Evodiamine Induces Cell Growth Arrest, Apoptosis and Suppresses Tumorigenesis in Human Urothelial Cell Carcinoma Cells

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Abstract. Background/Aim: Evodiamine, an indole alkaloid derived from Evodia rutaecarpa, exhibits pharmacological activities including vasodilatation, analgesia, anti-cardiovascular disease, anti-Alzheimer’s disease, anti-inflammation, and anti-tumor activity. Materials and Methods: This study analyzes the anti-tumor effects of evodiamine on cellular growth, tumorigenesis, cell cycle and apoptosis induction of human urothelial cell carcinoma (UCC) cells. Results: The present study showed that evodiamine significantly inhibited the proliferation of UCC cells in a dose- and time-dependent manner. Also, evodiamine suppressed the tumorigenesis of UCC cells in vitro. Moreover, evodiamine caused G2/M cell-cycle arrest and induced caspase-dependent apoptosis in UCC cells. Finally, we demonstrated that evodiamine exhibits better cytotoxic than 5-fluorouracil, a clinical chemotherapeutic drug, for UCC cells. Conclusion: Evodiamine induces growth inhibition, tumorigenesis suppression, cell-cycle arrest, and apoptosis induction in human UCC cells. Therefore, this agent displays a therapeutic potential for treating human UCC cells and is worthy for further investigation.

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Key Words: Evodiamine, urothelial cell carcinoma, apoptosis, cell-cycle arrest.
Natural compounds extracted from traditional Chinese herbs have been reported to exert anti-tumor potential and/or to enhance the effects of chemotherapeutics. Evodiamine, a quinoline alkaloid, is originally identified from the traditional Chinese herb *Evodia rutaecarpa* Bentham, and has exhibited multiple biological activities, including anti-cardiovascular disease, anti-Alzheimer’s disease, anti-obesity, anti-nociceptive, anti-nociception, anti-inflammation, anti-microbial, anti-influenza A virus and anti-tumor activity (8, 9). Evodiamine’s anti-tumor activity has been implicated in numerous tumors in humans, including esophageal squamous carcinoma, lung cancer, breast cancer, hepatoma, colon cancer, prostate cancer, bladder cancer, pancreatic cancer, gastric cancer, ovarian cancer, melanoma, glioblastoma and T-lymphoma (10-23).

Cell-cycle regulation is involved in the evodiamine mediated tumor suppression. Moreover, apoptosis and autophagy are also exhibited during evodiamine treatment in various human cancers (8). In addition, several pathways are shown to induce evodiamine-mediated cellular death, including NF-κB modulation, reactive oxygen species (ROS), phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway and extracellular signal–regulated kinases (ERKs) pathways (8). Evodiamine also has been identified to be the inhibitor of topoisomerases I and II and it may contribute to the anti-tumor property of evodiamine in human cancers (8, 24). Although anti-human urothelial cell carcinoma (UCC) effects of evodiamine have been reported to be involved in TRAIL-mediated apoptosis in two cell lines, the same is not clear regarding the effects of evodiamine on apoptotic signaling pathway and the cell cycle regulation (23). In the present study, we confirmed the anti-tumor properties in the human UCC cells by examining the cellular proliferation, tumorigenesis, cell cycle regulation and apoptosis and related signal pathways. Our data indicate that evodiamine could be a potential therapeutic agent for treating human UCC.

**Materials and Methods**

*Urothelial cell carcinoma cell lines and cell culture.* Two human UCC cell lines, 5637 and HT1197, obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) were used in the study (25). 5637 cells were generated from grade II patient, which carried *PIK3CA, FGFR3* wildtypes and *TERT* and *p53* mutations. H1197 cells were derived from grade IV patient, which carried *p53* wildtype, and *PIK3CA, FGFR3,* and *TERT* mutations. 5637 and HT1197 cells were maintained in minimum essential medium (MEM) (Gibco BRL, Grand Island, NY, USA) and RPMI1640 medium (Gibco) with 10% FBS (Gibco), respectively. Both cell types were incubated at 37˚C in 5% CO2 according to BCRC’s recommendations of cell culture conditions. Cell proliferation assay. UCC cells (5×10³/well) were seeded in 96-well cell culture plates and grown with the above mentioned medium. Cells were treated with medium only (containing 0.01% DMSO as the negative control) or medium containing various concentrations of evodiamine (Sigma, St. Louis, MO, USA). After treatment, the cell viability was determined by Cell Counting Kit-8 (CCK-8) (Sigma) per the manufacturer’s instructions. In addition, a pan-caspase inhibitor, Z-VAD-fmk (BioVision, Mountain View, CA, USA), was used to evaluate the caspase-dependent apoptosis on affecting the cellular viability of UCC cells using CCK-8 analysis (26-28).

