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Dietary flavonoids, luteolin and quercetin, inhibit invasion of cervical cancer by reduction of UBE2S through epithelial–mesenchymal transition signaling†

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We previously reported that the dietary flavonoids, luteolin and quercetin, might inhibit the invasiveness of cervical cancer by reversing epithelial–mesenchymal transition (EMT) signaling. However, the regulatory mechanism exerted by luteolin and quercetin is still unclear. This study analyzed the invasiveness activation by ubiquitin E2S ligase (UBE2S) through EMT signaling and inhibition by luteolin and quercetin. We found that UBE2S expression was significantly higher in highly invasive A431 subgroup III (A431-III) than A431-parental (A431-P) cells. UBE2S small interfering (si)RNA knockdown and overexpression experiments showed that UBE2S increased the migratory and invasive abilities of cancer cells through EMT signaling. Luteolin and quercetin significantly inhibited UBE2S expression. UBE2S showed a negative correlation with von Hippel-Lindau (VHL) and a positive correlation with hypoxia-induced factor (Hif)-1α. Our findings suggest that high UBE2S in malignant cancers contributes to cell motility through EMT signaling and is reversed by luteolin and quercetin. UBE2S might contribute to Hif-1α signaling in cervical cancer. These results show the metastatic inhibition of cervical cancer by luteolin and quercetin through reducing UBE2S expression, and provide a functional role for UBE2S in the motility of cervical cancer. UBE2S could be a potential therapeutic target in cervical cancer.

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Introduction

Flavonoids isolated from plants are widely reported to inhibit the functions and cell signaling transduction of multiple kinases.[1,2] They also repress proliferation, invasion, angiogenesis, and apoptosis of tumor cells.[3–10] Quercetin and luteolin are the most important flavonoids found in black tea, linden flower, sage, rosehip, violet carrot juice, grape molasses, onions, apples, red wine, olive, oil, berries, fruit skin, buckwheat, red pepper and tomato skin.[11–13] In our previous reports, luteolin and quercetin were the most efficient flavonoid molecules in reversing the progression of the epithelial–mesenchymal transition (EMT).[14]

The ubiquitin E2S ligase (UBE2S), ubiquitin carrier protein, is an E2 ubiquitination ligase that helps E1, E2, and E3 ligases link ubiquitin with target proteins, which then targets them towards proteasome degradation.[15–17] The ubiquitin-proteasome pathway was documented to have a critical role in tumor formation and progression.[18] The extent of UBE2S, which is highly expressed in several types of tumors, was correlated with the degree of tumor burden in esophageal cancer patients.[19] UBE2S messenger (m)RNA is highly expressed in cervical cancer patients and is associated with the growth and aggressiveness of cervical tumor cells.[20] Patients with papillary renal cell carcinoma (PRCC), a common kidney cancer, exhibited markedly higher expression levels of UBE2S that were found to be associated with Hif-1α signaling.[21]

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UBE2S overexpression in highly invasive A431 subgroup III (A431-III) cells was employed to probe the impact of the flavonoid antioxidants, luteolin and quercetin, on the presumptive regulatory role of UBE2S in the stability of Hif-1α and cell motility. Plant flavonoids are widely recognized facile modifiers of cell-surface transduction and potent inhibitors of multiple cognate kinases[1] and also of tumor cell proliferation, invasion, angiogenesis, and apoptosis. Intriguingly, they are also able to readily reverse the EMT. They have also been documented to impair PI3 K/Akt/mammalian target of rapamycin (mTOR)/Hif-1α signaling,[22–27] which is particularly relevant to the theme of the present investigation.

The von Hippel-Lindau (VHL) tumor suppressor has been reported to target Hif-1α, resulting in degradation of Hif-1α under aerobic conditions. Under hypoxia conditions, VHL is unable to access Hif-1α to accomplish this degradation, and such defunct VHL functionality consequently primes the constitutive activation of Hif-1α.[28,29] UBE2S was identified as a Hif-1α regulatory gene, and its protein directly targets VHL for degradation.[30] A high level of UBE2S expression in tumor cells allows VHL to be targeted for degradation, which consequently results in the increased release of Hif-1α and allows the entry of Hif-1α into nuclei and activation of downstream gene transcription, including that of UBE2S. This positive regulatory relationship between UBE2S and Hif-1α in tumor cells may contribute to greater proliferation, angiogenesis, and metastasis of cancers.[30]

Previously we obtained a highly invasive A431-III subline, which exerts higher expression levels of matrix metalloproteinase (MMP)-9 and various EMT markers[31,32] compared to A431 parental (A431-P) tumor cells. Using this subline, we were able to demonstrate that the dietary flavonoids, luteolin and quercetin, could inhibit the invasive capacity of A431-III cells by reducing the EMT and inhibit RPS12 expression by the reduction of c-Myc.[33] In addition, both luteolin and quercetin were found to inhibit the phosphorylation of Akt and GSK3β.[14]

However, understanding of the mechanisms of their major inhibitory role in gene regulation in cancer cells remains lacking and needs to be further elucidated. Herein, we designed experiments to elucidate the modulatory role of these two flavonoids upon Hif-1α and VHL, in highly invasive A431-III cells. In the current investigation, we elucidated the expression of UBE2S at the mRNA and protein expression levels in both A431-III and A431-P cells. UBE2S overexpression and small interfering (si)RNA knockdown experiments were then performed with the aim of mapping the regulatory function of UBE2S on the stability of Hif-1α and its contribution to cell motility. We were particularly interested in assessing the repercussions of UBE2S overexpression and siRNA knockdown in order to gain insights into the role of UBE2S in the stability of Hif-1α and cell motility. Hif-1α and VHL expression levels were positively and negatively correlated with UBE2S expression, respectively. Cell migration was also attenuated by siRNA knockdown and enhanced by UBE2S overexpression.

