Original Article
Synergistic effects of snail and quercetin on renal cell carcinoma Caki-2 by altering AKT/mTOR/ERK1/2 signaling pathways

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Abstract: Renal cell carcinoma has become the most common subtype of kidney cancer, and has the highest propensity to manifest as metastatic disease. Because of lack of knowledge in events that correlated with tumor cell migration and invasion, few therapeutic options are available. Therefore, in current study, we explore the anti-tumoral effect of a potential chemopreventive natural product, quercetin, combined with anti-sense oligo gene therapy (inhibiting Snail gene). We found that either one of them had the remarkable effects in suppressing cell proliferation and migration, inducing cell cycle arrest and apoptosis in a ccRCC cell line, Caki-2 cells. The combination of both means provides even strong suppressive effects toward these ccRCC cells. Our study, for the first time, provides the possibility of using a novel treatment for renal cancer, by combining natural product and gene therapy.

Keywords: Snail, quercetin, renal cell carcinoma, AKT/mTOR/ERK1/2

Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common malignant tumor in kidney, and more than 20% of patients after curative surgery develop server metastasis during follow-up [1, 2]. The cancer cells are capable of invading tissue barriers composed of basement membrane and extracellular matrix, which causes tumor metastasis. Epithelial-mesenchymal transition (EMT), changes of polarized epithelial cells to migratory, sarcomatoid phenotypic cells, is considered to be the crucial event during malignant tumor progression and metastasis. The indispensable transformation of epithelial cells into mesenchymal cells results in their acquisition of interstitial cell characteristics. Therefore, the cells become invasive. The hallmark of EMT is the loss of cell adhesion molecule E-cadherin, and regulators for EMT served as E-cadherin repressors (Valastyan and Weinberg, 2011) [3].

Snail is a zinc-finger transcription factor, which specifically targets E-cadherin promoter region, E-box sequence (CAGGTG), serving as an important transcriptional inhibitory protein and a repressor of E-cadherin [4]. Naturally, it has been largely confirmed that in tumorigenesis and metastasis, EMT affects numerous transcription factors, including Snail. Snail expression has been observed in many malignant tumors and is related with server invasion and metastatic potential of the tumors, indicating that Snail a key player in the induction of tumor invasion and metastasis [4, 5]. In ccRCC, it also has been reported that Snail is detectable with positive associations with primary tumor stage and nuclear grade. The down-regulation of Snail in RCC cell line, 786-O, led to decreased expression of MMP2/9 and increased level of E-cadherin, along with suppression of cell invasion [6, 7]. Mechanistically, Snail has been shown to bind to DNA binding domain of p53 and diminishes the tumor suppressive function of p53. All these preliminary data provide a new therapeutic approach as new cancer strategies.

Cancer prevention using natural products has become a new trend in cancer control, as high consumption of certain natural products is
believed to contribute to decreased risks of several types of cancer [8-10]. Quercetin (3,3',4',5,7-pentahydroxyflavone), a natural flavonoid, is found in many plant-based foods, such as apples, tea, capers, parsley and a number of berries. The potential chemopreventive effects of quercetin have been identified to be associated with various mechanisms including anti-oxidative activity, regulation of cell cycle and apoptosis, inhibitory effects enzymes that activate carcinogens, modification on key signal transduction pathways and interaction with cell receptors and other proteins [11, 12]. In addition, epidemiological studies have proposed the anti-tumoral effect of quercetin, and in particular, on pancreatic cancer [13, 14]. However, the exact effect of quercetin in mediating cancer cell migration or the formation of EMT is largely unveiled. Recently, quercetin was identified as the inhibitor of Snail-p53 binding, mainly through interfering with CK1/GSK-3 mediated Snail phosphorylation [13]. Therefore, we propose here that quercetin execute the anti tumor effect through interfering with Snail expression/phosphorylation, and explore the potential synergistic effects of inhibiting Snail and applying quercetin. In present study, we analyzed the effects of quercetin on proliferation and metastasis abilities of clear renal cell line, Caki-2, as well as the synergistic effects of slicing Snail and quercetin. This synergistic effect is observed not only in inhibiting cellular proliferation, causing cell cycle arrest, but also in suppressing cancer cell migration and promoting its apoptosis. Our research provides the potential novel treatment, by using the combination of natural product and Snail shRNA, as an integral part of cancer control.