**Colony formation analysis.** To determine the long-term effects of evodiamine exposure, UCC cells (300 cells/well) were plated in a 6-well culture dish and were pre-incubated with or without evodiamine for 72 h. After pre-treatment, cells were allowed to grow for 14 days to form colonies, and then the colonies were stained with crystal violet for further counting (27).

**Cell cycle analysis.** Cells (1×10⁵) were seeded in the plated and were further starved in serum-free medium for synchronizing cell cycle progression. Following starvation, cells were treated with DMSO or various concentrations of evodiamine at different time intervals. After treatment, cells were harvested and fixed with 70% methanol at 4˚C overnight. Fixed cells were further incubated with RNAse (10 mg/ml) and propidium iodide (PI; 1 mg/ml) (Sigma) at room temperature (RT) in the dark for 30 min. DNA content was analyzed using FACScan (Becton–Dickinson, San Diego, CA, USA) with ModFit LT 3.3 software. Moreover, the markers of cell cycle involving cyclin A (*GeneTex, Hsinchu City, Taiwan*), cyclin B1 (*GeneTex,* cd2c (Cell Signaling, Danvers, MA, USA) and phosphor-cdc25c (ser216) (Cell Signaling) were determined by Western blot analysis with specific antibodies (26, 29).

**Apoptosis analysis.** Effect of evodiamine on cell apoptosis was determined using fluorescein isothiocyanate (FITC)-labelled annexin-V/PI double staining (Sigma). Cells (1×10⁶) were treated with or without various concentrations of evodiamine for 24, 48, and 72 h. After incubation, cells were incubated with FITC-labelled annexin-V and PI for 15 min at RT. The intensity of annexin-V or PI fluorescence was analyzed by FACScan (Becton–Dickinson), and 10,000 cells were evaluated in each sample. Furthermore, the mechanism underlying evodiamine-mediated apoptosis, the activations/cleavages of Bid (Epitomics, California, CA, USA), Bcl-XL (Epitomics), PARP (Cell Signaling), caspase-3 (Cell Signaling), -8 (Cell Signaling), and -9 (Cell Signaling) were determined by Western blot with specific antibodies (26, 28, 30).

**Statistical analysis.** Data are presented as mean±standard deviation. Statistical evaluation was carried out by unpaired Student’s *t*-test with *p*<0.05 considered as statistically significant.

**Results**

*Evodiamine inhibited cell proliferation and colony formation ability of human UCC cells.* To evaluate the anti-tumor activities of evodiamine on UCC cells, 5637 and HT1197 cells were incubated with DMSO (a vehicle) or various concentrations of evodiamine. The cellular viabilities were detected after treatment. Figure 1A and B exhibited that evodiamine suppressed the proliferation of human UCC cells in a time- and dose-dependent manners. The cellular morphology was also examined through
microscopy (data not shown). The half-maximal inhibitory concentration (IC$_{50}$) of evodiamine in 5637 cells was 223, 1, and 0.5 μM at 24, 48 and 72 h, respectively. Moreover, the IC$_{50}$ in HT1197 cells was 38, 3 at 24 h, and 2.5 μM at 48 and 72 h, indicating that evodiamine can effectively inhibit the growth of UCC cell lines, and that 5637 cells were more sensitive to the evodiamine treatment (Figure 1). Additionally, to confirm the anti-tumor activities of evodiamine in 5637 and HT1197 cells, the carcinogenic property of UCC cells was determined by colony formation assay of 5637 and HT1197 cell that were treated with vehicle or evodiamine. Evodiamine could suppress tumorigenesis in a dose-dependent manner (Figure 2A and B). 5637 cells were more sensitive to evodiamine treatment than HT1197 cells, which was consistent with the anti-proliferation finding, suggesting that evodiamine is useful for treating human UCC cells.

**Evodiamine induced cell cycle arrest at G$_2$/M phase.** Since, evodiamine significantly reduced the proliferation of 5637 and HT1197 cells, the effect of evodiamine on cell cycle regulation and cell death was further investigated. Figure 3A and B show that evodiamine treatment caused cell cycle arrest of 5637 and HT1197 cells at G$_2$/M phase in a dose-dependent manner. To further evaluate the involvement of G$_2$/M checkpoint regulators, our data showed that expression of the cell cycle related proteins cdc2, cyclin A, and cyclin B1 were reduced and phospho-cdc25c (Ser216) was increased on UCC cells after evodiamine treatment (Figure 3C and D), indicating that evodiamine interfered with G$_2$/M regulators for arresting cell-cycle progression, which may contribute to inhibit proliferation and induce apoptosis of UCC cells.