Methods

Materials

A431-P cells (human epidermal carcinoma cells) were obtained from ATCC (Manassas, VA, USA). A431-III cells were isolated in our laboratory from A431-P tumor cells using a Boyden chamber.[31] RPMI-1640 and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Anti-UBE2S, anti-Hif-1α, and anti-VHL antibodies were obtained from GeneTex (Irvine, TX, USA). Anti-GADPH and anti-β-actin antibodies were purchased from Santa Cruz (Capitola, CA, USA). The anti-mTOR antibody was obtained from Cell Signaling (Beverly, MA, USA). Polymerase chain reaction (PCR) forward and reverse primers were purchased from Purigo Biotech (Taipei, Taiwan). Luteolin (purity ≥95%) was purchased from Toronto Research Chemicals (North York, ON, Canada). Quercetin (purity ≥95%) was purchased from Nacalai Tesque (Kyoto, Japan). Agarose and DMSO were purchased from E. Merck (Darmstadt, Germany). Epidermal growth factor was obtained from Upstate Biotechnology (Lake Placid, NY, USA) and dissolved in RPMI-1640 medium. Unless otherwise indicated, all other reagents were obtained from Sigma (St Louis, MO USA). Luteolin and quercetin were dissolved in 100% DMSO, and their concentrations were adjusted to 100 mM for stock solutions.

Cell culture

A431-P and A431-III cells were described in our previous report.[24] A431-P and A431-III cells were incubated in a 5% CO_2 air atmosphere at 37 °C with a RPMI-1640 medium (Gibco, NY, USA) containing 10% FBS (Gibco, NY, USA).

Preparation of cell lysates

Cultured cells (1×10^6 cells per well) were seeded onto 6-well plates overnight and washed three times with phosphate-buffered saline (PBS) before harvesting. These cells were then lysed in gold lysis buffer containing 20 mM Tris-HCl (pH 7.9), 1 mM EGTA, 0.8% NaCl, 0.1 mM b-glycerolphosphate, 1 mM sodium pyrophosphate, 10 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg mL^{-1} aprotinin, and 10 μg mL^{-1} eupeptin. Any insoluble material was then removed by centrifugation at 14 000*g* for 20 min at 4 °C. Protein concentrations were quantified using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). All samples were stored at −80 °C for further study.

Western blot analysis

Cell lysates (20 μg) were mixed with a sample buffer and boiled for 5 min. They were then separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Next, the membrane was blocked using PBS containing 5% bovine serum albumin (BSA) for 1 h at room temperature, which was followed by incubation with a primary antibody overnight at 4 °C. After washing with

TBST containing 20 mM Tris-HCl (pH 7.6), 0.8% (w/v) NaCl, and 0.25% Tween-20, the blot was incubated with a secondary antibody conjugated with horseradish peroxidase (HRP) (Millipore, Billerica, MA, USA). Finally, the membrane was washed with TBST, and the presence of immunoreactive bands was detected using an enhanced chemiluminescence (ECL) reagent kit (Millipore) followed by exposure to Fujifilm (Tokyo, Japan). ImageJ software (http://rsb.info.nih.gov/ij/index.html, NIH, USA) was used to measure the relative quantification of the ECL signals present on the X-ray film.

UBE2S complementary (c)DNA construction and transfection

The full-length cDNA encoding UBE2S was isolated from the human cervical epithelial cancer cell A431-III subline cDNA by reverse-transcription (RT)-PCR using specific primers (UBE2S-F, 5′-CCA TGA ACT CCA ACG TGG AGA ACC-3′; UBE2S-R, 5′-AGG AAG AGA GCC CAC TAC AGC-3′); the product was then cloned into the pGEMT-Easy vector (Promega, San Luis Obispo, CA, USA) which was followed by characterization *via* DNA sequencing. Next, the pcDNA3-UBE2S plasmid was constructed to include the coding region of UBE2S by transferring the appropriate region from the pGEMT-Easy plasmid to the pcDNA3 plasmid. Next, A431-P cells (1×10^6 cells per well) were seeded onto 6-well plates overnight and transfected with the pcDNA3-UBE2S and pcDNA3-HA plasmids using the Xfect transfection reagent (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. After transfection for 5 h, the medium was changed to fresh medium, and incubation continued for an additional 24 h. Cells were then either treated or not (control) with a plant flavonoid constituent (10 or 20 μM luteolin or 20 or 40 μM quercetin) for 24 h in order to determine the influence of these compounds on expressions of key proteins.

Transfection of siRNA

UBE2S siRNA (Life Technologies, Taipei, Taiwan) and control siRNA (Life Technologies, Taipei, Taiwan) were dissolved in RNase-free water provided by the manufacturer to a stock concentration of 20 μM. A431-III cells (1×10^6 cells per well) were seeded onto 6-well plates overnight and then transfected with 40 nM of siRNA using the Lipofectamine 2000 transfection reagent (Life Technologies, Taipei, Taiwan) following the manufacturer's instructions. All assays were performed 48 h after transfection.

Quantitative real-time (q)PCR

Total RNA was extracted from the cell lines using a High Pure RNA Isolation Kit (Roche, Basel, Switzerland) and then 1 μg of total RNA was reverse-transcribed using a MMLV High Performance Reverse Transcriptase kit (Epicentre, Madison, WI, USA). Quantification of the transcript levels of various target genes was performed using the LightCycler system (Roche), commercial SYBR Premix Ex Taq (Takara Bio, Shiga, Japan), and specific primer sets.