Materials and methods

Materials

Quercetin (> 95% purity) was purchased from Sigma (Cat# Q4951 St. Louis, MO), dissolved in DMSO, aliquoted and stored at -20°C. The antibodies anti-VEGF, anti-VEGFR2, anti-COX2, anti-CD147 were purchased from Abcam (Cambridge, MA). The anti-E-Cadherin was obtained from Santa Cruz Biotech (Santa Cruz, CA). The other antibodies including anti-HIF-1, anti-AKT, anti-mTOR, anti-ERK1/2, anti-GAPDH, phosphor-specific anti-AKT (Ser473), anti-mTOR (Ser2448), anti-ERK (Thr202/Tyr204) were obtained from Cell Signaling (Beverly, MA). All other chemicals were obtained either from Sigma or Fisher Scientific (Pittsburgh, PA).

Cell culture and treatment

Human clear cell renal cell carcinoma (ccRCC) cell line Caki-2 is purchased from ATCC (HTB47). It displays epithelial morphology and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum plus antibiotics (100 units penicillin /ml and 100 μg streptomycin/ml). The cells were cultured at 37°C under a humidified 5% CO₂ environment.

Generation of snail shRNA constructs

Vector pCMV-G&NR-U6 was purchased from JRDUN Biotechnology Co. (Shanghai, China), and packing plasmids, including VSG-G, pol/gag was obtained from Addgene. Three sequences of short hairpin RNA, including sh275, sh689 and sh447, were designed to target human SNAIL gene, following the rules suggested at http://www.rockefeller.edu/labheads/tuschl/sirna.html and using the software available at http://jura.wi.mit.edu/bioc/siRNAext/ following the guidelines provided (ref). A BLAST search was also performed with the candidate sequences against the human genome to avoid non-specific gene silencing. 3 hairpin oligos were then cloned into expression plasmid pCMV-G&NR-U6 via BamHI and HindIII. The ligation product was transformed into E.Coli XL-1 Blue and selected with Ampicillin. This is a 2nd generation lentiviral vector that expresses shRNA under the human U6 promoter. A CMV-GFP reporter cassette is included in the vector to monitor expression. All 3 viral shRNA constructs were tested in vivo by quantitative real-time PCR to choose the one with the most efficient silencing effect. Snail shRNA (Sh275) (AGATGAGGACAGTGGGAAA) was chosen as having the best silencing effect, and used in all following experiments.

Cellular proliferation assay

Cell proliferation assays were performed with a Cell Counting Kit-8 (Dojindo, Japan). Caki-2 cells were plated in 24-well plates in triplicate at about 5 × 10⁴ cells per well. Then cells were treated with various treatment conditions and the numbers of cells per well were measured by the absorbance (450 nm) of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-
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5-(2,4-isulfophen-yl)-2H-tetrazolium, monosodium salt) at the indicated time points.

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR) analyses

Total RNA from cells was isolated by using Trizol (Invitrogen, #15596). Reverse transcription was performed with RevertAid First Strand cDNA Synthesis Kit (Thermo, K1622). Quantitative PCR was performed with SYBR Green PCR Master Mix (Thermo, F-415XL) on Applied Biosystems 7300 Fast Real-Time PCR System. Sequences of primers used are listed as the following: GAPDH (sense: 5’-CACCCACTCCTCCAC-CTTTG-3’, anti-sense: 5’-CCACCACCGTCTTGTGTTGAAG-3’), Snail (sense: 5’-TTCCCTGAAGCTGGC- TGTCTG-3’, anti-sense: 5’-TGGCCTGAGGGTTTCAGTAG-3’). Data were normalized to GAPDH mRNA content, by using efficiency (2-ΔΔCt) method (Livak and Schmittgen, 2001).

