Transcriptional Expression of Bcl-2, Her2, VEGF, and hTERT in Caki-1 Human Renal Cancer Cells Modulated by *Cornus mas* Extract

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Received 2020 September 18; Revised 2020 October 09; Accepted 2020 October 21.

Abstract

**Background:** Herbal medicines, particularly those rich in polyphenolic compounds, have been proposed to be chemotherapeutic factors, which can modulate several pathways associated with cancer. To gain mechanistic insights into the anti-proliferative impacts of *Cornus mas* extract (CME), this study investigated the expression changes of several prominent genes, which involved in malignancy with therapeutic potential.

**Objectives:** The aim of the study was to determine the anticancer potential of CME on the main regulatory genes in renal carcinogenesis.

**Methods:** To perform the research, Caki-1 cancer cells were incubated for 72 h with 250 µg/ml of CME upon the cells with ribonucleic acids (RNAs) extracted for identified alterations of human telomerase reverse transcriptase (hTERT), vascular endothelial growth factor (VEGF), human epidermal growth factor receptor 2 (Her2), and B-cell lymphoma-2 (Bcl-2) gene expressions by a quantitative reverse transcription-polymerase chain reaction. The changes in protein expression were analyzed by the western blot method. Cell apoptosis was detected using the flow cytometry technique.

**Results:** *Cornus mas* extract caused down-regulated Bcl-2 as an anti-apoptotic 4.34-fold gene expression. Moreover, Her2 oncogene messenger RNA expression was inhibited by 250 µg/ml concentration of ~10-fold CME. The antitumor activity of CME was pronounced in its potent anti-angiogenic potential, as CME resulted in a striking decrease in ~125-fold expression of VEGF compared to the untreated control. In contrast, CME led to ~2.6-fold up-regulation of hTERT in Caki-1 cancer cells.

**Conclusion:** Overall, various molecular pathways were formed to interplay with Caki-1 cells, which depended on the active phenolic compound of CME. It is recommended to perform further studies to investigate the effect of unique polyphenols of the total extract of CME to establish an effective strategy for renal cancer treatment.

**Keywords:** *Cornus mas* extract, Bcl-2, Her2, hTERT, Renal cancer, VEGF

1. Background

Cancer is a hyperproliferative disorder, which affects public health and results in proliferation, angiogenesis, inhibition of apoptosis, irreversible cell change, invasion, and metastasis (1). The incidence of renal cancer (RCa) is extensively affected by racial/ethnic, genetic factors, and country origin, in which the rate of incidence in Western countries is higher than in Asian countries (2,3). Caki-1 is an RCa cell line with potential metastatic activity, which is useful in studying the curative interventions in progressive RCa cells (4).

The majority of cancers are often diagnosed upon having reached an end-stage in which extensive chemotherapy is used. Although chemotherapy may transitiorly decelerate neoplasm development, eventually these cancer cells often lose effectiveness and develop a drug-resistance property. Moreover, some chemotherapeutic agents may not be appropriate for long-term administration because of severe side effects(5). Therefore, it is important to develop novel strategies that inhibit the progression/invasive of cancer cells. In this regard, phytochemicals and their synthetic derivatives have intrigued huge attention and reduced the severity or improvement of several human disorders, including cancer. Pharmacological effects of most phytochemical compounds are exerted via a multi-targeted approach, such as dysregulation of multiple genes, as well as correlated cell signaling pathways at diverse stages of initiation, progression, and metastasis (6).

Dietary factors play a crucial role in the expansion/inhibition of RCa (2); in this regard, the effects of herbs on ameliorating diseases, including cancers, are undeniable. Diets rich in flavonoids have been correlated with a lower incidence of morbidity and mortality rates related to RCa (7). Cornelian cherry (*Cornus mas* L.) is a plant of an extensive natural range that has been already known in garden cultivation for years. Its bark, leaves, flowers, and fruits have been recognized and appreciated in folk medicine for a long time, especially in Asia (8). Cornelian cherry is a flowering plant in the dogwood species native to Southern Europe and Southwestern Asia. Fresh cornelian cherry fruits possess twice as
The aim of the study was to identify the anticancer effect of CME treatment on the Bcl-2, Her2, VEGF and hTERT expression in renal cancer cells.

3. Methods

3.1. Preparation of the extract

The leaves were shade-dried and ground into powder with mortar and pestle. The prepared powder was kept in tight containers and protected completely from light. The process of hydroalcoholic extraction was carried out by macerating 100 g of the powdered dry plant in 500 mL of 80% ethanol for 48 h at room temperature. Subsequently, the macerated plant material was extracted with 80% ethanol solvent by percolator apparatus (2-liter volume) at 25°C. The extracted plant leaves were removed from the percolator, filtered through a 0.22 μm syringe filter, and dried under reduced pressure at 37°C with the rotary evaporator. The concentrated leave extracts of the plant were dissolved in Phosphate Buffered Saline (PBS) and filtered to obtain a stock solution of 10 mg/ml.