**Evodiamine induced apoptosis in human UCC cells.** Based on the cell cycle analysis, evodiamine not only caused G$_2$/M arrest but also increased the ratio of sub-G$_1$ cells, the effect of evodiamine on the apoptosis of UCC cells were further verified by annexin V and PI staining (Figure 4A and B). Annexin V positive+PI negative fraction indicates the early apoptotic cells, and annexin V+PI double positive fraction indicates the late apoptotic cells. The results showed that evodiamine induced apoptosis in 5637 and HT1197 cells in the dose- and time-dependent manner. Due to the apoptotic effect of evodiamine, the underlying molecular mechanism of the apoptosis of UCC cells was explored. Figure 4C and D show that evodiamine enhanced cleavage of caspases-8, -9, and -3 and PARP. Furthermore, Figure 4C and D demonstrates that evodiamine decreased the expression of Bid in a dose- and time-dependent manner, which suggests the increase of the truncated Bid protein that can promote apoptosis in 5637 and HT1197 cells. Moreover, the expression of Bcl-XL, a pro-survival Bcl-2 member protein, was also dose- and time-dependently suppressed after the treatment of evodiamine at 48 and 72 h in 5637 and HT1197 cells. These results (Figure 4) indicate that both intrinsic and extrinsic pathways are involved in evodiamine-mediated cellular apoptosis in human UCC cells.

To further assay the impact of caspase-dependent apoptosis in evodiamine-mediated anti-tumor effect, Z-VAD-FMK, a pan-caspase-inhibitor, was used to elucidate caspase-mediated apoptosis under evodiamine treatment. Figure 5A and D show that the cleavages of caspase-3, -8, and PARP were reduced under Z-VAD-FMK treatment in both 5637 and HT1197 cells. Moreover, the reduction of cellular viability mediated by evodiamine was significantly reversed under Z-VAD-FMK treatment in 5637 cells (Figure 5B and C) but not HT1197 cells (Figure 5E and F), suggesting that evodiamine stimulated...
caspase-dependent apoptosis is a critical event in the viability of human UCC cells, but the existence of caspase-independent mechanism might also contribute to evodiamine-mediated cytotoxicity in different human UCC cells.

Evodiamine exhibited better anti-tumor activity than 5-fluorouracil. Previous and present studies demonstrated that evodiamine has an anti-cancer activity in various human cancer and UCC cells. We further evaluates the therapeutic potency of evodiamine and the clinical chemotherapeutic agent 5-fluorouracil (5-FU), a common chemotherapeutic agent used in several human cancer treatments. We, therefore, compared the anti-tumor activity of evodiamine and 5-FU in 5637 cells after the treatment of 24 and 48 h. The result showed that low dose of evodiamine (1 μM) exhibited similar effect with a higher dose of 5-FU (10 μM) regarding viability suppression in human UCC cells after 24 or 48 h treatments (Figure 6). However, there was no significant synergistic effect between the combination of evodiamine with 5-FU in UCC cells (Figure 6). These findings indicate that evodiamine is an alternative therapeutic agent and might be better than 5-FU in human UCC treatment.

Evodiamine regulated signaling pathways in human UCC cells. To evaluate the signaling pathways involved in evodiamine-treated human UCC cells, 5637 and HT1197 cells were incubated with evodiamine, and the mitogen-activated protein kinase (MAPK) signaling changes were determined by western blot. The data revealed that evodiamine treatment could strongly elevate the phosphorylation levels of P38 and JNK in both cell lines (Figure 7); however, enhancement of phosphorylation of ERK was observed in HT1197 cells but not in 5637 cells after 48 h treatment, (Figure 7). Here, our data suggest that evodiamine could modulate MAPK signaling pathways, which may involve in the regulation of cell proliferation or apoptosis of UCC cells.
Discussion

In the present study, we demonstrated an anti-tumor ability of evodiamine in human UCC cells through inducing G₂/M cell-cycle arrest and both intrinsic and extrinsic apoptotic pathways. Among them, the growth inhibition as well as tumorigenesis inhibition and apoptosis induction on 5637 cells were more susceptible to evodiamine treatment (Figures 1, 2 and 5). However, HT1197 cells were more susceptible for a short-term treatment (24 h) of evodiamine than 5637 cells (Figure 1). The anticancer activity of evodiamine has also been demonstrated in various cancers, including lung cancer, breast cancer, hepatoma, melanoma, oral cancer, colon cancer, cervical cancer, etc., and our findings in this
Figure 4. Continued
study are similar with these reports (10-22). It has also been reported that evodiamine suppresses human UCC cells through a TRAIL-mediated apoptosis (23). In the present study, the regulation of evodiamine in tumorigenesis, cell cycle and MAPK signaling pathways in human UCC cells was demonstrated. Our findings and others strongly suggest that evodiamine can be a potential therapeutic agent for treating human UCC cells.

