Original Paper

Cyanidin Curtails Renal Cell Carcinoma
Tumorigenesis

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Key Words

Renal cell carcinoma • Cyanidin • Apoptosis • Migration • Invasion

Abstract:

Background/Aims: Cyanidin is an anthocyanin found in many foods. Although its variable antioxidant levels are well-documented, little is known about its effects on renal cell carcinoma (RCC) tumorigenesis. This study, therefore, investigated the effects of cyanidin on the proliferation, migration, and invasion of renal cell carcinoma lines and demonstrated, for the first time, significant inhibitory effects of cyanidin on RCC tumorigenesis. **Methods:** RCC cells were treated with different doses of cyanidin and the effects were tested by Cell Counting Kit-8 reagent, clone formation assay, transwell assay, and flow cytometry. Moreover, the cyanidin-mediated mechanism that curtailed tumorigenesis was analyzed by RNA sequencing (RNA-seq). Sequencing data from The Cancer Genome Atlas (TCGA) were used to compare the expression of both early growth response protein 1 (EGR1) and selenoprotein W (SEPW1) in RCC and tumor-free adjacent normal tissue samples. Real-time PCR (RT-PCR) and/or western blot were used to assess the expression of E-cadherin, cleaved-caspase3, Bcl2, p62, and ATG4. **Results:** We found significantly greater induction of cell-cycle arrest, apoptosis, and suppression of RCC cell invasion and migration at concentrations of 25 μ M and 100 μ M than at a concentration of 50 μ M. It was also discovered, first through RNA-seq then confirmed by RT-PCR, that cyanidin (100 μ M) inhibited RCC carcinogenesis through EGR1 and SEPW1. TCGA data indicated that the expression level of EGR1 was lower and that of SEPW1 was higher in RCC tumor tissue than in normal tissues. Moreover, western blot and/or RT-PCR indicated that cleaved-caspase3 was enhanced and E-cadherin was inhibited by cyanidin treatment. Furthermore, western blot and RT-PCR also showed regulation of p62 and ATG4, which are associated with autophagy. Cyanidin *in vivo* significantly inhibited the growth of xenografts in nude mice. **Conclusions:** The results of this study showed the therapeutic potential of cyanidin for the treatment of RCC and the prevention of recurrence and metastasis.

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Introduction

Renal cell carcinoma (RCC) accounts for 2-3% of all malignant diseases in adults, and is the most common type of kidney cancer. Currently, surgical resection of the kidneys remains the most effective treatment for RCC because almost all RCC pathological types are resistant to chemotherapeutic and radiation therapies. However, roughly one-third of patients with RCC are diagnosed during the later phases when the cancer has progressed to the point that significant opportunities for surgical treatment have already been missed. Although immunotherapy is currently a key treatment for the late phase of the disease, the response rate is less than 20% [1], and while the responses of metastatic RCC to sorafenib and sunitinib are better [2, 3], the overall survival rate remains poor. Therefore, alternative anti-tumor agents and methods that are highly efficient are strongly needed. The identification of novel therapeutic molecules for RCC will help to provide an understanding of the pathogenesis that leads to tumorigenesis and improve outcomes for patients.

Recently, numerous natural products have served as important sources for the development of potent anti-tumor drugs [4, 5]. Flavonoids have unique and varied chemical structures and include a large variety of polyphenolic compounds that occur naturally in plant foods [6]. Extensive studies have shown that the consumption of dietary flavonoids affords significant anti-tumor effects [7-10]. Moreover, there have been multiple studies performed at the cellular level to investigate the effects of flavonoids on tumor proliferation, migration, and invasion [11-13]. This study focused on cyanidin, which belongs to a branch of the flavone compounds. Cyanidin is a well-defined anthocyanidin compound that occurs in fruits and vegetables [14] and has various biological functions such as antioxidant and anti-inflammatory activities [15-17]. Cyanidin is not the most common dietary anthocyanidin, however, and it occurs naturally in its glycosylated form, namely, cyanidin-3-glucoside (C3G). After intake, C3G can be metabolized by oral epithelial cells and absorbed by the gastric epithelium (1-10%), where it is gut-transformed and enters the bloodstream (<1%) and urine (about 0.02%) [18]. After oral administration, the predominant flavonoid species in the gastrointestinal (GI) mucosa and liver is C3G, whilst after IV dosing the majority of anthocyanins are C3G metabolites, which are mostly products of methylation and glucuronidation. Cyanidin is a minor metabolite in the gut [19]. The effects of anthocyanidins on chronic diseases, carcinomas, and health promotion have been intensively studied over the last few decades [14, 20-24], but the impact of cyanidin on renal cell carcinoma has not yet been revealed.