3.2. Cell culture

In this study, the human renal cancer cell line (i.e., Caki-1) was bought from the Cell Bank of Shanghai, China. All cells were cultured in Roswell Park Memorial Institute 1640 with +2 mM L-glutamine supplemented with 10% fetal bovine serum (Gibco Carlsbad, USA) and 1% penicillin/streptomycin (Invitrogen, USA) and incubated in a humidified atmosphere of 5% (vol/vol) CO2 and 95% air (vol/vol) at 37°C.

3.3. Treatment of cells

Caki-1 cells were plated in 24 well plates (100,000 cells/500 μl/well) and treated with 0 (control) and 0.25 mg/mL concentration of CME for 72 h at 37°C. The basis of 250 μg/mL of CME was assessed using our previous study, which displayed nearly 55% growth inhibition of Caki-1 cells.

3.4. Ribonucleic acid isolation and quantitative reverse transcription polymerase chain reaction

To isolate total ribonucleic acid (RNA) from cells, the AccuZolTM reagent was applied according to the manufacturer’s instructions protocol (Bioneer, Korea). Ribonucleic acid underwent deoxyribonuclease treatment (Fermentas, Hanover, USA) and was transcribed to complementary DNA (cDNA) by RevertAid™ First Strand cDNA Synthesis Kit (Fermentase, Hanover, USA). Following, cDNA was detected by an iCycler IQ Multicolor Real-Time PCR Detection System (Bio-Rad, Foster City, USA) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA). The sequence of primers used in

Figure 1. Chemical structure of the main anthocyanins present in Cornus mas extracts.
the qRT-PCR reaction is listed in Table 1. Polymerase chain reaction amplification was conducted at 95°C for 3 min, followed by 45 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, and a final incubation at 72°C for 5 min. All experiments were carried out in triplicates. Raw data were analyzed with the comparative Ct method using β-actin as an internal reference gene.

3.5. Western blotting

The cells were lysed with RNA immune-precipitation assay lysis buffer (Beyotime, China). The protein concentration was detected by BCA Protein Assay Kit (Beyotime, China). In this regard, 35 μg protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis method and then transferred to polyvinylidene fluoride membranes. Afterward, 8% skim milk dissolved in Tris-buffered saline containing 0.2% Tween was used to block the membranes for 2 h at room temperature, with subsequent incubation by specific primary antibodies for overnight. The membranes were washed and then incubated with the secondary antibody alkaline phosphatase-conjugated at room temperature for 2 h. Beta-actin was applied on the same membrane as a loading reference. Subsequently, membranes were visualized using an enhanced chemiluminescence system (Bio-Rad Laboratories, Hercules, California).

3.6. Cell apoptosis measurement

At the end of the treatments, cells were washed with PBS. Transfected cells were detached with 0.25% trypsin and centrifuged at 2500 × g for 3 min at room temperature. Cell pellets were suspended in 10 μl of fluorescein isothiocyanate (FITC)-labeled Annexin V-FITC/Propidium Iodide solution for 20 min at dark in room temperature and then analysis was carried out on FACS Calibur system (Becton Dickinson Immunocytometry Systems, CA).

3.7. Statistical analysis

All quantitative results presented in this study are mean ± SEM of three identical tests made in three replicates. The statistical analysis between the groups over time was carried out by analysis of variance (i.e., ANOVA) followed by Duncan’s test. A p-value of < 0.05 was considered significant. The collected data were analyzed by GraphPad Prism software (version 6.01; San Diego, CA).

4. Results

4.1. Cornus mas extract inhibited Bcl-2, Her-2, and VEGF expression in Caki-1 cancer cells

Based on the results, 250 µg/ml concentrations of CME reduced messenger RNA (mRNA) level of the Bcl-2 as an anti-apoptotic gene (Figure 2A). Moreover, Her-2 mRNA level showed an approximately 10-fold decrease in Caki-1 renal cancer cells, compared to the negative control (P<0.01; Figure 2B). The vascular endothelial growth factor is the main pro-angiogenic factor involved in tumor progression. Vascular endothelial growth factor expression was radically inhibited by the treatment of 250 µg/ml concentration of CME in Caki-1 cells, compared to that of untreated control (P<0.01). Furthermore, the relative fold change decreases in VEGF expression Caki-1 (~125-fold; Figure 2C).