In vitro wound-healing migration assay

Both A431-P (1×10^6 cells per well) and A431-III cells (1×10^6 cells per well) were seeded onto 6-well plates overnight and transfected with either UBE2S siRNA or the full-length UBE2S expression vector was plated onto six-well culture plates in RPMI-1640 containing 10% FBS. After 24 h, manual scratching with a pipette tip and subsequent washing with PBS wounded the cell monolayers. The monolayers were then incubated at 37 °C for 24 h. The monolayers were photographed at 0 and 24 h after wounding using phase-contrast microscopy and an Olympus IX70 camera (Tokyo, Japan) in order to capture the size of the wound. Experiments were performed in triplicate for each treatment group.

Cell viability assay

A431-III cells (10^5 cells per well) were seeded onto 48-well plates overnight and then incubated with different concentrations of luteolin, quercetin (10, 20, and 40 μM) and 0.1% DMSO (0 μM) as control for an additional 24 h. The medium was removed, and cells were washed with PBS three times. An MTT assay was performed by treating with MTT (5 mg mL^{-1}) containing RPMI-1640 with 10% FBS at 37 °C for 4 h. The medium was then removed, and 200 μl DMSO was added to dissolve the precipitate. The absorbance was measured using a micro-plate reader (Bio-Tec, Winooski, VT, USA) at a wavelength of 570 nm.

Gelatin zymography

Conditioned media were collected from samples and subjected to 8% SDS-PAGE with 0.1% gelatin (Sigma). After electrophoresis, the gel was washed twice with PBS with 2.5% Triton X-100 for 60 min and then incubated with reaction buffer (50 mM Tris-HCl at pH 8.0, containing 5 mM $CaCl_2$ and 0.02% NaN_3) at 37 °C for 24 h. The gel was stained with Coomassie Blue R-250 in 10% acetic acid and 20% ethanol for 1 h and then de-stained with de-staining buffer (10% acetic acid and 20% ethanol). The clear zone on the gel indicated the enzyme activity of gelatinase.

Statistical analysis

Results from three independent experiments are expressed as the mean ± standard deviation (SD). For comparison between two groups, statistical analysis was determined by an unpaired Student's *t*-test. For comparison of more than two groups, one-way ANOVA followed by Tukey's test was used. A probability of $p < 0.05$ was considered significant as *; $p < 0.01$ is indicated as **; $p < 0.001$ is indicated as ***.

Results

UBE2S was more highly expressed in A431-III than A431-P cells

We previously demonstrated that A431-III cells exhibit greater invasive and migratory capacities than A431-P cells, and that these properties are related to higher MMP-9 expression.[31] The A431-III cell line provided us with a reliable and sensitive

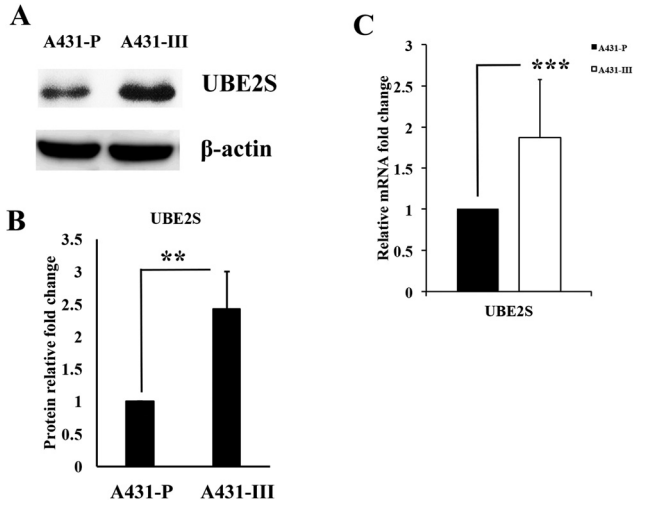

Fig. 1 Highly invasive A431-III cells express higher UBE2S than A431-P cells. (A) Comparison of the expression of UBE2S in A431-III and A431-P cells by western blotting. (B) Data represent the mean (SD) of three different experiments. Statistical significance was determined with a *t*-test (**$P < 0.01$) in (A). (C) Comparison of the transcripts of UBE2S in A431-III and A431-P cells by a qPCR. Results from three independent experiments are expressed as the mean ± standard deviation (SD). *** Indicates a significant difference compared to the control ($P < 0.001$).

model that allowed an analysis of invasiveness and identification of potential migratory mechanisms, because we could compare A431-III cells with A431-P cells. With the aim of assessing the expression of UBE2S in A431-III cells, we compared the levels of protein expression and mRNA transcription of UBE2S in A431-III and A431-P cells by western blot and qPCR analyses, respectively. Total protein samples from A431-III and A431-P cells were collected, and the level of UBE2S protein expression was evaluated by employing an anti-UBE2S antibody and Western blotting. The UBE2S protein level was higher in A431-III cells than in A431-P cells (Fig. 1A and B). The expression level of UBE2S mRNA in A431-III cells was greater than A431-P cells (Fig. 1C). These results suggest that UBE2S is more highly expressed in A431-III cells than in A431-P cells in cervical cancer.

Higher UBE2S increases the migratory and invasive abilities of cervical cancer cells

In earlier reports, we documented that A431-III cells exhibit an elevated migration capacity and greater invasion potential compared to A431-P cells. To further characterize the migratory abilities of these two cell lines and ascertain whether these are related to UBE2S expression, we employed a wound-healing experiment to assess the cell migratory ability after siRNA knockdown of UBE2S in A431-III cells and after overexpression of UBE2S in A431-P cells. The cell migratory ability was determined at 24 h post-wound-healing. The diminished expression of UBE2S in A431-III cells after siRNA knockdown significantly inhibited cell migration compared to control siRNA knockdown (Fig. 2A). On the other hand, overexpression of UBE2S in A431-P cells appreciably increased the cell migratory potential (Fig. 2C). To evaluate whether the expression of UBE2S was related to the invasiveness of cervical cancer, we prepared a trans-well assay to analyze the invasiveness of cancer cells. The invasive ability of A431-III cells was reduced by UBE2S siRNA knockdown (Fig. 2B) compared to control siRNA knockdown. Overexpression of UBE2S in A431-P cells raised invasive ability compared to control (vector only) (Fig. 2D). These data pose the intriguing possibility that elevated levels of UBE2S expression in A431-III cells trigger significantly enhanced cell motility in cancer cells.