We therefore investigated the effects of cyanidin on RCC and demonstrated its role in RCC cell proliferation, invasion, apoptosis, and the cell cycle *in vitro*. The results of our investigations showed that cyanidin-induced cell-cycle arrest and apoptosis and the suppression of RCC cell invasion and migration were significantly greater at concentrations of 25 μ M and 100 μ M than at concentrations of 50 μ M. Moreover, the RNA-seq results indicated that cyanidin (100 μ M) increased the expression of EGR1 and decreased the expression of SEPW1 in RCC cell lines compared with a control group and confirmed by RT-PCR. Previous research has shown that quercetin (a flavonoid) induces apoptosis in colon carcinoma cells via EGR1 [25], and that the silencing of SEPW1 mRNA expression causes cells to accumulate in the G0/G1 phase of the cell cycle via P53 and p21 [26]. The present study demonstrated that cyanidin mediated its anticancer effects via EGR1 and SEPW1 and provided a mechanistic basis for the anticancer effects on RCC cells. Furthermore, western blot assay indicated that both the cleavage of the apoptotic mediator caspase3 and inhibition of E-cadherin activation were enhanced, while additional western blot and RT-PCR revealed the regulation of both P62 and ATG4, which are associated with autophagy. *In vivo*, cyanidin (0.5 mg/mouse) significantly inhibited the growth of xenografts in the nude mouse model; however, the anti-proliferation activities of cyanidin and its underlying mechanisms on cancer cells remain largely unknown. If the mechanisms can be elucidated in detail, they may provide future guidance for the development of cyanidin as a potent anticancer agent.

Materials and Methods

Protocol approval

All experiments were carried out in strict accordance with the regulations and guidelines for experimental research issued by the Third Military Medical University, as well as the protocol of the Institutional Animal Care and Use Committee of the Third Military Medical University.

Cell lines, reagents, and antibodies

The 786-O and ACHN cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Line 786-O was grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) in a humidified chamber with 5% CO₂ at 37 °C; ACHN cells were grown in MEM supplemented with 10% FBS. FBS was purchased from Gibco (Life Technologies, CA, USA). The penicillin-streptomycin solution was Hyclone (Thermo Fisher Scientific, Massachusetts, USA). The cell strain was purchased from Corning (Corning, NY, USA). Cyanidin was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Antibodies against MMP-9, Cyclin D1, E-cadherin, HIF2A, Bcl2, cleaved caspase-3, LC3, and p62 were purchased from Cell Signaling Technology (Danvers, MA).

Cell proliferation assay

The 786-O and ACHN cells were seeded (2×10^3 per well) into 96-well plates and left to adhere overnight. The medium was removed the following day and fresh medium, either alone or with cyanidin (0, 0.5, 1, 2, 5, 10, 25, 50, 75, or 100 μM) was added. The cultures were incubated for either 24 h, 48 h, or 72 h. Floating cells were then removed and 100 μL of fresh medium containing 1/10 (v/v) Cell Counting Kit-8 reagent (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was added to each well before incubation for a further 4 h. After incubation, the absorbance was measured using a 96-well plate reader at 450 nm. Wells containing CCK-8 reagent without cells were used as blank controls. Cell proliferation was assessed by the absorbance values according to the manufacturer's protocol.

Clone formation assay

Cells were seeded at a density of 200 cells on 6-well plates and maintained in a complete medium containing 10% FBS with different concentrations of cyanidin for 12 days. Colonies were fixed with methanol and stained with 0.1% crystal violet (Beyotime Institute of Biotechnology, Shanghai, China). Visible colonies were photographed and counted manually.

Apoptosis assay

The cyanidin-induced apoptosis of 786-O and ACHN cells was detected by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit (KGA, KeyGEN, Biotech, City, China) according to the manufacturer's instructions. The rate of apoptosis was analyzed using FACSCalibur Flow Cytometer (Becton Dickinson and Beckman-Coulter, San Jose, CA, USA) at 488 nm, and the data were analyzed by flow cytometry (MoFlo, Beckman, CA, USA).

Cell cycle detection assay

On reaching 60-80% confluence, cells were treated with different amounts of cyanidin (0, 25, 50, or 100 μM) for 48 h. The cells were collected and washed twice with phosphate-buffered saline (PBS), fixed with 70% ethanol for 1 h at 4 °C, washed again with PBS, and re-suspended with propidium iodide (PI) solution (0.05 mg/mL) containing RNase. Next, they were incubated at room temperature in the dark for 30 min. The DNA content was analyzed using a flow cytometer (MoFlo, Beckman); the populations of cells in each phase of the cell-cycle were determined using Flow Jo 10.6 for Windows (Tree Star, Ashland, OR, USA).