4.2. Cornus mas extract overexpressed human telomerase reverse transcriptase expression in Caki-1 cancer cells

Compared to the untreated control, CME enhanced hTERT mRNA and protein expression to values of ~2.6-fold in Caki-1 cancer cells (P<0.01; Figure 2D).

4.3. Cornus mas extract modulated vascular endothelial growth factor gene expression the most and human telomerase reverse transcriptase the least in Caki-1 cancer cells

The order of relative transcript down-regulation of different oncogenes in renal cancer cells were obtained as follows: VEGF (1=125 fold) > Her2 (1=10 fold) > Bcl-2 (0.235=4.34 fold) > hTERT (2.6-fold overexpression). In addition, it was found out that VEGF with 125-fold down-regulation and hTERT with 2.6-fold up-regulation were respectively the most and the least affected genes in Caki-1 cancer cells.

4.4. Cornus mas extract changed expression levels of hTERT, VEGF, Her2, and Bcl-2 proteins in Caki-1 cells after treatment

The protein expressions of Bcl-2, Her2, VEGF, and table 1 sequence of primers used in quantitative reverse transcription polymerase chain reaction

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-cell lymphoma-2</td>
<td>Forward: 5'-CTGCAAGCAGGCTGGCCTTT-3'</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGCCTGCTGGTCTCCCTCCTC-3'</td>
<td></td>
</tr>
<tr>
<td>Human epidermal growth factor 2</td>
<td>Forward: 5'-GCCGCTCCCTGCTAGGTGTCA-3'</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TCTCCGGAGGCTGTAAGTTG-3'</td>
<td></td>
</tr>
<tr>
<td>Vascular endothelial growth</td>
<td>Forward: 5'-CTCCTGGAGCTGTAAGTTG-3'</td>
<td>92</td>
</tr>
<tr>
<td>factor</td>
<td>Reverse: 5'-CTCCTGGAGCTGTAAGTTG-3'</td>
<td></td>
</tr>
<tr>
<td>Human telomerase reverse</td>
<td>Forward: 5'-GGAGCTCTGCGTTGAGCCTC-3'</td>
<td>96</td>
</tr>
<tr>
<td>transcriptase</td>
<td>Reverse: 5'-GGAGCTCTGCGTTGAGCCTC-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5'-AACCTGTGTTAGCCCTT-3'</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AACCTGTGTTAGCCCTT-3'</td>
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</tbody>
</table>
Figure 2. The graph represents the relative expression of Bcl-2, Her2, VEGF, and hTERT genes upon normalization with β-actin. The data are illustrated as mean ± SEM values from three replicate experiments. Statistical significance was identified by analysis of variance, followed by Duncan’s test (**P<0.01, *P<0.05 in comparison to control). Cornus mas extract modulates the expression of Caki-1 in RCa cells. (A) B-cell lymphoma-2 anti-apoptotic gene expression was significantly inhibited, (B) Cornus mas extract resulted in considerable inhibition of Her2 oncogene expression, (C) Transcriptional expression of the main pro-angiogenic factor-VEGF was markedly suppressed by CME, and (D) human telomerase reverse transcriptase expression in Caki-1 cells was significantly overexpressed upon treatment with 0.25 mg/ml concentration of CME.

Figure 3. The expression levels of hTERT, VEGF, Her2, and Bcl-2 proteins in Caki-1 cells after treatment by CME. Western blot Representation of target proteins and β-actin as an internal control.

hTERT were determined by the western blot method. Based on the result, CME significantly decreased Her2, VEGF, and Bcl-2, and increased hTERT in comparison to the control group (Figure 3).

4.5. Cornus mas extract induced apoptosis in Caki-1 cells
To investigate the role of CME in the apoptosis of Caki-1 cells, the researchers determined the apoptosis rate of 48 after treatment. Flow cytometric results showed that CME dramatically enhanced apoptosis (Figure 4).

5. Discussion
Notwithstanding the development of novel treatment approaches, the role of herbal products, particularly those rich in antioxidant activities, have permanently been regarded as safer agents that could
Apoptosis is defined as an ordered and orchestrated programmed cell death with a crucial role in eradicating damaged or senile cells (19). Some important regulatory apoptotic proteins have been recognized, such as Bcl-2 and Bcl-2-associated X protein (Bax) that are anti-apoptotic and pro-apoptotic factors, respectively (20). B-cell lymphoma-2 is located principally on the outer layer of mitochondria and its up-regulation prevents cells from undergoing apoptosis. *Cornus mas* extract causes down-regulation of Bcl-2 in carcinoma cells by reversing its inhibitory effect on the release of cytochrome c from mitochondria into the cytosol and results in the initiation of apoptosis (21). Disruption of this pathway contributes to a pathological aggregation of cells and a perpetuation of abnormal gene expression, which can generate cancer development and progression (19). Lee et al. (22) revealed that the apoptotic processes induced by flavonoid-derived quercetin were necessary for exerted functional role mediated by the dissociation of Bax from B-cell lymphoma-extra large and the activation of caspase families in human RCa cell line (i.e., LNCaP). This perspective is in line with our findings regarding the anti-Bcl-2 activity of ANTs of CME.

