SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Study of the relationship between angiogenesis markers and miRNAs in human samples of patients with renal carcinoma, and search for therapeutic targets related to angiogenesis pathways by using in vitro studies with human CAKI-2 and A-498 kidney cancer cell lines

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, on the 28th of March 2025 at 13:00.

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1. Introduction

Renal cell carcinoma (RCC) is the most common neoplasm of the kidney in adults, accounting for about 3% of all malignancies, and has the highest mortality rate of over 40%. At the time of diagnosis, metastases developed in approximately 20–30% of patients with RCC, and also, after curative surgery for localized RCC, another 30% of patients developed metastases in follow-up studies. The current system used to predict the prognosis of patients diagnosed with renal cancer does not accurately predict the natural outcome of the disease, and because of this, there is an urgent need to find novel molecular biomarkers that can be used for the early diagnosis and evaluation of the prognosis.

MicroRNAs are conserved, small (18~22 nucleotides), non-coding RNAs that play a major role in different molecular pathways by regulating gene expression. In stable microvesicles, apoptotic bodies, or membrane-free carriers, miRNAs in human body fluids may have diagnostic biomarker roles and also indicate the prognosis of cancer diseases. Based on the expression profiles, miRNAs can be indicators in distinguishing tumorous and normal tissues, even classifying tumors by histological type. They also have the ability to balance between pro- and antiangiogenic processes and are able to modify the appropriate course of events in angiogenesis. Based on related studies, miRNAs represent potential therapeutic targets for the treatment of pathological neovascularization-related diseases due to their influence on multiple different pathways.

While previous studies have illustrated the role of miRNAs in renal cancer, many of their functions are not fully understood.

Tyrosine kinase inhibitors, sunitinib, sorafenib, temsirolimus, and an immunoadjuvant, bevacizumab, have improved clinical outcomes in randomized trials. However, there are newly discovered molecules that are effective in renal cancer therapy and may help overcome multidrug resistance, which is critical for successful therapy.

Shikonin is isolated from the roots of the Chinese medical herb *Litho-spermium erythrorhizon*. Recently, emerging evidence has illustrated that shikonin has high efficacy against a series of human cancer cell lines in vitro and in vivo, with minimal toxicity to non-malignant human cells. Shikonin was reported to induce apoptosis, necrosis, or necroptosis in various cell lines, through signal regulation and molecular targets, also, it shows similar potency toward drug-sensitive and drug-resistant cancer cell lines.

Certain studies have demonstrated the use of shikonin as a potential therapeutic agent to treat human glioblastoma by regulating miRNA expression profiles. Recent evidence has

indicated the role of miR-21 in the development and progression of human tumors. In numerous human cancer types, miR-155 is overexpressed and demonstrates an oncogenic role. Previous studies have revealed that miR-155 has an important role in the progression of clear cell renal cell carcinoma.

Shikonin, as a potential therapeutic compound for renal carcinoma, has become an exciting and interesting topic, however, its effect and anticancer mechanism on kidney cancer cells have not been thoroughly investigated.

2. Aims

In the present study, we compared the expressions of relevant RCC miRNAs, such as hsa-miR-15b-5p, hsa-miR-99b-5p, hsa-miR-181a-5p, and other angiogenesis-related genes, in both renal tumors and adjacent normal renal tissues from patients with RCC. In addition, our investigation sought to describe specific miRNAs and their roles in the progression of the angiogenesis process of renal cancer, as well as the potential prognosis of RCC. Utilizing statistical strategies, we correlated the expression patterns of the miRNAs of interest with clinicopathological parameters, including clinical stage and histology, to confirm our findings, and we aimed to describe the interactions of miRNAs and their targets as putative representatives of renal angiogenesis.

The effect and the anticancer mechanism of shikonin on kidney cancer cells have not been thoroughly investigated. Thus we aimed to study whether shikonin alone could suppress the growth of human kidney cancer cells in vitro. One assumption is that some strong oncogenic miRNAs might have an epigenetic regulation on the process; therefore, we investigated the biological role of miR-21 and miR-155 on the underlying molecular mechanisms of shikonin in two human renal cancer cell lines in vitro. We also intended to describe the effect of shikonin on the genes involved in the apoptotic pathways leading to cancer cell death.