Wound healing assay

A wound healing assay was used to assess the capacity of the cells to migrate and invade. When cells reached 90-95% confluence, a wound was generated by scratching the surface of the plates with a pipette tip. The floating cells were removed, and the adherent cells were incubated with various concentrations of cyanidin (0, 25, 50, or 100 μM) for 24 h until the wound healed. The gap distances at different time points were normalized to the distance immediately after the scratch was made.

Transwell cell migration and invasion assay

Transwell (Millipore, Billerica, MA, USA) assays were used to measure migration. A total of 4×10^4 786-O and ACHN cells were seeded into the upper chamber in serum-free media with different concentrations of cyanidin (0, 25, 50, or 100 μ M). The medium was further supplemented with 10% serum, which was added to the lower chamber (it contained the appropriate concentration of cyanidin). After 24 h of incubation the cells were fixed and stained using 0.05% crystal violet. The numbers of cells that migrated from the upper to the lower chamber were counted in five random fields of view per experiment and the assays were performed in triplicate.

For the invasion assay, a mixture of Matrigel (Millipore) and serum-free cell culture medium (1:10) was added to the upper chamber of the 24-well transwell plates, incubated at 37 °C for 4 h for gelling, and kept at 4 °C for 24 h. RPMI 1640 culture medium plus 20% FBS (600 μ L/well) was added to the bottom well. Cells (4×10^4 in 100 μ L) were subsequently seeded in the upper chamber with an 8- μ m pore membrane and incubated at different concentrations of cyanidin (0, 25, 50, or 100 μ M) for 24 h. The cells in the membrane were fixed with 4% PFA for 10 min, stained with 0.1% crystal violet for 5 min, and counted.

RT-PCR

The total RNA was isolated using a TRIzol Reagent (Life Technologies, CA, USA). Single-strand cDNA was prepared from 1 μ g of total RNA using reverse transcriptase with oligo(dT) primer according to the manufacturer's instructions (Takara Bio, Japan). Each cDNA sample (2 μ L) was subjected to RT-PCR amplification using specific primers for Bcl2, Bim, Bix, MM9, CD47, ATG4, and LC3B, as detailed in Table 1. The data were collected and analyzed using the $2^{-\Delta\Delta C_t}$ method. The values were normalized to those of GAPDH and then compared with the experimental controls.

Detection of reactive oxygen species

The dichloro-dihydro-fluorescein diacetate (DCFH-DA; Beyotime) method was used to measure intracellular reactive oxygen species (ROS) levels. The cells were harvested following treatment with cyanidin and washed once with ice-cold PBS before treatment with DCFH-DA (at a final concentration of 10 μ M in a serum-free medium). After incubation at 37 °C for 20 min in the dark, the cells were washed twice with PBS. The intracellular accumulation of ROS was measured by flow cytometry and the median fluorescence intensity values were calculated. All experiments were performed in triplicate.

Western blot

Cells were lysed by lysis buffer containing 10 mM Tris, pH 7.2, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and 1% deoxycholic acid. The supernatants were collected after centrifugation at 12000 $\times g$ at 4 °C for 5 min. Protein concentration was determined using a BCA protein kit (Beyotime) and whole lysates were mixed with 4 $\times$ SDS loading buffer (125 mmol/Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/L DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Protein samples were heated at 100 °C for 10 min. For the western blot, 30 μ g of protein samples