Cell cycle flow cytometric analysis

For FACS analysis, Caki-2 cells were harvested in 0.25% trypsin solution, fixed in cold 75% ethanol, and stored at -20°C for 16 hours. Fixed cells were subsequently washed once with cold PBS, treated with RNase A (100 μg/ml) in PBS and 50 μg/mlpropidium iodide (PI) for 30 min in the dark. DNA content of cells was quantified in a BD Biosciences FACSCalibur with 10,000
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**Apoptosis analysis**

The apoptotic cells were detected using annexin V-FITC along with PI solution, by flow cytometry assay according to manufacturer’s instructions. Briefly, Caki-2 cells were treated under various conditions as indicated. Then, cells were gently washed twice with annexin-binding buffer. When cells appear detached from the plate, cells were released with gentle tapping, collected and suspended in cold binding buffer and stained with annexin V FITC and PI solution. Analysis was conducted for 20,000 cells using a flow cytometer with CellQuest software. In each of the graphs, the bottom right quadrant represents cells in early apoptosis.

**Transwell invasion assay**

Transwell invasion assay was performed as described before. Basically, Caki-2 cells (10⁵ cells/transwell) along with indicated treatment (shRNA or quercetin or both) were seeded into the upper compartment of invasion chambers. The bottom chambers were filled with normal RPMI 1640 medium. After 24 hours incubation, migrated cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet in 2% ethanol. Following termination of the invasion assay, images were obtained under an inverted microscope. Image J (NIH, Bethesda, MD) was used to count the number of invaded cells (cell counter tool).

**SDS-PAGE and Western blotting assays**

Cultured cells were collected on ice with 0.5-1 ml of IP buffer and then lysed by sonication for 10 sec. Cell lysates were centrifuged for 45 min at 13,000 g to remove cellular debris. Total proteins in cell lysates were separated by 10-12% SDS-PAGE. Western blotting assay was performed by probing with antibodies according to manufacturer’s instructions. Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000) or anti-mouse IgG (1:10,000) using chemiluminescence reagent kit (ECL).

**Statistical analysis**

All the data were analyzed using GraphPad Prism 5.04 software (GraphPad Software, La Jolla, CA). One-way ANOVA and Student-Newman Keul’s test were used for more than two compared groups and paired Student t test was used for comparison between two groups. Unless otherwise specified in the Figure Legends, the data are presented as the mean ± SEM of at least three determinations. Asterisks indicate the degree of significant differences, *P < 0.05, **P < 0.01, ***P < 0.001.

**Results**

**Generation of snail shRNA and knock-down of snail in renal cancer cells**

pCMV-G&NR-U6 shRNA delivery system (Figure 1A) is appealing tool which maximize the efficiency of knock down specific gene expression in cells, by utilizing powerful human U6 promoter and expressing GFP under pCMV promoter as a monitoring tool. Here, we designed 3 sets of short hairpin RNA (sh275, AGATGAGGAC-AGTGGGAA; sh689, CTCAGATGTCAAGAAGTAC; sh447, GCTCGAAAGGCCTTCAACT) and inserted them into HindIII/BamHI site respectively (Figure 1B). After successfully cloning and confirmed by sequencing, DNA constructs were
transfected into 293T cells and the viruses were collected and saved in -80°C for further use. We compared the knockdown efficiency by examining the mRNA expression levels in Caki-2 cells that were infected by individual Snail shRNA construct (Figure 1C). We finally chose sh275 as the most effective shRNA for knocking down Snail in Caki-2 cells, for all the following experiments. The mRNA expression level of Snail in Caki-2 with sh275 was only 30% compared to cells infected with scramble shRNA, suggesting an effective knocking down of Snail in Caki-2 cells. Western blotting assay was also performed here, showing about 60% reduction in Snail protein expression when sh275 viruses were applied, compared to the ones in control and scramble shRNA infected cells (Figure 1D), further confirmed the effectiveness of Snail shRNA (sh275) in RCC Caki-2 cells. The Snail sh275 shRNA were used in all the following experiments.