3. Materials and methods

3.1. Clinical sample collection

Tumorous specimens and adjacent normal kidney tissues were isolated from 20 patients with histologically proven kidney cancer who underwent surgical resection at the University of Debrecen, Department of Urology. From the collected cancer samples, all were diagnosed as primary tumors without any evidence of metastases. Tumors were staged using the TNM staging system of the Union for International Cancer Control, and histological grade was

determined according to World Health Organization criteria. Local invasion of the tumor cells was assessed using T staging, and lymphatic status was recorded as positive or negative. After the initial study metrics were collected, the tumor tissues were immediately frozen in liquid nitrogen and stored at −80 °C until further processing.

3.2. Cell cultures

The A-498 and CAKI-2 human renal carcinoma cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Both cell lines were maintained at 37 °C in a humidified atmosphere under 5% CO_2 /95% air condition.

3.3. Chemicals

Shikonin and sunitinib (purity > 98%) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), dissolved in DMSO, and stored as a stock solution in aliquots at −20 °C. In the cell proliferation assay, the drugs were applied between 2.5 and 40 μM DMSO was used as a vehicle in all experiments. Concentrations of the drugs used in further cell culture experiments were optimized according to the cell proliferation curves.

3.4. Detection of cell proliferation activity

Cell Titer Blue Assay (Promega, Madison, WI, USA) was used for cell proliferation analysis. For the assay, A-498 and CAKI-2 cells (10^4/well) were seeded in 96-well plates. All the cells were plated in their complete growth media 24 h before the experiments. The following day, the complete growth media was removed and replaced with the media containing increasing concentrations of the drugs (sunitinib or shikonin) for dose-response analysis. After the treatments, the cells were further incubated with the drugs over 72 h at 37 °C in the cell culture incubator with 5% CO_2. The viability of the cells was detected every 24 h. The Cell-Titer Blue reagent (Promega) was added to the cells and, thus, the plates were incubated for 2 h at 37 °C in the cell culture incubator. Fluorescence intensity was measured using the BioTek Plate Reader system (BioTek, Winooski, VT, USA).

3.5. Clonogenic cell survival assay

The assay was performed on A-498 and CAKI-2 cells treated with sunitinib and shikonin. After the preparation of a single-cell culture suspension, 3–5 × 10^3 cells/well were plated in six-well plates and seeded overnight. The next day, the cells were treated with different concentrations of shikonin (1–20 μM) or sunitinib (2.5–40 μM) (this range of concentrations was based on cell proliferation activity results). The control group of the cells was cultured in the complete cell culture media with 0.01% DMSO instead of the drugs. Afterward, the cells were allowed to grow for another 14 days. The media containing drugs was replaced with fresh media and drugs every third day. After 14 days, the experiment was terminated by removing the media. The cells were gently washed with Phosphate-Buffered Saline (PBS), fixed in methanol: acetic acid (3:1), and stained with 0.1% Crystal Violet solution (Sigma-Aldrich, St. Louis, MO, USA). For quantification of the colonies, the cells were resuspended in 2% Sodium Dodecyl Sulfate (SDS) solution. The absorbance was measured by Biotek plate reader system (BioTek, Winooski, VT, USA).

3.6. Caspase-3 and -7 Activity Assay

Caspase-3 and caspase-7 activities were measured using the Caspase-Glo 3/7 assay (Promega, Madison, WI, USA) following the instructions outlined by the manufacturer. CAKI-2 and A-498 cells (10^4) were plated into 96-well plates and treated with increasing concentrations of shikonin (2.5–10 μM). Forty eight hours after the treatment, the reaction was completed and fresh media was added to each well of the plate. Afterward, the cells were lysed in 25 μL Caspase-Glow® reagent and the plates were shaken for 30 min at room temperature (RT). Also, 100 μL of the lysate was transferred to a 96-well white wall plate and luminescence was measured on a BioTek plate reader (BioTek, Winooski, VT, USA).

3.7. RNA Extraction and Quality Determination

Complete RNA was isolated for measurements related to our angiogenesis and miRNA studies, as well as for our in vitro studies. Total RNA was extracted from tumorous kidney cancer and paired adjacent normal kidney tissues using the Qiagen RNeasy Mini isolation kit (Invitrogen, Life Technology, Carlsbad, CA, USA), according to the manufacturer's protocol. RNA concentration and purity were determined by NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity also was analyzed using the

Agilent 2100 Bioanalyzer (Agilent Technolo-gies, Santa Clara, CA, USA). For further molecular biology analyses, RNA was stored at −80 °C.