Table 1. Nucleotide sequences of primers used for RT-PCR reactions

Gene	Forward	Reverse
Atg3	5'-ATGGGAGTTGGCGAAGGCAAGT-3'	5'-AGTCCACGTATCGAAGACAGC-3'
Atg4	5'-TATTGGTGGCAAACCTAAACAGTC-3'	5'-TTGGGAGAAGGGCAGTGGAATGA-3'
Atg5	5'-GCAGATGGACAGTTGCACACA-3'	5'-GAGGTGTTTCCAACATTGGCTCA-3'
Atg7	5'-CGTTGCCACAGCATCATCTTC-3'	5'-CACTGAGGTTCCACATCCTTGG-3'
Atg9	5'-GCTTCTCAAGGAGCAGGTTCA-3'	5'-CCACATTTGCGATAAGGCTCAGG-3'
Atg10	5'-CTACGGAAGAGAACCAGGAGCT-3'	5'-CTGGTAGAGGTTCTTTGCTGC-3'
Atg12	5'-GGGAAGGACTTACGGATGTCTC-3'	5'-AGGAGTGTCTCCACAGCCTTT-3'
LC3B	5'-CTGAGATTGGTGTGGAGACG-3'	5'-CGGTGATAATAGAACGATACAAGG-3'
Bak1	GACGCCCATTCCTGGAAACT	CTTGCCCGAAGCCATTTT
Bax1	TGCTTCAGGGTTTCATCCAG	GGCGGAATCATCTCTG
Bim1	ACTCTCGGACTGAGAAACGC	CTTCACTCCGTGATTGCCT
Bcl2	TGTGGAGAGCGTCAACCGGGAG	GCAAGCTCCACAGGGCCAAA
HIF1A	5'-TCCAAGAAGCCCTAACGTGT-3'	5'-TGATCGTCTGGCTGTGTAA-3'
HIF2A	5'-TGGAGACACTGGCACCCTAT-3'	5'-GCTGTCTGTGAAACTTGTCC-3'
CD47	5'-TGCTATACTCTGTTCTGGGGA-3'	5'-TCTCATCCATACCACGGATCT-3'
MMP9	5'-CACCTCGAACTTTGACAGCG-3'	5'-CCCTCAGTGAAGCGGTACAT-3'
CXCR4	5'-GGTGGTCTATGTTGGCGTCT-3'	5'-GTCAATGGGGTAGAAGCGGT-3'
SEPW1	5'-GTCTGTTTATTGTGGCGCTTGAG-3'	5'-CCACGTAGCCATCGCCTTTCTTC-3'
ERG1	5'-CCCCGGCCACCACCTCATAC-3'	5'-ACGGGGAGGGGAAGCCACTG-3'
GAPDH	5'-GAAGGTGAAGGTCGGAGT-3'	5'-GAAGATGGTGATGGGATTTC-3'

were subjected to SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane, and incubated first with rabbit antibodies against E-cadherin, Bcl2, cleaved caspase-3, HIF2A, p62, LC3, and actin (all from Cell Signaling Technology) overnight at 4 °C, followed by another 2 h incubation with a secondary antibody (1:5000). GAPDH served as a loading control. The secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Densitometric analysis of the western blots was performed with NIH Image J software (Bethesda, MD, USA). The protein levels were first normalized to actin and subsequently to the experimental controls.

RNA-seq and analysis

Total RNA was prepared from 786-O cells via RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Library preparation and sequencing on an Illumina HiSeq2000 instrument were performed by Genewiz Inc. (New Jersey, USA). Target genes were verified by synthesizing cDNA using a qScript cDNA Synthesis Kit (Quantabio, Maryland, USA) according to the manufacturer's instructions. The RT-PCR mixtures contained 2x QuantiTect SYBR Green PCR Master Mix (Qiagen), 10x QuantiTect primer assay mix, and synthesized cDNA. Amplification was performed in triplicate for each sample on a RealPlex MasterCycler (Eppendorf, Hamburg, Germany). Relative expression levels were normalized to GAPDH levels. Eleven low-quality nucleotides from the 5' ends of the reads were trimmed for RNA-Seq data analysis. The resulting 40-nucleotide single-ended reads were mapped to the human genome (UCSC mm10) by using TopHat with NCBI Ref-Seq genes as the reference and up to two possible mismatches. Read duplicates were removed using the same tools to reduce possible biases caused by RT-PCR. Cufflinks was used to estimate gene expression levels (transcript counts). DESeq2 (27) was used to test whether the genes were differentially expressed between the wild-type and cyanidin groups. The Benjamini-Hochberg method, which controls the false discovery rate (FDR), was used to correct for multiple comparisons. Alterations of genes shown in the volcano maps were all significant (FDR, 0.1). These genes were clustered into functional groups by means of DAVID bioinformatics tools (NIH). Differential expression of genes selected from each category was verified by quantitative RT-PCR.

Patient data mining from The Cancer Genome Atlas

In this study, we collected sequencing data of RCC from The Cancer Genome Atlas (TCGA) dataset (<https://tcga-data.nci.nih.gov> and <https://genome-cancer.ucsc.edu>) containing 523 RCC tissues and 100 tumor-free adjacent normal tissues. The TCGA sequencing data was used to obtain the expression of both EGR1 and SEPW1 in RCC and tumor-free adjacent normal tissue samples. The expression levels of EGR1 and SEPW1 were analyzed using the TCGA portal. Overall survival rates of patients with high and low levels of EGR1 and SEPW1 were derived using the Kaplan-Meier survival plot.

Xenograft assays in nude mice

All animal handling and experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the Third Military Medical University, China (approval no. SYXK20070002). All efforts were made to minimize animal suffering and to reduce the number of animals used. Four-week old male BALB/c nude mice (n = 10) were used in this study and maintained at room temperature (25 ± 2 °C) under a standard 12h/12h light-dark cycle in a specific pathogen free experimental animal room and fed a sterilized diet. ACHN cells were re-suspended to 2 × 10⁶ cells/100 µL in PBS and implanted subcutaneously into the flank region of 6- to 7-week-old male nude mice weighing 18-25 g. When tumors reached 40-50 mm³ in volume, the animals were randomly assigned to two groups (n = 5), receiving either intraperitoneal (IP) injections of 100 µL PBS twice a week or IP injections of cyanidin (6 mg/Kg in 100 µL PBS) twice a week. Tumor volumes were measured twice a week and once the control tumors reached 1,000 mm³ the animals were humanely euthanized in accordance with ethical study requirements. At the end of the study, all animals were sacrificed with an overdose of CO₂ and the tumor tissues were extracted for immunostaining and weighing.