Snail shRNA and quercetin synergistically inhibit the proliferation of renal cancer cells

Caki-2 cell line is human clear cell renal cancer cell carcinoma (ccRCC), and it displays epithelial morphology. The Caki-2 cells have a loss-of-function mutation in von Hippel-Lindau tumor-suppressor protein and are known to form tumors in immunocompromised mice. This cell line is a useful preclinical model for studying renal cancer [15]. Natural product, quercetin, has been shown to cause bi-directional modification on proliferation of certain cancer cells. For example, quercetin, at a relatively high concentration (> 50 uM), causes a significant decrease in cell proliferation of colon carcinoma cell lines; however, at lower concentrations (< 20 uM), a subtle but significant stimulation of cell proliferation was observed for mammary adenocarcinoma, as well as colon cancer cell lines. Therefore, certain higher concentration of quercetin often is used to inhibit the growth of human cancer cells. Here, we first investigated the possible anticancer activity of this drug on Caki-2 renal cancer cells. At the dose of 10 µg/ml (33 µM), quercetin caused a slight but significant reduction (11% in 24 hours and 18% in 48 hours) in cellular proliferation on Caki-2 cells (Figure 2), measured by CCK-8-WST-8 proliferation assay. Slicing Snail, a transcription inhibitor factor for E-cadherin, also showed the similar degree of suppression on cellular proliferation. Remarkably, the combination of both caused the further inhibitory effects on Caki-2 cellular proliferation, 18% in 24 hours and 25% in 48 hours. The control groups, both control (DMSO only) and Scramble shRNA, did not cause any reduction on cellular proliferation, suggesting the specific effectiveness.

Slicing snail and applying quercetin synergistically cause the cell cycle arrest

As cell cycle is normally linked to cellular proliferation, to further understand the mechanism

![Graph](image-url)
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Figure 4. Synergistic effect of Snail and quercetin on promotion of the Caki-2 cell apoptosis. A. Caki-2 cells were treated with Snail shRNA and quercetin (10 µg/ml) as indicated. Cells were then harvested and co-stained with FITC-conjugated annexin V and PI for 15 minutes, and analyzed by flow cytometer as described in Methods. B. Histogram represents mean ± SE of percentage of cell population in apoptotic phase in three independent experiments. Significant difference from control cells is indicated by *P <0.05 and **P < 0.01.
that the inhibitory effects of knocking-down Snail and quercetin on cancer cell proliferation, we investigated the effects of slicing Snail and using quercetin on cell cycle progression (Figure 3). Caki-2 cells with Scramble shRNA or Snail shRNA were treated with 10 µg/ml quercetin for 24 hours, and then cells were collected and stained with PI solution prior to flow cytometry analysis. As expected, Compared to controls, Caki-2 cells treated with quercetin or infected with Snail shRNA had a significantly increased cell population in G1 phase, less cells in S phase or G2 phase, indicating a G1/S cell cycle arrest. Applying both Snail shRNA and quercetin, more cells were found in G1 and much less cells were in S phase, as compared to either Snail shRNA or quercetin treatment alone. This synergistic effect between Snail shRNA and quercetin is similar to what we observed in proliferation assay, suggesting that suppressing renal cell proliferation by both Snail shRNA and quercetin is very like through inhibiting cell cycle progress.

Both snail shRNA and quercetin promote apoptosis in ccRCC

Cellular proliferation, differentiation and death are the basic and essential processes in multicellular organisms. Especially, in cancer cells, uncontrolled proliferation can sometime be associated with a high level of apoptosis, suggesting that cell proliferation and apoptosis pathways are closely linked [16]. To further access the anti-tumorigenic features of Snail shRNA and quercetin, we sought to find out if Snail shRNA and quercetin have any effects on renal cancer cell apoptosis (Figure 4). We utilized Annexin-V staining, which detects phosphatidylserine (PS) when it is flipped and translocated from the inner to the outer leaflet of the membrane in the intermediate stages of apoptosis. Highly fluorescent annexin V conjugates provide quick and reliable detection methods for studying apoptosis. There were nearly no apoptotic cells that can be detected in parental Caki-2 cells or cells treated with Scramble shRNA. Snail shRNA could largely enhance apoptotic cell population by about 10%, and treatment of quercetin alone had even bigger effects on promoting apoptosis in renal cancer cells (16% apoptotic cells). Moreover, the combination of both Snail shRNA and quercetin remarkably showed a synergistic effect on inducing apoptosis in renal cancer cells (25% apoptotic cells). These data provide an inverse relationship between proliferation and apoptosis in renal cancer cells treated by Snail shRNA or/and quercetin, and partially explains that suppressed cellular proliferation by Snail shRNA or/and quercetin is very likely due to enhanced apoptosis.