To further investigate cell motility signaling of cervical cancer cells promoted by UBE2S, we analyzed the expressions of EMT-related markers (E-cadherin, N-cadherin, vimentin, snail, and MMP-9) by UBE2S siRNA knockdown in A431-III cells and cDNA overexpression in A431-P cells. The expression levels of N-cadherin, vimentin, snail, and MMP-9 were reduced and that of E-cadherin was elevated by UBE2S siRNA knockdown in A431-III cells (Fig. 3A and B). In UBE2S cDNA overexpression, opposite results were obtained (Fig. 3C and D). The enzymatic activity of MMP-9 was used to evaluate the motility of tumor cells. We also analyzed the digestive activity of MMP-9 using a zymography experiment. MMP-9 activity was significantly reduced by UBE2S siRNA knockdown in A431-III cells (Fig. 3E) and raised by UBE2S cDNA overexpression in A431-P cells (Fig. 3F). These data suggest that UBE2S promotes the motility of cervical cancer cells through EMT signaling and MMP-9 expression.

Luteolin and quercetin inhibited UBE2S expression

We previously documented that the flavonoids, luteolin and quercetin, are able to inhibit metastasis and invasiveness by reversing the EMT,[14] inhibition of Src/FAK/Cortactin signaling,[34] and mTOR/c-Myc/RPS12 signaling[30] in A431-III cells. To evaluate the inhibition effect of luteolin and quercetin on UBE2S in A431-III cells, we performed an MTT assay. A431-III cells showed respectively 100%, 99%, 76%, and 31% cell variabilities and 100%, 101%, 117%, and 86% cell viability after treatment with 0.1% DMSO (0 μM), and 10, 20, and 40 μM of luteolin and quercetin (Fig. 4A). To further elucidate the effects of luteolin and quercetin on UBE2S expression, we treated A431-III cells with 0.1% DMSO (Control), 10 or 20 μM of luteolin or with 0.1% DMSO (Control), 20 or 40 μM of quercetin for 24 h to characterize the protein levels of UBE2S. As suggested above, luteolin and quercetin significantly reduced the protein level of UBE2S (Fig. 4B–E). The mRNA levels of UBE2S and Hif-1α in A431-III cells were detected using a qPCR after treatment with 0.1% DMSO (Control), 20 μM of luteolin, and 40 μM of quercetin, which were significantly effective concentrations at reducing the protein levels of UBE2S. The mRNA levels of UBE2S were significantly decreased (Fig. 4F). These findings indicate that both luteolin and quercetin are able to repress expression of UBE2S in A431-III cells.

UBE2S was correlated with VHL and Hif-1α expressions

In previous reports, highly expressed UBE2S degraded VHL and improved Hif-1α stability in cancer cells. UBE2S is also