Immunohistochemistry

Tumors excised from the nude mice were fixed in 4% paraformaldehyde, dehydrated through a graded series of ethanol solutions, embedded in paraffin, cut into 4-mm sections, and stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC), the sections were incubated with a primary antibody

versus Ki-67 (Cell Signaling Technology) overnight at 4 °C. The sections were subsequently incubated with biotinylated goat and rabbit antibodies IgG for 20 min at room temperature, and then 30 min with streptavidin-conjugated HRP. Diaminobenzidine- H_2O_2 was used as a substrate for peroxidase.

Statistical analysis

All data are representative of at least three experiments, which were performed in triplicate unless otherwise indicated. All statistical analyses were carried out using the GraphPad Prism 6.0 statistical software package (GraphPad Software Inc., LaJolla, CA). Data are expressed as the mean $\pm$ standard deviation. One-way analysis of variance followed by Student-Newman-Keuls post hoc tests were used to determine the significance of the differences between the results. A p-value of <0.05 was regarded as significant.

Results

Cyanidin inhibits the growth and migration of RCC cells

To investigate the effect of cyanidin on the proliferation of human RCC cell lines, 786-O and ACHN cells were treated with different concentrations of cyanidin and CCK-8 assays were performed. The corresponding IC₅₀s for 786-O and ACHN cell lines were 120.6 μ M and 96.96 μ M, respectively (Fig. 1A). Cyanidin treatment of 786-O and ACHN cells decreased cell viability in a concentration-dependent manner, whereas cell viability was higher at 50 μ M than at 25 μ M. The effect of cyanidin was also tested on colony formation, another type of proliferation assay, in which 200 of each RCC cell type (786-O and ACHN) were seeded into 6-well plates and cultured with cyanidin at concentrations of 0, 25, 50, or 100 μ M for 14 days. As shown in Fig. 1B-D, cyanidin significantly inhibited colony formation of both 786-O and ACHN cells in 25 and 100 μ M.

The possibility of whether cyanidin would inhibit the migratory and invasive ability of RCC cells was also explored. Cyanidin concentrations of 0, 25, 50, and 100 μ M were chosen for these experiments. In a wound healing assay, both 786-O and ACHN cells were cocultured with different concentrations of cyanidin for 24 h. Compared with the control group, cyanidin inhibited the migratory ability of 786-O and ACHN cells (Fig. 2A-D). Moreover, a concentration-independent inhibitory effect was observed, whereby migration decreased less at 50 μ M than at 25 μ M (Fig. 2B and 2D). Similarly, in a transwell migration and invasion assay (Fig. 2E-H), cyanidin inhibited the ability of RCC cells to migrate. These data suggest that cyanidin inhibits the proliferation and migration of RCC at a broad range of concentrations. Furthermore, cyanidin significantly curtails RCC tumorigenesis via concentration-independent effects.

Cyanidin induces RCC cell apoptosis and cell cycle arrest

The study also investigated whether the suppression of cell growth by cyanidin was due to an impact on cell apoptosis. Cell death in 786-O and ACHN was measured by flow cytometry based

Fig. 1. Cyanidin inhibits RCC-cell proliferation and colony formation in vitro. (A) The IC₅₀ values at the indicated concentrations of cyanidin in 786-O and ACHN cells were detected by the CCK-8 assay and absorbance was read at 450 nm. (B-D) A colony formation assay was performed to detect the proliferation of 786-O and ACHN cells. * $p < 0.05$ (compared with the 0 μ M group), ** $p < 0.01$, *** $p < 0.001$.

Fig. 2. Cyanidin induces anti-metastatic effects. Cells were treated with cyanidin (0, 25, 50, or 100 μ M) for the indicated times (24 or 48 h). (A-D) Wound healing, (E, F) transwell migration, and (G, H) invasion assays were performed. Red lines represent the borders of the wounds. The pictures shown are representative of three separate experiments. Error bars represent standard deviations. * $p < 0.05$, ** $p < 0.01$.

