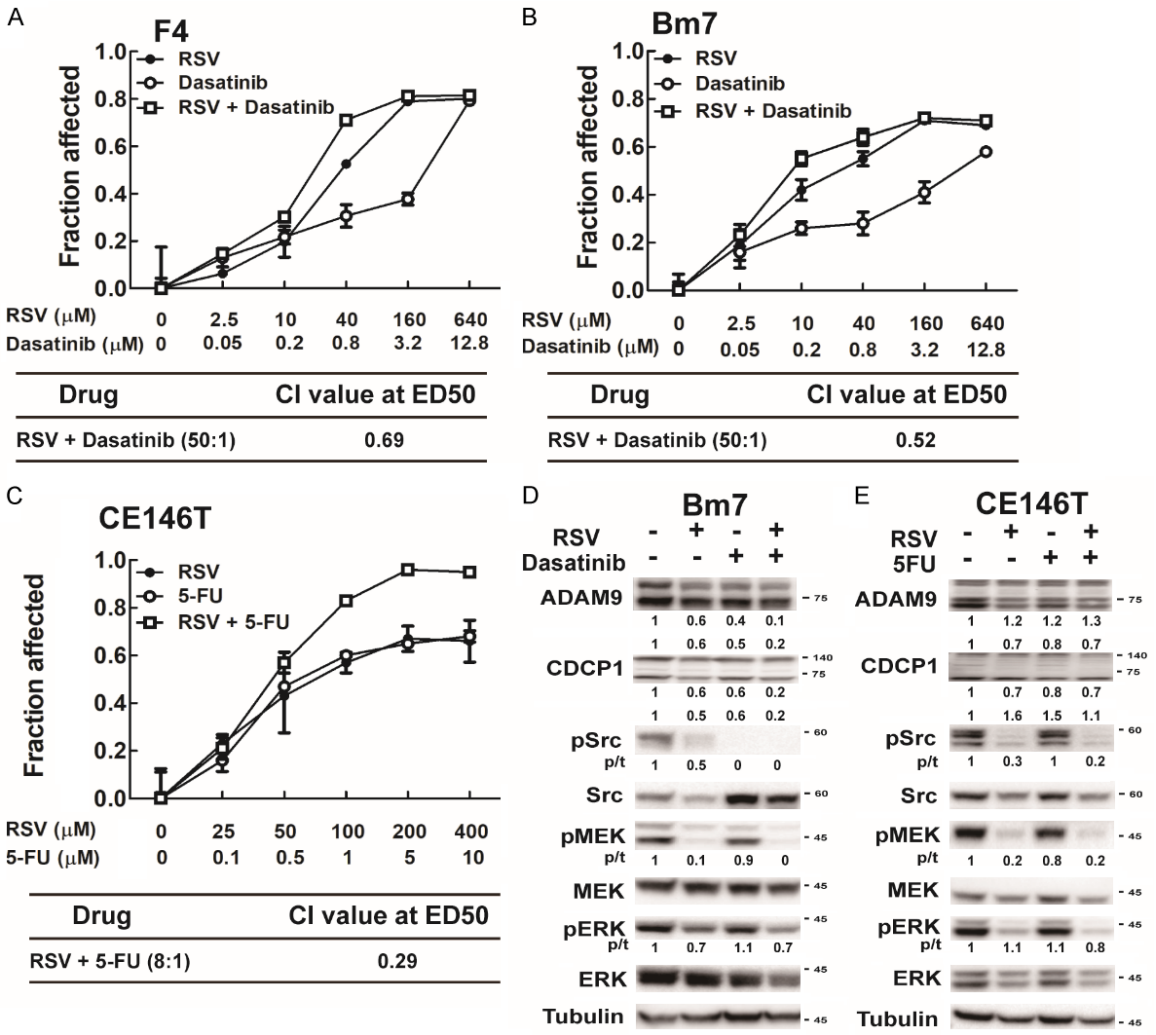

Figure 5. The combination of RSV with chemotherapy drugs has synergistic therapeutic effects. (A, B) Combination treatment with RSV and dasatinib in F4 (A) and Bm7 (B) cancer cells for 72 hours. (C) Combination treatment with RSV and 5-FU in CE146T cells for 72 hours. The cytotoxic effects were examined using MTT assays. Dose-dependent cytotoxic fraction (top); combination index at 50% inhibition (ED50) (bottom). (D) Western blot analysis of the indicated proteins in Bm7 cells after RSV and/or dasatinib treatment for 24 hours. (E) Western blot analysis of the indicated proteins in CE146T cells after RSV and/or 5FU treatment for 24 hours. p/t indicates the quantified ratio of phosphorylated proteins to total proteins.

strong synergistic anticancer effect in CE146T cells (**Figure 5C**). The combination of RSV and dasatinib strongly decreased the levels of pSrc, pMEK, and pERK in Bm7 lung cancer cells (**Figure 5D**). Similar results were detected with the combination of RSV and 5-FU in CE146T cells (**Figure 5E**). Together, RSV treatment provides synergistic effects with dasatinib and 5-FU treatment in lung cancer and ESCC cells, respectively.