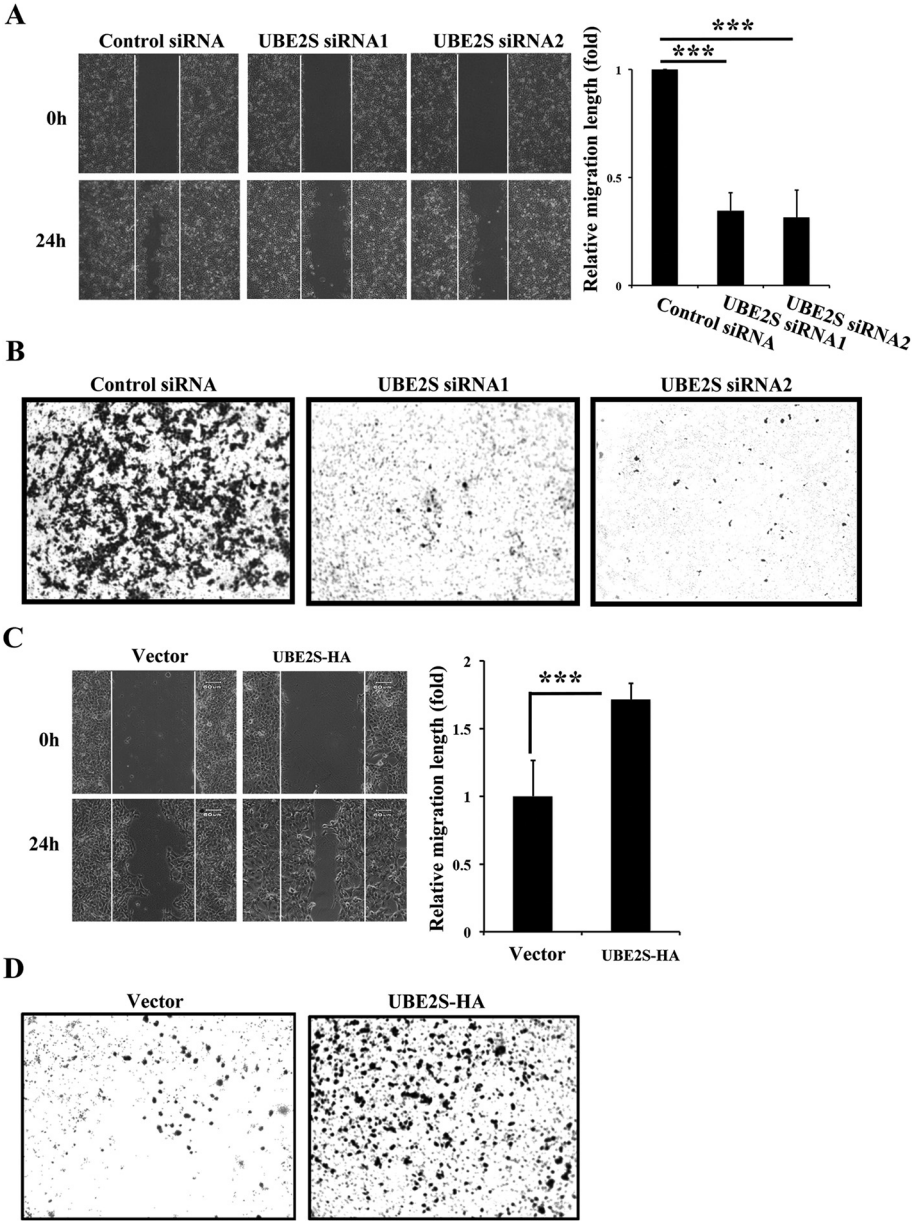

Fig. 2 UBE2S increases the migratory and invasive abilities of cervical cancer cells. A wound-healing experiment was used to analyze cell migration of A431-III cells after UBE2S siRNA knockdown (A) and UBE2S overexpression in A431-P cells (C). A trans-well assay was used to analyze the invasive ability of A431-III cells by UBE2S siRNA knockdown (B) and UBE2S overexpression in A431-P cells (D). The migration area was calculated and is presented on the right side. Data represent the mean (SD) of three different experiments. Statistical significance was determined by a one-way ANOVA with Tukey's test (***$P < 0.001$) in (A) and *t*-test (***$P < 0.001$) in (C).

transcriptionally activated by Hif-1α which contributes to a positive regulation of hypoxia signaling.[30] To test the correlations of UBE2S with VHL and Hif-1α in cervical cancer, we compared the protein levels of VHL and Hif-1α in the two A431 cell lines. Hif-1α was more highly expressed in A431-III than A431-P cells, while VHL showed opposite results (Fig. 5A and B). UBE2S was overexpressed in A431-P cells after transfection with pcDNA3-HA (vector) and pcDNA3-UBE2S (UBE2S-HA) plasmids. The protein level of Hif-1α significantly increased with UBE2S overexpression compared to control (vector only) (Fig. 5C and D). In contrast, VHL protein expression was decreased (Fig. 5C and D). We further knocked down UBE2S using two siRNAs in A431-III cells. The UBE2S protein level was significantly lower compared to control siRNA (Fig. 5E and F). Hif-1α protein expression was also diminished after UBE2S siRNA knockdown compared to control siRNA knockdown. In contrast, the protein level of VHL in A431-III cells increased with UBE2S siRNA knockdown compared to control siRNA (Fig. 5E and F). These findings suggest that UBE2S helps stabilize Hif-1α in cervical cancer.