Snail shRNA and quercetin suppresses the invasion and migration capabilities in ccRCC-Caki-2 cells

Migration and invasion of renal cancer cells are essential processes during renal carcinoma metastasis. To evaluate the invasion capability in vitro of Snail shRNA and quercetin on RCC Caki-2 cells, we examined the effect of Snail shRNA and/or quercetin on the invasion and migration abilities by Transwell assay (Figure 5). The data suggested that both Snail shRNA and quercetin significantly abolished the invasion and migration abilities of Caki-2 cells. The average numbers of cells invaded into the lower chamber were 253 and 376, when cells were infected with Snail shRNA or quercetin alone respectively, compared to 877 cells in control group and 846 cells in scramble shRNA group. The cell number decreased to average of 156/well when both Snail shRNA and quercetin were applied. Our results strongly suggested that either knocking down of Snail or quercetin treatment resulted in the suppression of the migration and invasive capabilities in renal cancer Caki-2 cells. More importantly, the combination of Snail shRNA and quercetin showed a significant and even more remarkable reduction on renal cancer cell migration and invasion, implying the potential synergistic effects of Snail shRNA and quercetin on renal cancer metastasis.

Snail shRNA and quercetin regulates the expression of matrix proteins and Akt/mTOR/ERK signaling pathways

Snail has been long known as a direct repressor of E-cadherin, an epithelial adhesion protein and significantly inhibited in more than 80% of renal cell carcinoma patients [17]. Down regulation of Snail significantly elevates the protein expression level of E-cadherin in RCC Caki-2 cells (Figure 6B), confirming the role of Snail as a suppressor of E-cadherin. Interestingly, even though treatment of quercetin alone does not increase E-cadherin level,
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the combination of using Snail shRNA and quercetin results in a dramatic increase in E-cadherin protein expression. This result implied a strong synergistic effect of both means in regulating E-cadherin, potentially provides a better therapeutic option for treating renal cell carcinoma patients. Cyclooxygenase (COX)-2 is overexpressed in many types of cancers including renal cell carcinoma [18]. In clinic, Snail and COX2 are shown previously to be associated with various tumor grade and survival rate of patients with gliomas [16]. Here, for the first, we showed that inhibition of Snail also inhibits the expression of Cox2 protein, and quercetin even shows greater inhibition upon Cox2 expression. The combination of both Snail shRNA and quercetin inhibits the expression of Cox2 equal to quercetin alone, suggesting that Snail shRNA might be upstream to quercetin’s effect on regulation of Cox2. Hypoxia-inducible factor (HIF), a transcription factor that respond to changes in oxygen level in cells or environment, is constitutively activated in the majority of clear cell RCC (ccRCC), through inactivation of the von Hippel-Lindau (VHL) gene [19]. Similar to E-cadherin, Snail shRNA alone inhibits expression of HIF-1 but quercetin alone does not have such effect. Interestingly, the greater effect is observed on inhibiting expression of HIF-1 when both Snail shRNA and quercetin were applied. Similar inhibitory effects is observed on the expression of Vascular endothelial growth factor (VEGF), a signal protein stimulating vasculogenesis and angiogenesis, and its receptor protein VEGFR2, which suggested that Snail and quercetin is very likely to play important roles in regulating vasculogenesis and angiogenesis processes in renal cell carcinoma. Associated with VEGF, CD147, extracellular matrix metalloproteinase inducer, is also shown to be positive in majority of patients with various stages of RCC [20]. Snail shRNA alone, or the combination of Snail shRNA and quercetin, is able to suppress the expression of CD147 to the similar extent, further confirming the role of Snail and quercetin in regulating RCC metastasis. All the above data demonstrated that Snail and quercetin are involved in signaling pathways that play important roles in mediating RCC tumorigenesis and metastasis.