In addition, evaluations of novel therapeutic agents that have less toxicity in normal cells for treatment are important and challenging tasks. Regarding the medical safety profile, evodiamine has been reported to be with low toxicity in human normal peripheral blood mononuclear cells (PBMC) and in mice (31, 32). Moreover, a randomized, double-blind clinical trial in premenopausal women, the evodia extract including evodiamine 6.75 mg and rutaecarpine 0.66 mg was administered and no significant adverse effects was observed in serum tests or in the self-questionnaire (33). This study suggests that evodiamine is safe to used in humans.

Evodiamine induced cell-cycle arrest at G2/M phase in human UCC cells was firstly observed in our study (Figure 3). It also reported in various cancers post-treatment with evodiamine including human leukemic T-lymphocytes, prostate cancer, cervix carcinoma, thyroid cancer, colorectal cancer, ovarian cancer, and lung cancer (10, 11, 14, 15, 22, 34-38). In addition, we and others have demonstrated apoptosis in human UCC and other cancers after evodiamine treatment. Evodiamine induces a TRAIL-related apoptosis has been reported in human UCC cells (253J and T24) (23). In our study, both intrinsic- and extrinsic-apoptotic pathways were involved in UCC cells during evodiamine treatment (Figure 4). However, 5637 cells were more susceptible to evodiamine stimulation than 1197 cells in viability, tumorgenesis, G2/M cell cycle arrest and apoptosis. It may need to explore that the genetic diversity between these human UCC cells this variation. Among them, only HT1197 cells show a mutation in FGFR3, others exhibit wildtype FGFR3 (39). TRAIL expression in human UCCs has been demonstrated to positively correlate with FGFR3 expression (40). Mutations in the extracellular domain (codons 248, 249) or within the transmembrane domain and its vicinity (codons 372, 375, 382, 393) of FGFR3 lead to receptor dimerization in the absence of ligand (41). Mutations in FGFR3 result in higher risks of cancer recurrence and progression (42). Moreover, it has been reported that FGFR3 signaling promotes the activation of PI3K/Akt/mTOR pathway for suppressing apoptosis (41). Therefore, the activations between FGFR3 and TRAIL signals in evodiamine-induced extrinsic-caspase apoptotic pathway are needed for further investigation.

In addition, blocking the activation of caspases with Z-VAD-FMK in 5637 and HT1197 cells, the total cell number was rescued significantly in 5637 cells only (Figure 5). These data suggests that in addition to caspase-dependent...
Figure 5. Evodiamine mediated apoptosis plays an anti-cancer effect is cell type dependent in human urothelial cell carcinoma cells. (A, B, and C) 5637 and (D, E, and F) HT1197 cells were co-incubated with evodiamine without or with Z-VAD-FMK (a pan-caspase inhibitor) for 48 h, and the expressions of activated form of caspases were detected by Western blot. GAPDH was used as a loading control. Moreover, cell viabilities of these cells were also investigated with microscopy and examined with CCK-8 analysis. Data shown represent mean±SD (**p<0.001) of three independent experiments.
apoptosis, the cell-cycle regulation may play the key role in evodiamine-mediated anticancer effect in HT1197 cells. However, a comprehensive investigation of the anti-tumor activity of evodiamine in all of the human UCC cell lines as well as in mice is also recommended.

MAPK signals including ERK, P38, and JNK involving in cell differentiation, proliferation, and apoptosis are well known. In the present report, we demonstrated that evodiamine treatment significantly elevated the activations in p38 and partially in JNK pathways in 5637 and HT1197 cells, and only decreasing activation of ERK pathway in 5637 cells (Figure 7), and our findings is similar with others (11, 18, 32, 43). It is worth further investigation for the roles of MAPK pathways involved in evodiamine regarding anticancer effects in human UCCs. Therefore, it is worthy to further investigate the different involvement of MAPK pathways on evodiamine activated anticancer effects in various human UCCs.

In the present study, we demonstrated that treatment with evodiamine could inhibit cellular proliferation, tumorigenesis, cell cycle progression, and induce caspase-dependent apoptosis in human UCC cells. An intrinsic caspase-dependent apoptosis with or without an extrinsic caspase-dependent apoptosis pathway were exhibit in human UCC cells after incubation with evodiamine. Therefore, evodiamine is suggested to be a chemotherapeutic agent for human UCCs and is worthy of further investigation in clinical therapeutics.

**Acknowledgements**

This study was supported by grants from Chang Gung Memorial Hospital, Chaiyi, Taiwan (CMRPG6D0091-2), and Ditmanson Medical Foundation Chia-Yi Christian Hospital, Chiayi city, Taiwan (R104-35). Furthermore, we thank the Conjoint Laboratory and the Flow Cytometer Room of the Department of Medical Research, Chang Gung Memorial Hospital, Chaiyi, Taiwan for their provision of the necessary apparatus, facilities, and flow cytometric analysis of this study.

**References**