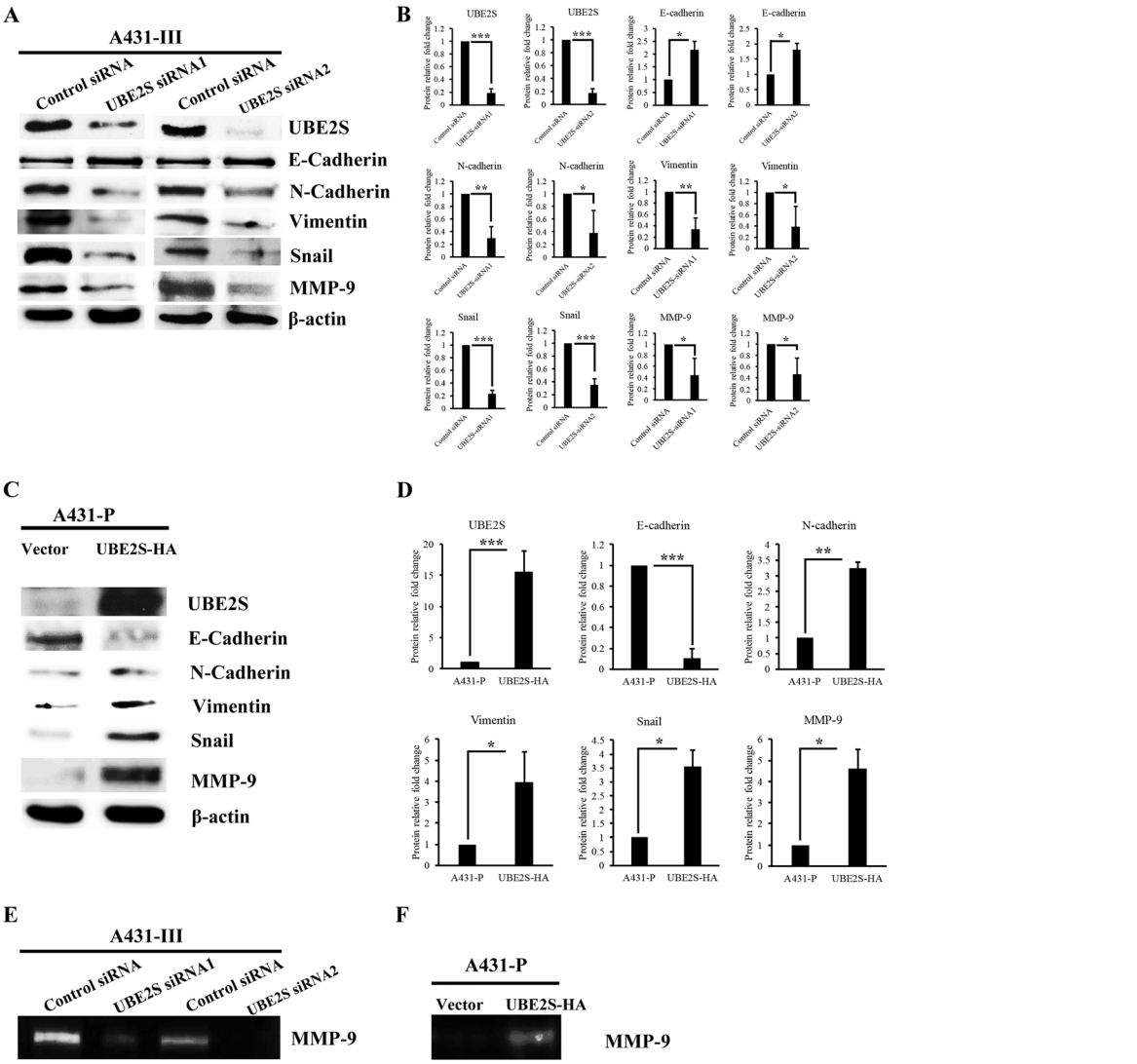

Fig. 3 UBE2S activates epithelial–mesenchymal transition (EMT) signaling and matrix metalloproteinase (MMP)-9 activity. (A) Protein expressions of EMT markers were analyzed after UBE2S siRNA knockdown in A431-III cells by western blotting. (B) Data represent the mean (SD) of three different experiments. Statistical significance was determined with a *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) in (A). (C) Overexpression of UBE2S in A431-P cells and analyzed EMT markers by western blotting. (D) Data represent the mean (SD) of three different experiments. Statistical significance was determined with a *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) in (C). (E) MMP-9 secretion was analyzed in UBE2S siRNA in A431-III cells (C) and overexpression in A431-P cells (F) by zymography.

Discussion

Herein, we showed that UBE2S enhanced the migration and invasion potential of cervical cancer cells through EMT signaling, and this effect could be inhibited by the flavonoids, luteolin and quercetin. UBE2S contributed a cardinal role to modulate the Hif-1α/VHL pathway.

The role of UBE2S in modulating cancer cell malignancy is poorly characterized. Using A431 parental tumor cells (A431-P) and a highly invasive derivative subline, namely A431-III,[31] we established a model system to systematically probe tumor invasion mechanisms. This model led us to find pronounced expressions of MMP-9 and several EMT markers, and all of which were associated with elevated invasion and an enhanced EMT[32] in A431-III cells compared to those of A431-P cells. To explore whether the observed higher malignancy of A431-III cells is a direct consequence of elevated UBE2S, we assessed UBE2S expression in these two cell types. Higher UBE2S gene transcript and protein levels were observed in A431-III cells than in A431-P cells. UBE2S overexpression and siRNA knockdown experiments furnished further evidence that UBE2S increased the migration and invasion of cervical cancer cells *via* EMT signaling. In contrast, the migratory and invasive abilities of A431-III cells were nullified by UBE2S siRNA knockdown. It is plausible that this regulation contributes to tumor cell malignancy.

Our previous findings illustrated that treatment with either of the two flavonoids, luteolin and quercetin, was able to

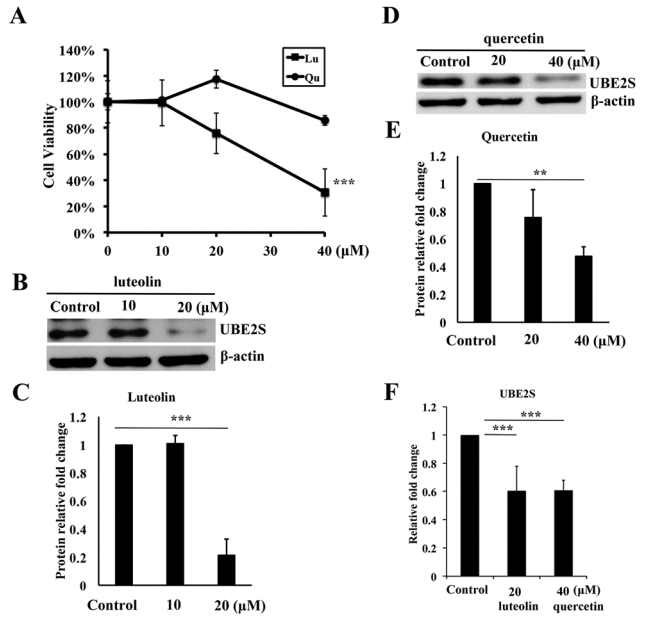

Fig. 4 Luteolin and quercetin inhibited UBE2S expression in A431-III cells. (A) Cell viability analysis of A431-III cells treated with luteolin and quercetin for 24 h using an MTT assay. Data represent the mean (SD) of three different experiments. Each experiment was performed in duplicate. Statistical significance was determined with a one-way ANOVA with Tukey's test (***P < 0.001). Western blot analysis of UBE2S expression levels in A431-III cells after either 0.1% DMSO (control), 10 and 20 μM of luteolin (B) or 20 and 40 μM of quercetin (D) treatment for 24 h. The protein level of UBE2S in (B) and (D) was measured by ImageJ software. Data represent the mean (SD) of three different experiments. Statistical significance was determined with a one-way ANOVA with Tukey's test (**P < 0.01, ***P < 0.001) (C, E). (F) Quantification of the transcript levels of UBE2S by a qPCR with 0.1% DMSO (control), 20 μM of luteolin, and 40 μM of quercetin treatment for 24 h. Data represent the mean (SD) of three different experiments. Statistical significance was determined with a one-way ANOVA with Tukey's test (***P < 0.001).