Akt/mTOR and ERK signaling pathways have been shown to participate in renal cancer proliferation and metastasis. mTOR is frequently dysregulated and selected as a potential target for RCC patients [21]. Akt directly phosphorylates mTOR and plays a critical role in the proliferation, survival and motility of RCC. Therefore, here, we investigate the effect of Snail and

Figure 5. Inhibition of Snail and quercetin treatment act synergistically to suppress Caki-2 cell migration. A. Caki-2 cells were first plated in the top compartment of invasion chambers. After Snail shRNA and quercetin treatment, migrated cells were then fixed and stained with crystal violet. Then, the images of cells in bottom chambers were taken under an inverted microscope. B. Cells numbers were counted by Image J. Histogram represents mean ± SE of three independent experiments. Significant difference from control cells is indicated by *P < 0.05 and **P < 0.01.
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Figure 6. Inhibition of Snail and quercetin treatment alters the Akt/mTOR/ERK signaling pathways. ccRCC Caki-2 cells were treated as described above. The expression of E-cadherin, COX2, HIF-1a, VEGF/VEGFR2, CD147, and the phosphorylated or total Akt, mTOR, ERK1/2 proteins was detected by Western blot. GAPDH was used as a control for sample loading.

Discussion

In recently years, quercetin has been studied extensively as a potential anti-cancer agent with a minimal toxicity or carcinogenicity [12]. Major molecular mechanisms of action of quercetin include down-regulation of mutant p53 protein, causing G1 phase arrest in various cancer cells, functioning as a tyrosine kinase inhibitor. Quercetin was the first tyrosin kinase inhibitor that even passed human phase I trial, which makes quercetin envisioned as a possible anti-cancer drug without the cytotoxic side-effects with conventional chemotherapy. Contradictory to the previous studies, Kuanchou Chen, Robert Peng, and colleagues [22] note that quercetin, along with another antioxidant ferulic acid, appeared to aggravate, but not suppress kidney cancer in diabetic laboratory rats. Similar study was published recently, which showed that general antioxidants, N-acetylcysteine and vitamin E, remarkably increased tumor progression in lung cancer mouse models [23]. Though the effects of antioxidant could
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be cell/tumor type specific, these surprising results inspired us to further investigate the role of quercetin in suppressing or promoting renal cancer using specific RCC cell line and study the potential molecular mechanisms. Here, in current research, we found that quercetin is capable of inhibiting the proliferation rate and migration of ccRCC Caki-2 cells, causing the G1 cell cycle arrest, inducing apoptosis of ccRCC Caki-2 cells, all of which might related with Akt/mTOR/ERK signaling pathways. Inhibition of Snail by shRNA has the similar effects on ccRCC Caki-2 cells, and the combination of both Snail shRNA and quercetin marks even greater effects. These data suggest that quercetin alone or Snail shRNA, or the combination of both can significantly inhibit the growth and migration of RCC, and the potential molecular mechanisms might involve Akt/mTOR/ERK signaling pathways.

Quercetin has been intensively studied in the past for its anti-tumor properties. Currently, in our research, we first revealed that inhibition of Snail and exposure of cells to quercetin resulted in a significant reduction in proliferation rate starting at 24 hours. At 48 hours, Snail shRNA alone or quercetin alone leads to 30% reduction, and the combination of both Snail shRNA and quercetin leads to more than 40% reduction in proliferation. The results again confirmed in agree with most previous studies, that in most types of cancer, including renal cancer, quercetin indeed displays an inhibitory effect on cellular proliferation. The reduction in cellular proliferation could results from inhibition/arrest of cell cycle progression, and/or due to advanced cell death or apoptosis. Cell cycle analysis by FACS-PI staining showed that either Snail shRNAor quercetin cause significantly cell cycle arrest at G1/S phase, and meanwhile, 10-15% cells were detected positive in Annexin V/PI staining, v.s. nearly 0% in control groups. Consistently, the combination of Snail shRNA and quercetin cause the 20% more cells arrest at G1/S phase, and 15% more cells undergoing apoptosis. Here, we concluded that treatment of ccRCC Caki-2 cell line with either Snail shRNA or quercetin causes a significant reduction in cell proliferation, a strong cell cycle arrest at G1/S phase with a concomitant induction of apoptosis. The combination treatment caused a remarkable and greater suppression in proliferation and cell cycle progress, with a stronger apoptotic effect. Furthermore, we assessed the effects of Snail shRNA or quercetin on migration ability of ccRCC Caki-2 cells, by transwell assay. Consistently, we found that either Snail shRNA or quercetin causes a significant reduction on cell numbers that pass through the gel, nearly 70% reduction by Snail shRNA and 60% reduction by quercetin when compared to control groups. More reduction, over 80%, was observed when both Snail shRNA and quercetin were applied to cells.