Fig. 3. Cyanidin promotes apoptosis and induces G1 arrest in RCC cells. (A-C) Flow cytometry was used to analyze apoptosis in 786-O and ACHN cells treated with cyanidin. (D-G) Flow cytometry was used to analyze cell cycle arrest in 786-O and ACHN cells treated with cyanidin.

on annexin V and PI staining. The percentage of apoptotic cells gradually increased after treatment with cyanidin in a concentration-independent manner (Fig. 3A-C). Cyanidin was found to affect late apoptosis in a dose-dependent manner; however, the early apoptosis rate appeared relatively unaffected by the different concentrations.

The effect of cyanidin on cell cycle arrest was determined by flow cytometry. After cyanidin treatment for 48 h at 0, 25, 50, and 100 μM , most cells arrested mainly at phase G1-M stage, independently of the concentration. Fig. 3D-G show that cyanidin induced cell cycle arrest. These results indicated that cyanidin inhibited the growth of RCC cells by inducing apoptotic death and cell cycle arrest.

Fig. 4. Cyanidin increases EGR1 expression and decreases SEPW1 expression. (A) Volcano map of RNA-seq data. (B) Gene Ontology analysis of RNA-seq data. (C) Kyoto Encyclopedia of Genes and Genomes analysis of RNA-seq data. (D-G) RT-PCR confirmed that EGR1 was upregulated and SEPW1 downregulated in 786-O cells treated with cyanidin (100 μM). (H, I) EGR1 was downregulated and SEPW1 upregulated in an RCC tumor compared with normal kidney tissue based on TCGA data. (J, K) Kaplan-Meier analysis revealed that lower expression of EGR1 mRNA correlated with a lower survival rate in RCC.

Fig. 5. Effect of cyanidin on mRNA and protein expression in RCC cells. (A, B) Bcl2 mRNA decreased when cells were co-cultured with cyanidin. (C, D) HIF2A mRNA decreased when cells were co-cultured with cyanidin. (E) E-cadherin, HIF2A, Bcl2, and cleaved-caspase3 proteins were regulated when cells were cocultured with cyanidin. * $p < 0.05$ (compared with the 0 μM group), ** $p < 0.01$, *** $p < 0.001$.

Cyanidin increases the expression of EGR1 and decreases the expression of SEPW1

In an investigation into the mechanism of cyanidin-mediated inhibition of the proliferation and migration of RCC cells, mRNA expression RNA-seq (Illumina 2000) was used to determine the genes that showed changes in their expression levels. The genes most upregulated and most downregulated in the 100 μM cyanidin group were selected and compared with the control group. According to the observed fold-changes (>2) and significance level ($p < 0.05$), two genes (EGR1 and SEPW1) were screened (Fig. 4A and B). In addition, RT-PCR confirmed that EGR1 was upregulated and SEPW1 was downregulated in the 786-O cells treated with cyanidin (100 μM ; Fig. 4D-4G). Furthermore, under the special considerations that deserve the comparison of informatic data with the results presented here, we found that the expression level of EGR1 was lower and that of SEPW1 was higher in RCC tissues than normal tissues (Fig. 5H and I). Kaplan-Meier analysis revealed that the lower EGR1 mRNA expression correlated with a lower survival rate in RCC patients (Fig. 4J) while there was no statistically significant association between the expression of SEPW1 and the survival rates in RCC (Fig. 4K). Based on the RNA-seq results (Fig. 4A-C), it appears that the genes associated with cyanidin are mainly related to apoptosis and autophagy pathways. These results indicate that cyanidin activates the expression of EGR1 and inhibits the expression of SEPW1, while simultaneously inhibiting the proliferation and migration of RCC cells.

Effect of cyanidin on mRNA and protein expression in RCC cells

The study found that cyanidin upregulated EGR1 and downregulated SEPW1. EGR1 is a type of transcription factor containing zinc finger domains, and has been shown to be associated with apoptosis, metastasis, and autophagy in tumorigenesis [27, 28]. SEPW1 is a highly conserved small thioredoxin-like protein required for cell cycle progression and apoptosis. Previous studies have shown that quercetin (a flavonoid) induces apoptosis in colon carcinoma cells via EGR1 [25]; furthermore, it has been confirmed that EGR1 and SEPW1 are required for the progression of apoptosis, while EGR1 is also required for epithelial mesenchymal transition [29]. This study, therefore, sought to determine whether the signaling pathways of apoptosis and metastasis were directly influenced by cyanidin. We confirmed through RT-PCR that the expression of Bcl2 decreases when the cells are co-cultured with cyanidin (Fig. 5A and B), and cyanidin decreases the expression of Bcl2 and cleaved caspase-3 in western blot (Fig. 5E). These results indicated that, first, cyanidin possibly activates the apoptosis signaling pathway because both the HIF2A mRNA levels and protein expression of E-cadherin decreased after the co-culture of cells with cyanidin (Fig. 5C-E). Second, the results indicated that cyanidin may decrease the expression of proteins involved in metastasis signaling pathways.