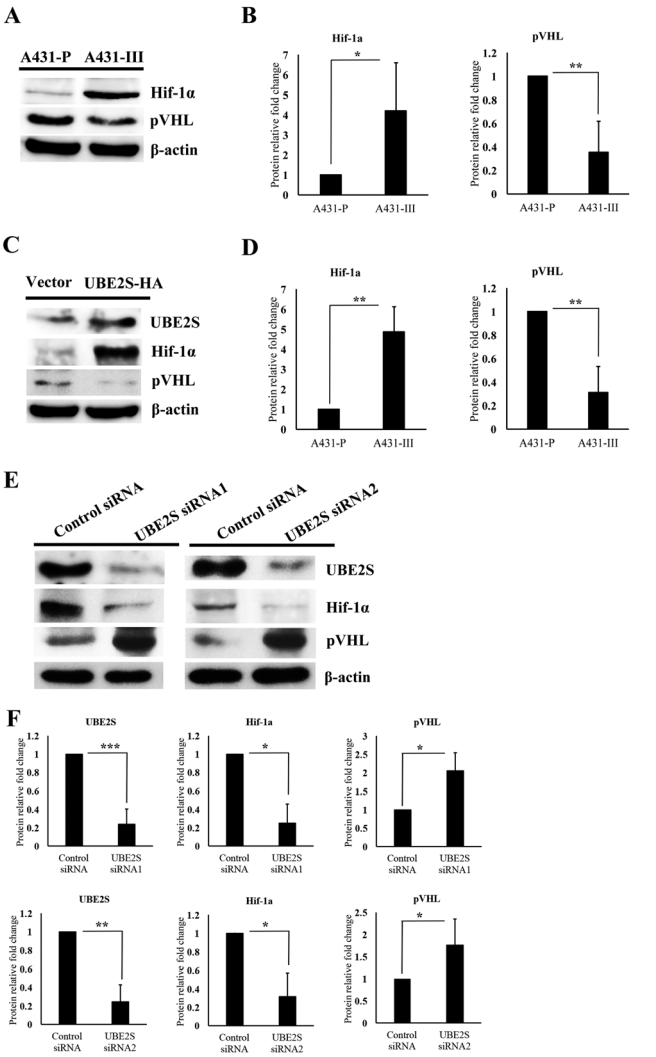

Fig. 5 UBE2S correlated with expressions of von Hippel-Lindau (VHL) and hypoxia-inducible factor (Hif)-1α. Protein levels of Hif-1α and VHL were analyzed by western blotting in A431-III and A431-P cells (A). The protein level of UBE2S in (A) was measured by ImageJ software. Data represent the mean (SD) of three different experiments. Statistical significance was determined with a t-test (*P < 0.05, **P < 0.01) (B). Protein levels of Hif-1α and VHL after UBE2S siRNA knockdown in A431-III cells were analyzed (C). The protein level of UBE2S in (C) was measured by ImageJ software. Data represent the mean (SD) of three different experiments. Statistical significance was determined with a t-test (**P < 0.01) (D). Protein expressions of Hif-1α and VHL were analyzed by western blotting after the overexpression of UBE2S in A431-P cells (E). (F) The protein level of UBE2S in (E) was measured by ImageJ software. Data represent the mean (SD) of three different experiments. Statistical significance was determined by a t-test (*P < 0.05, **P < 0.01, ***P < 0.001).

impair cell migration of A431-III cells through the inhibition of PI3K/Akt signaling.[14] In this study, we found that UBE2S contributed to cancer malignancy. The motility of cancer cells is a major cause of mortality in patients. The inhibition of the migration, invasion, and growth of cancer cells in distant organs is important in clinical treatment. Flavonoids isolated from plants, either total extracts or an identified single compound, contribute to the inhibition of cell motility and growth of cancer cells. Flavonoids identified from Korean *Citrus aurantium* L. were reported to inhibit the growth of non-small cell lung cancers *in vitro* and *in vivo*.[5] Total flavonoids from *Scutellaria barbata* inhibited the invasion of hepatocarcinoma *via* MMP/TIMP *in vitro*.[35] The flavonoid, diosmin, isolated from grape seeds and red wine, reduced metastasis of melanoma cells.[36] Total flavonoids of *Daphne genkwa* root inhibited the growth and metastasis of Lewis lung carcinoma.[37] The flavonoid, ampelopsin, inhibited the growth and metastasis of prostate cancer *in vitro*.[38] In our previous reports, the flavonoids, luteolin and quercetin, contributed to the repression of invasion and reversed the progression of EMT in cervical cancer.[14,34] In this study, we separately treated A431-III cells with these flavonoids. Both the protein expression and gene transcription of UBE2S decreased with either of these flavonoids. It is known that both luteolin and quercetin are inhibitors of multiple kinases and that such inhibition is able to block various signal cascades by hampering activation of key proteins. Reports on the modifying effects of flavonoids on

gene transcription in cancer cells are sparse. In the present study, we highlighted the downregulation by either luteolin or quercetin of UBE2S expression at both the mRNA and protein levels. In our previous investigations, we have suggested that luteolin and quercetin have the same ability to bind to ATP binding sites in PTK.[14,33,34,39–41] Luteolin and quercetin facilitate the following: inhibition of migration and invasion of A431-III cells through epithelial–mesenchymal transition signaling,[39] inhibition of Src/FAK/Cortactin signaling,[34] and mTOR/c-Myc signaling.[33] Combination treatment of luteolin and quercetin significantly reduced mRNA and protein levels of UBE2S (Fig. S1†). This inhibition might be related to blocking activation of EMT signaling.