3.8. Isolation of RNA from CAKI-2 and A-498 cells for in vitro studies

To the gene expression analyze at mRNA level, CAKI-2 and A-498 cells were treated with increasing doses of shikonin (2.5–10 μM) within 24, 48, and 72 h incubation periods, then total RNA was isolated. For total RNA extraction, NucleoSpin RNA/Protein commercial kit (Macherey-Nagel, Düren, Germany) was used.

3.9. Reverse Transcription PCR (RT-PCR)

cDNA was synthesized from 250 ng of RNA from each sample using the Tetro cDNA Synthesis Kit (Bioline, London, UK) according to the manufacturer's guidelines. The RT-PCR reaction was performed in a 20 μL volume using random hexamers. The run consisted of 35 cycles. To test for contamination, RT-NTC was incorporated into the reaction.

3.10. MicroRNA (miRNA) Specific Stem-Loop RT-qPCR Analysis

The expression of intracellular miRNAs, miR-21 and miR-155, were quantified by miRNA-specific Universal ProbeLibrary (UPL)-probe-based stem-loop RT-qPCR method. miRNAs transcription into cDNA via reverse transcription was performed from total RNA (10 ng) using miRNA-specific stem loop-RT primer (500 nM, Integrated DNA Technologies, Leuven, Belgium) and TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). miRNA quantification was performed by RT-qPCR using designed miRNA-specific forward primer (100 μM, Integrated DNA Technologies), universal reverse primer (100 μM, Integrated DNA Technologies), and UPL probe #21 (10 μM, Roche Diagnostics, Mannheim, Germany) with Taq polymerase (5 U/μL) and dNTPs (2.5 mM), (Thermo Scientific, Wilmington, DE, USA).

The RT-PCRs were carried out in triplicates on a QuantStudio 12 K Flex qPCR instrument (Applied Biosystems). For normalization, the small-nucleolar RNU-43 was measured as a reference gene.

3.11. TaqMan® miRNA Quantitative Real-Time PCR and Statistical Analysis

qRT-PCR was performed using TaqMan® microRNA assays (Life Technologies, Carlsbad, CA, USA). Complementary DNA (cDNA) for each miRNA of interest was

synthesized from total RNA (5 ng) using the TaqMan® microRNA Reverse Transcription Reagents (Invitrogen, Life Technology, Carlsbad, CA, USA) and specific reverse transcription primers (Life Technologies, Carlsbad, CA, USA). Real-time PCR was performed with TaqMan® probes in a CFX-96 Real-time PCR System (Bio-Rad Laboratories Inc., Hercules, CA, USA). All assays were performed in 96 well plates using triplicates. For each hsa-miR applied, the C_T values were determined using the SDS software (version 3.1) with automatic baseline and threshold settings. The data were loaded into the R statistical environment (v.2.14.0, Applied Biosystem, Foster City, CA, USA) and preprocessed. Triplicate C_T values were averaged and normalized to the geometric mean of RNU6, which was selected as an endogenous control based on geNorm27 and NormFinder (Department of Molecular Medicine (MOMA), Aarhus University Hospital, Aarhus N, Denmark). The normalized expression was calculated as $\log2|2-\Delta CT|$. C_T values > 36 were considered to be below the limit of detection.

3.12. Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was conducted using the iTaqTM Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA). The reaction was performed in a CFX-96 Real-Time System (Bio-Rad Laboratories Inc., Hercules, CA, USA) in a final volume of 20 µL. Following this, the relative value of mRNA was determined by the C_T technique using threshold cycle times for each mRNA. Triplicate C_T values were averaged and normalized to the average Cp mean of GAPDH, which were selected as endogenous control. The normalized expression was calculated as $\log_2 (2^{-\Delta CT})$.

3.13. In Silico miRNA Analysis for Target and Pathway Prediction

In the case of hsa-miR-15b-5p, hsa-miR-99b-5p, and hsa-miR-181a-5p chosen for the study, based on literature data, an in silico study was carried out by comparing miRNA-specific targets with the help of 3 databases: miRDB, TargetScan, and Tarbase. A search for angiogenesis signaling pathway-related target proteins was the main purpose of this process. Based on the database screening results, we determined the miRNA-target interactions and performed enrichment analysis.