Furthermore, Her2 is one of the receptor tyrosine kinase (RTK) pathway elements that the overexpression of Her2 is known to be one of the most leading causes of progression of different types of cancer (23). Andersson et al. showed that RTK targeting therapeutics may affect the RCa cancer cells phenotypic profile, which associates with treatment outcome. Additionally, their observation of such changes during therapy could be used for treatment monitoring and as an improved therapy outcome (23). Overexpression of Her2 is involved in the suppression of apoptosis, cell division, and enhanced cell motility. Our results showed that CME decreased mRNA expression of Her2 in Caki-1 (~10-fold), these findings suggest that the apoptotic potential of CME is partly mediated by simultaneous down-regulation of Bcl-2 and Her2 in Caki-1 cancer cells. Furthermore, Banerjee et al. (24) demonstrated that the combined treatment of curcumin with docetaxel led to the down-regulation of the expression of EGFR and Her2 resulting in the repression of the expression of phosphatidylinositol 3-kinase/protein kinase B pathway, which inhibited the expression of nuclear factor kappa-light-chain-enhancer of activated B cells, therefore blocking RCa cells proliferation as well as its inducing apoptosis.

Telomerase is an RNA-dependent DNA polymerase playing an important role in making a curative intervention in many cancers such as RCa. Human telomerase reverse transcriptase is the catalytic subunit of telomerase that is affected during RCa (1). Telomerase activity in healthy cells is low, while it is over-activated in the majority of malignancy, which confers this element as a rational target for anti-neoplastic agents (25). The expression of telomerase has been reported as a prominent characteristic of RCa. Ashtiani et al. (1) reported that achillea wilhelmsii extract could inhibit hTERT mRNA expression, which was enhanced in RCa, and exert an apoptotic effect on RCa cell line. Additionally, our
previous study on the anti-proliferative activity of Melissa officinalis extract against Caki-1 cancer cells revealed that hTERT expression is down-regulated in this cell line (26). Inversely, our evaluation of CME potential on hTERT expression showed that CME leads to ~2.6-fold up-regulation of hTERT in Caki-1 cancer cells. These discrepancies may be connected with the presence or absence of some specific polyphenols or doses applied for the assays, as well as the kind of studied cell/tissue.

Angiogenesis is recognized as an important hallmark of cancer (27). The delicate balance between inducers/inhibitors of angiogenesis leads to the control of angiogenesis. The angiogenesis inducers, such as angiogenin, placental growth factor, VEGF, basic fibroblast growth factor (bFGF), tumor necrosis factor-alpha, transforming growth factor (TGF)-alpha, TGF-β, interleukin-8, hepatocyte growth factor, and epidermal growth factor, are constantly produced during cancer growth. Among these angiogenesis inducers, VEGF and bFGF are especially crucial in tumor angiogenesis (28). Indeed, inhibitor agents of angiogenesis are divided into two major groups of direct (i.e., endothelial cells and cancer cells) or indirect (targetting cancer-associated stromal cells) (28). Vascular endothelial growth factor binds to its receptors through the mitogen-activated protein kinase (MAPK) signaling pathway, which results in stimulating endothelial cell proliferation. The results of a majority of studies reveal that VEGF plays a critical role in RCa angiogenesis, which indeed has been observed to be up-regulated in RCa cells, and a high level of VEGF is correlated with metastasis to other tissues (28). The investigations conducted in this regard indicated that VEGF expression increased only in metastatic tumor samples, while it experienced a decrease in primary neoplasms (29).

6. Conclusion

Based on the results, it can be concluded that treatment with 250 μg/mL of CME led to the down-regulation of Bcl-2, Her2, and VEGF expression, compared to the untreated control in Caki-1 cells. In contrast, CME leads to the up-regulation of hTERT in Caki-1 cancer cells. These findings create new perspectives into the anti-proliferative properties of CME, which can be allotted as a complementary treatment besides the principle therapies for RCa.

Footnotes

Conflict of Interests: The authors declare that there is no conflict of interest.

References