UBE2S is highly expressed in a variety of tumor cells, and several investigators notably reported that UBE2S regulates the expression of Hif-1α *via* degradation of VHL, which delivers Hif-1α to the proteasome for degradation.[19–21,30,42] Hif-1α expression is activated through PI3K/Akt signaling. Furthermore, the inhibition of this pathway can be accomplished by exposure to plant flavonoids.[23,43] In our findings, UBE2S was more highly expressed in A431-III than A431-P cells. The protein level of Hif-1α was also higher in A431-III cells than in A431-P cells. VHL expression was reversed. Overexpression of UBE2S in A431-P cells and siRNA knockdown in A431-III cells revealed the regulation of Hif-1α and VHL. We suggest that UBE2S/VHL/Hif-1α signaling also contributes to cancer malignancy in cervical cancer. Although Hif-1α was reported to increase EMT signaling in prostate cancer,[44] hepatocellular carcinoma,[45] and pancreatic cancer,[46] it has not been reported in cervical cancer. In this study, we provide significant evidence to correlate expressions of UBE2S and Hif-1alpha.

In previous reports, the IC_{50} of luteolin by 24 h exposure to cervical cancer cells (HeLa) was 15.41 μM.[40] The IC_{50} of luteolin in breast cancer cells (T47-D and BT-474) by 24 h exposure was approximately 50 μM.[41] In our previous investigation, luteolin and quercetin were able to significantly reduce the growth of A431 cells, HepG2 cells (liver hepatocellular carcinoma), MCF-7 cells (breast cancer) and MiaPaCa-2 cells (pancreas adenocarcinoma).[39] The concentration of luteolin and quercetin used in the present study was similar to those employed in our previous studies.

In certain studies, flavonoids have been detected in low (micromolar) concentrations in blood following the consumption of onions, apples and tea.[47–49] The consumption of flavonoids from plant foods (and in vegetarian populations) could be several hundred milligrams.[50,51] A previous study documented that luteolin inhibited tumor growth in c-Met-overexpressed patient-derived tumor xenograft models of gastric cancer consequent to treatment with luteolin (10 mg kg^{-1} ip daily) for 30 days.[52] Luteolin inhibited MPA-induced breast cancer cell xenograft tumor growth by treatment with 20 mg kg^{-1} when tumor was grown to 60 mm^3.[41] Combinations of luteolin (100 mg per 10 kg weight) and quercetin (70 mg per 10 kg weight) were employed in a clinical trial aimed at assessing their anti-inflammatory effects relevant to behavior in children with autism spectrum disorder.[53] These reports provide an idea on the effective concentrations of luteolin and quercetin in order to accomplish efficacy. They also indicate that the active concentrations of these dietary flavonoids might be achievable in plasma.[13] Pharmacokinetics and bioavailability of quercetins were analyzed. Administration of onion supplement or quercetin-4′-*O*-glucoside (both equivalent to 100 mg quercetin, 331 μM), as well as quercetin-3-*O*-rutinoside and buckwheat tea (both equivalent to 200 mg quercetin, 662 μM) glycosides to 12 healthy volunteers was used to analyze the quercetin concentration within 24 h. Quercetin was rapidly absorbed from the onion supplement and quercetin-4′-*O*-glucoside and peak plasma concentrations of 2.3 ± 1.5 μg mL^{-1} and 2.1 ± 1.6 μg mL^{-1} (mean ± SD) were reached after 0.7 ± 0.2 hours and 0.7 ± 0.3 hours, respectively. After the administration of buckwheat tea and rutin, plasma peak was only 0.6 ± 0.7 μg mL^{-1} and 0.3 ± 0.3 μg mL^{-1}, respectively.[54] The single intravenous (100 mg) dose of quercetin administration was studied in 6 volunteers. The predominant half-life was 2.4 ± 0.2 h after intravenous administration.[55] A phase I clinical trial of quercetin was processed. Quercetin was administered by short intravenous infusion at escalating doses from 60 mg m^{-2} to 1700 mg m^{-2} initially at 3-week intervals. The serum level achieved immediately above 1 μM being maintained up to 4 h after injection of quercetin was in the range of 200–400 μM at 945 mg m^{-2}. One patient with stage 4 metastatic ovarian cancer, liver metastases and minor response to chemotherapy was entered into the trial. Following treatment with two courses of quercetin at 420 mg m^{-2}, the cancer antigen (CA) 125 decreased from 290 units per ml to 55 units per ml. Following treatment with three additional courses of quercetin at 500 mg m^{-2}, the CA 125 decreased to 45 units per ml after 6 months of treatment.[56] In our results, the efficient concentration of luteolin and quercetin used to inhibit expression of UBE2S was 20 and 40 μM. However, since this concentration may not be consistently present in human plasma and cancer patients, the long-term prevention efficiency should be further evaluated.

In conclusion, our findings suggest that the elevated levels of UBE2S expression in A431-III cells are linked to enhanced malignancy by UBE2S through EMT signaling. The invasiveness of cancer cells was activated through EMT signaling and MMP-9 enzyme activity. The dietary flavonoids, luteolin and quercetin, are independently capable of disrupting increased malignancy by altering UBE2S expression. UBE2S contributes to Hif-1α signaling through reducing the expression of VHL. UBE2S should be a potential therapeutic target for cervical cancer.

Abbreviations

A431-III	A431 subgroup III
A431-P	A431 parental
BSA	Bovine serum albumin
ECL	Enhanced chemiluminescence

EMT	Epithelial–mesenchymal transition
FBS	Fetal bovine serum
Hif-1α	Hypoxia-induced factor 1α
HRP	Horseradish peroxidase
mTOR	Mammalian target of rapamycin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RT	Reverse transcription
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
UBE2S	Ubiquitin E2S ligase
VHL	von Hippel-Lindau

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