3.14. Tissue Lysate Preparation for Protein Array Analysis

Proteins from 8-8 adjacent tumorous and healthy renal cancer tissue samples were extracted with Lysis buffer 6 or Lysis buffer 17 (provided in the Proteome profiler Human Angiogenesis Array Kit) (ARY007, Bio-Techne, McKinley, MN, USA). The total protein content of the samples was quantified by the Bradford Method.

For each membrane supplied in the kit, 300 μg of protein extract was used for Human Angiogenesis Array analysis, according to the manufacturer's protocol. The signal of the protein spots was visualized by chemiluminescence measurement using the reagent supplied in the kit. ChemiDoc Imaging System (Bio-Rad, Her-cules, CA, USA) was used for the membrane visualization and for the quantification of band density. The intensity score of each duplicated array spot was measured with the Image Lab software (version 5.2.1., Bio-Rad, Hercules, CA, USA), and the average intensity was calculated by subtracting the average background signal.

3.15. Western blot Detection of Proteins

The cells were treated with 5 μM shikonin for 24, 48, and 72 h. After the treatment, the cells were washed with PBS and lysed in protein lysis buffer (M-PER, Thermo Fisher Scientific, Waltham, MA, USA), containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Protein quantification of cell lysate was performed using Bicinchoninic Acid (BCA) reagent (Thermo Fisher Scientific, Waltham, MA, USA). Samples were diluted with 4x Laemmli buffer and boiled at 95 °C for 8 min. Equal volumes (40 μg) were loaded on 10% or 14% sodium dodecyl sulfate–polyacrylamide gel and run by electrophoresis (SDS-PAGE). The protein lysates were separated corresponding to the molecular weight. As a molecular weight marker, Precision Plus Protein Kaleidoscope Standard (BioRad Laboratories Inc., Hercules, CA, USA) was used. The proteins from the gels were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Burlington, MA, USA). The blots were probed with specific primary antibodies, followed by horseradish pe-roxidase (HRP)-tagged anti-mouse IgG or anti-rabbit IgG secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA). The signal was detected by chemiluminescence technique. For each sample, band intensities were normalized to beta-actin or Hypoxan-thine Phosphoribosyltransferase (HPRT) (Sigma-Aldrich, St. Lous, MO, USA) or total ERK in the case of pERK (Cell Signalling, Danvers, MA, USA).

3.16. Statistical Analysis

All experiments were performed at least three times. The evaluation and generation of mean values, the associated standard deviation, and normalization in percent were performed by Microsoft Excel (Office Professional Plus 2016, Microsoft, Redmond, WA, USA). Statistical significances were calculated with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) with two-way ANOVA test. Differences were considered statistically significant at a p-value ≤ 0.05.

Using the TaqMan miRNA assay (Life Technology, Carlsbad, CA, USA), hsa-miR-15b-5p, hsa-miR-99b-5p, and hsa-miR-181a-5p expression levels were quantified. For each dataset, the correlation coefficient (r) between variables (expression and grade) was calculated using Spearman's (nonparametric) method. The precision of the correlation was characterized by a 95% CI of r. If the correlation was found to be statistically significant, linear regression was performed, and its precision was visualized by the 95% confidence bands around the related best-fit straight line. The p value less than 0.05 was considered to be statistically significant. The levels of significant differences were the following: $p < 0.05$ (*), $p < 0.0021$ (**), $p < 0.0002$ (***), and $p < 0.0001$ (****). All statistical analyses were performed with GraphPad Prism 9.5.1 for Windows (GraphPad Software Inc., La Jolla, CA, USA) and Microsoft Excel 365 (Microsoft Co., Redmond, WA, USA).

4. Results

4.1. Clinicopathological Characteristics of the Patients

A histopathological examination of each specimen (N = 20) was performed to confirm the presence of cancer with minimal mixed nonmalignant tissue. According to the pathologist's overview, the samples used in the study were classified as clear cell renal cell carcinoma (ccRCC): 16 cases (78%), papillary type: 2 cases (10%), and chromophobe type: 2 cases (10%). Within a histological type, all the tissues were analyzed according to Fuhrman Grades: 62.5% (13 samples) of the samples were classified as Grade 2 (low-grade), 20% (4 samples) as Grade 1 (low-grade), and 15% (3 samples) as Grade 3 (high-grade). Eight (40%) of the examined kidney tissue samples were isolated from male patients, and 12 samples (60%) originated from female patients.