Discussion

In this study, we demonstrate that RSV can reduce cell migration in ESCC and lung cancer cells. Silencing ADAM9 increases cancer cell viability with a higher IC50 value for RSV in ESCC and lung cancer cells. RSV reduces ADAM9 protein levels by increasing the ubiquitin-proteasome degradation pathway but has little effect on RNA expression. Moreover, the combination of RSV and clinical drugs, such as dasatinib and 5-FU, provides synergistic therapeutic effects in lung and ESCC cancer cells. We reveal a new finding that RSV plays a role in cancer treatment by promoting oncogenic ADAM9 protein degradation and sensitize the cancer cells to drug treatment (**Figure 6**).

Resveratrol has been reported to inhibit human ovarian cancer progression and angiogenesis

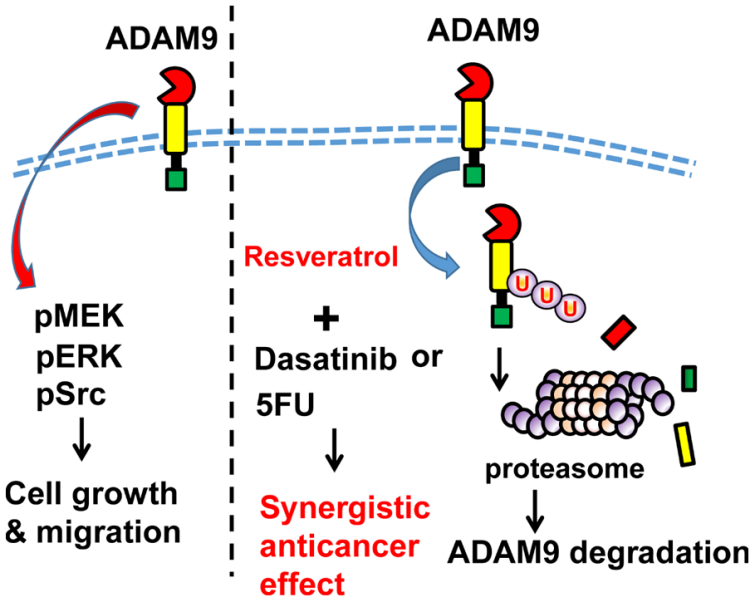

Figure 6. The mechanism of RSV-mediated anticancer effects through promoting ADAM9 protein degradation. Combined RSV with clinical chemotherapeutics can provide synergistic anti-cancer effects.

by inhibiting HIF-1α and VEGF proteins through multiple mechanisms, including translational regulation and proteasomal degradation [27]. However, the effect of resveratrol on the induction of oncoprotein degradation is still elusive. We showed that ADAM9 protein levels were decreased when cancer cells were treated with resveratrol and that they could be rescued by adding the protease inhibitor MG132. Moreover, resveratrol reduces the ADAM9 level mainly through posttranslation modification via ubiquitination. Although the mechanism by which resveratrol enhances the proteasome degradation pathway is still unclear and requires further study, the capacity of resveratrol to mediate oncoprotein degradation provides a new direction for investigating cancer therapy.

Many studies have reported that oral administration of resveratrol is safe because of efficient absorption and quick metabolism in rodents and humans. In surgically resected colon tissue from colorectal cancer patients who ingested 1.0 g of resveratrol daily for 7 days before surgery, resveratrol was detectable in the range between 8.3 and 674 nmol/g tissue [28]. Although the maximal resveratrol concentrations in plasma of humans administered 1.0 g resveratrol, which is lower than the dose used in in vitro studies for cancer treatment, is in the micromolar range, there are still much higher levels of glucuronide and sulfate conjugates of resveratrol in the plasma [29]. Based on the evidence that resveratrol has been reported to possess a significant anticancer property in various preclinical animal models [30], repeated administration of resveratrol might result in its accumulation in the tissues, including the parental form, the metabolites, and the in vivo conjugates, and may provide sufficient chemopreventive effects. We have demonstrated that the combination treatment of lung cancer and ESCC cells with RSV and dasatinib or RSV and 5FU, respectively, revealed synergistic effects. This indicates that RSV and chemotherapy combination treatment could reduce the drug concentration required and increase the effects of treatment. Therefore, RSV could be used as a sensitizing agent for chemotherapy in clinical applications.

Acknowledgements

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Disclosure of conflict of interest

None.

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