Cyanidin-reduced ROS and autophagy

Many previous reports have shown that cyanidin influences ROS and inhibits oxidative-stress-induced autophagy. This study further explored whether an increase in EGR1 induced by cyanidin would cause a decrease in ROS and whether EGR1 influences the autophagy signaling pathway. Since 786-O and ACHN exhibit different levels of sensitivity to cyanidin treatment, this study further investigated the redox state of the cells by estimating their ROS levels in both the control and cyanidin groups. The ROS levels were determined using the

Fig. 6. RCC reduced autophagic flux in RCC 786-O and ACHN cells. (A, B) Cells were treated with the indicated doses of cyanidin for 24 h and the levels of ROS were examined by flow cytometry. (C, D) Cells were treated with the indicated doses of cyanidin for 24 h and the levels of autophagy-related genes were determined by RT-PCR. (E) Cells were treated with the indicated doses of cyanidin for 24 h and the levels of p62 and LC3 were measured by western blot. (F, G) Cyanidin attenuates enhanced chemosensitivity during cisplatin treatment.

fluorescent probe DCFH-DA, in which the intensity of fluorescence is proportional to the levels of intracellular oxidant species. As shown in Fig. 6A and B, the introduction of cyanidin caused a concentration-dependent decrease of ROS production.

To further determine the effect of cyanidin on autophagy and the association of cyanidin with ROS and autophagy, genes involved in the autophagy signaling pathway were measured via qRT-PCT and western blot. It was concluded that ATG4 and p62 were downregulated while LC3-II decreased (Fig. 6C-E). Furthermore, evidence to date has shown that autophagy is rapidly activated when cancer cells are treated with chemotherapeutic drugs and plays a significant role in protecting the survival of cancer cells [30, 31]. The results of this study indicate that cyanidin increases the chemosensitivity of RCC to cisplatin treatment (Fig. 7F and G).

These data suggest that the increase in EGR1 in renal cell carcinoma cells is associated with ROS levels, and EGR1 may influence autophagy signaling pathways. This supports the findings of previous studies which have suggested a relationship between EGR1 and ROS in gastric cancer [32, 33].

Cyanidin reduced tumor growth in vivo

We investigated whether cyanidin could inhibit renal cell carcinoma cells *in vivo*. The test results revealed that a high concentration of cyanidin (100 μ M) modulated RCC growth *in vitro*. ACHN cell suspensions (1×10^6) were injected into the right axillae of nude mice

Fig. 7. Cyanidin regulates RCC growth in vivo. (A, B) ACHN cell suspensions (1×10^6 cells) were injected into the left axillae of nude mice (indicated by arrows), 100 μ L PBS was given twice a week to the control group and IP injections of cyanidin (6 mg/Kg in 100 μ L PBS) to the cyanidin group (n = 5 in each group). Tumors were harvested and visualized after 4 weeks. Images of xenograft tumors from the ACHN cells treated with cyanidin. (C) Tumors grew more slowly in the cyanidin group. (D) Tumor weights were compared between the control and cyanidin groups. (E) Histopathology of xenograft tumors. The tumor sections of the control and cyanidin groups were H&E stained and IHC stained using antibodies against Ki-67. * $p < 0.05$, ** $p < 0.01$.

and the results revealed that a high concentration of cyanidin inhibited renal cell carcinoma growth and reduced the volumes (Fig. 7A-C) and weights of the tumors (Fig. 7D). Moreover, the expression of Ki67 was lowered (Fig. 7E). The data agree with the results of *in vitro* proliferation assays and suggest that a high concentration of cyanidin in cells inhibits the growth of renal cell carcinoma. Therefore, our study indicated that cyanidin suppressed the proliferation and migration potential of RCC cells.