In various tumors, tumorigenesis, invasion and metastasis are forming a complicated network, involving many molecular signaling pathways, cellular factors and other tumor-promoting factors. Particularly in renal cancer, tumor invasion and metastasis, in addition to the biological activity of kidney cell transformation and migration, currently receive remarkable attention. The treatment of renal cell carcinoma is becoming extremely difficulty, as metastatic kidney cancer is more common in patients now days. Thus, investigating renal cancer metastasis and infiltration are becoming focal points in kidney cancer research. Previous studies have demonstrated that mesenchymal-epithelial transition in cancer infiltration during migration is closely related with development of epithelial-mesenchymal transition (EMT). Numerous transcription factors, including Snail, affect EMT in tumorigenesis. Here, in our research, in specific ccRCC Caki-2 cell line, we provide the consistent data showing that Snail shRNA promotes E-cadherin expression, and quercetin further enhances this effect. The up-regulation of E-cadherin will potential results in alterations to the cell morphology of cancer, preventing structural instability of organization and the destruction of the connective tissues, delaying the renal tumor invasion and metastasis, which has already been shown in various stages of cancer, such as breast ductal carcinoma. The results from previous studies demonstrate a high level of Snail expression in ccRCC with a positive detectable rate of 82.96% compared with those in the normal tissues. The tumor stage, classification, invasion and metastasis are correlated with the expression of Snail. Hence, Snail, to a certain extent, may reflect the ability of the tumor cells to invade, particularly in renal cell carcinoma. Inhibiting Snail by using specific Snail shRNA provides an efficient and potential means to treat ccRCC patients. Besides E-cadherin, COX2 and VEGF are also
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considered positive makers for ccRCC, in which previous studies showed that 63.4% for COX2, and 68% for VEGF of ccRCC patients showed positive expression. And also various clinicopathologic features, including sizes and stages of tumor, are associated with COX2 and VEGF expression. Inhibitory effects of Snail shRNA and/or quercetin on COX2 and VEGF expression further confirmed the anti-tumor effects of Snail shRNA and/or quercetin, especially in renal cell carcinoma. The Akt/mTOR signaling pathway is a highly deregulated pathway in most of cancers. mTOR exists in two complexes, mTORC1 and mTORC2. Akt phosphorylated at T308 inhibits TSC1/2 complex to activated mTORC1; mTORC2 is recognized as the kinase phosphorylating Akt at S473. Previous reports show that in ovarian cancers, E-cadherin down-regulation mediated by Insulin-like growth factor-1 requires the Akt/mTOR signaling pathway [24]. As Snail and/or quercetin enhance E-cadherin expression level, we consistently found that Akt/mTOR signaling pathway was also regulated by Snail and/or quercetin. Inhibition of Snail significantly down regulated the ratios of p-Akt/Akt and p-mTOR/mTOR, thus resulting in the suppression of Akt/mTOR signaling pathway. ERK signaling pathway was also disrupted by Snail shRNA and/or quercetin treatment, as the p-ERK/ERK ratio was significantly reduced. Therefore, our data suggest that Snail and/or quercetin might regulate proliferation, cell cycle, cell migration, as well as apoptosis via Akt/mTOR/ERK.

In summary, we demonstrated that inhibition of Snail and/or quercetin treatment inhibited cellular proliferation, cell cycle progression and migration, and induced apoptosis in ccRCC Caki-2 cells, by simultaneously modulating the expression of E-cadherin, COX2, HIF-1, VEGF/VEGFR2, CD147, and Akt/mTOR/ERK signaling pathways. Our findings provide new insights into the molecular mechanisms of Snail/quercetin-mediated proliferation inhibition and apoptosis induction, suggesting that the combination of natural products and gene therapy may be a novel therapeutical strategy for the prevention and treatment of human renal cancer.

Disclosure of conflict of interest

None.

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