Discussion

The availability and efficacy of chemotherapy for renal cell carcinoma is currently limited; therefore, new and effective agents for renal cell carcinoma treatment are urgently needed. Anti-cancer agents extracted from natural and organic herbs have attracted increasing attention in recent years. Cyanidin has been established as an effective anti-oxidative agent that has proven protective effects on the human cardiovascular system, nervous system, diabetes, retinal degeneration, and carcinogenesis [34-38]. Increasing evidence suggests that cyanidin inhibits tumor initiation, promotion, and progression [23, 24, 39-41]. Like other common dietary flavonoids, it is assumed that cyanidin will also have an effect on the growth and migration of RCC. One previous study showed that cyanidin has an anti-tumor effect in prostate cancer [23], while another indicated that the anti-tumor role of cyanidin is due to the suppression of cyclooxygenase-2 and attenuation of PGE2 production [40]. The effect of cyanidin on renal cell carcinoma had not been previously investigated; however, this study revealed for the first time that cyanidin exerts a direct anti-tumor effect on the growth and migration of RCC. The results indicated that low (25 μ M) and high concentrations (100 μ M) of cyanidin played a significant role in curtailing RCC carcinogenesis, although moderate concentrations (50 μ M) of cyanidin had no obvious impact. It was also discovered in this study, using RNA-seq and confirmed by RT-PCR, that cyanidin inhibited RCC carcinogenesis through EGR1 and SEPW1 concentration-independently. Furthermore, under the special considerations that required the comparison of bioinformatic data with the results presented here, we found that in RCC tissues, the expression level of EGR1 was lower and that of SEPW1 was higher than in normal tissues (Fig. 5H and I). The transcriptional factor of EGR1 is expressed in response to various extracellular signals such as growth factors, cytokines, irradiation and various kinds stressors and it has been confirmed that EGR1 plays a significant role as a tumor suppressor in breast, brain, and lung cancer and inhibits growth and transformation when overexpressed [42-44]. SEPW1, a mammalian archetype of the selenoprotein W-like family, is an ubiquitous 9 kDa Sec-containing protein with glutathione-dependent antioxidant activity [45] that promotes tumor growth in breast cancer [26]. The silencing of SEPW1 mRNA expression causes cells to accumulate in the G0/G1 phase of the cell cycle via P53 and p21 [26]. This study found that cyanidin curtails RCC carcinogenesis by targeting EGR1 and SEPW1.

This study mainly focused on three important signaling pathways during carcinogenesis, namely, apoptosis, autophagy, and metastasis. The results convincingly showed that both low and high concentrations of cyanidin have inhibitory effects on these three pathways during RCC carcinogenesis. They were also found to enhance cleavage of the apoptotic mediator caspase-3 and enhance the inhibition of E-cadherin activation. Further exploration revealed regulation of ATG4, which is associated with autophagy in RCC. In addition, a significant number of reports have shown that cyanidin influences the biological system through redox reactions. Cancer cells often have high levels of intrinsic oxidative stress due to increased formation of ROS, which are known to be associated with many diseases through the activation of different signaling-regulated transcriptional pathways. ROS are also associated with tumor initiation, promotion, and progression [46]. The results of this study indicated that cyanidin decreased the intracellular ROS levels. Recent studies have also demonstrated that ROS are involved in the induction of autophagy in some cell types [47], while others have suggested that antioxidants may have the ability to inhibit autophagic cell death by suppressing the generation of intracellular ROS [48, 49]. Our findings that showed

increased expression of autophagy markers in the H₂O₂ group indicated that the increase in cell death is related to autophagy. The results further demonstrated that oxidative-stress-induced autophagic cell death in RCC cells can be reduced by cyanidin. Additionally, accumulating evidence suggests that autophagy is rapidly activated when cancer cells are treated with chemotherapeutic drugs, which is a phenomenon that plays a significant role in cancer survival [30, 31]. Furthermore, our results indicated that cyanidin increases the chemosensitivity during cisplatin treatment.

There are, however, certain limitations to this study. The mechanism by which cyanidin increases EGR1 expression and decreases SEPW1 expression in RCC cells remains unknown, and further investigations are required to explore the potential underlying mechanisms. In addition, it is also possible that EGR1 and SEPW1 represent other targets involved in this therapy. An in-depth examination may contribute to a full understanding of their roles in the control of RCC cell invasion.

Conclusion

In summary, we found that cyanidin inhibits the proliferation and migration of RCC cells in a concentration-independent manner. Cyanidin exerts this function by increasing the expression of EGR1 and decreasing that of SEPW1. The data revealed the previously unappreciated roles of EGR1 and SEPW1 in the regulation of cyanidin-mediated cell invasion and migration, and it is concluded that cyanidin may have therapeutic potential to curtail the carcinogenic process and attenuate the chemosensitivity of RCC.

The dose-independent effect of cyanidin on carcinogenesis provides new insight into the consumption of this compound in daily life. It is worth investigating the relationship between the optimum intake of cyanidin and the carcinogenesis of RCC because different doses may exhibit different protective effects.

Acknowledgements

This work is supported by National Natural Science Foundation of China (Nos. 81170705 and 81230017). We thank Dr. Qingjian Wu for the constructive comments and for editing the manuscript. We are grateful to the authors and publications not cited in this review due to space restrictions.

LL, JX, and ZY conceived and designed the experiments; XL, DZ, YH, and QL performed the experiments; LL, YH, YW, XL, and XL analyzed the data; LL and QW contributed reagents/materials/analysis tools; and LL and XL wrote the paper.

Disclosure Statement

The authors have no competing interests to declare.

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