4.2. Angiogenesis Related miRNAs Expression

All of the three miRNAs examined were downregulated in tumorous tissues compared to adjacent normal healthy tissues. A significant difference ($p \leq 0.05$) was found in the expression levels of miRNAs between tumorous and paired healthy tissues of patients with RCC, which was the most significant in the expression of hsa-miR-99b-5p ($p < 0.0001$) and hsa-miR-180a-5p ($p = 0.0090$) between the healthy and tumorous samples.

4.3. Correlation of Patients' miRNAs and Tumor Stages

The patients were included in different pathological stages (grades 1, 2, and 3) of RCC. For all three miRNA types in all cases, we found a statistically significant negative correlation between relative expression and pathological grade of the holding tissue. The correlation and the statistical significance were the strongest, and the determination was the most precise when Q = 1% (medium "aggressiveness") was chosen for the outlier identification. The results obtained by linear regression matched the results of the correlation analysis. According to Spearman correlation, p values were deter-mined for each miRNA examined as follows: $p = 0.033$ for hsa-miR-15a-5p, $p < 0.0001$ for hsa-miR-99b-5p, and $p = 0.0133$ for hsa-miR-181a-5p.

4.4. Correlation of Patients' miRNAs and Lymph Nodes

In total, most of the patients had negative lymph node status as metastasis was not detected. Only in one of the patients, regional lymph node metastases was described. Therefore, the correlation between the expression of hsa-miR-15b-5p, hsa-miR-99b-5p, and hsa-miR-181a-5p and lymph node status was not observed.

4.5. In silico miRNA Target Database Analysis

In silico analyses of three distinct miRNA databases were performed in search of the angiogenesis pathway-specific targets on three different databases, including miRDB, Tarbase and TargetScan. According to database analyses for hsa-miR-181a-5p, we found 32 common targets, including VEGF, TIMPs, and MMPs. Similarly, for hsa-miR-15b-5p, our search identified 21 validated targets, among which VEGF, FGF-1, and FGFR1 were found to be relevant. In addition, VEGF and TIMPs were found to be commonly validated for hsa-miR-99b-5p. These database analyses revealed that all three studied miRNAs could be involved as epigenetic factors in the pathological process of renal cancer angiogenesis.

4.6. Evaluation of the Angiogenesis Array

A Human Angiogenesis Array has the ability to detect angiogenesis biomarkers in the protein lysate of tumorous and adjacent healthy kidney cancer tissues. After chemiluminescence detection, the spot intensity showed the expression of specific proteins placed on the array. Based on the results obtained from eight healthy and eight tumorous kidney tissues, the average spot density was analyzed and calculated using the Chemidoc Image Analyser (Bio-Rad, Hercules, CA, USA). The evaluation of angiogenesis arrays showed an increase in the expression of angiogenic proteins, such as ANG, in tumorous samples, while a slight decrease in the expression of MMP-9 was observed during the screening. In tumorous tissues, TIMP-1 also showed a slight decrease compared to healthy samples. Based on the protein array analyses, most likely one of the main angiogenic markers, VEGF, has a very low expression level (not even visible) in healthy tissues. However, in tumorous samples, we could observe significant amount of VEGF.

4.7. Results of Real-Time qRT-PCR

All the 20 paired healthy and tumorous kidney samples were analyzed with the help of gene-specific primers for VEGF-A, FGF-1, ANG, MMP-9, and TIMP-1. The expression of HIF-1α, MMP-2, and TIMP-2 also was analyzed by real-time qRT-PCR. As indicators of both physiological and pathological conditions of angiogenesis, vascular endothelial growth factor receptor 1 (VEGFR-1) and vascular endothelial growth factor receptor 2 (VEGFR-2) were also measured.

ANG, VEGF, and HIF-1α were significantly upregulated ($p \leq 0.05$) in the tumorous samples and significantly downregulated ($p \leq 0.05$) in paired healthy tissues. This slight discrepancy in the results obtained by qRT-PCR and the angiogenesis array may be a result of the low representative number of the samples used for screening the main angiogenesis markers in RCC samples.

Real-time PCR analysis verified the results of the Human Angiogenesis Array. The downregulation of FGF-1 was shown in tumorous tissues, and the expression of VEGFR-1, -2, and -3 receptors was also detected both in tumorous and adjacent healthy tissues of RCC. Additionally, observing results in the light of the pathological grades of the samples. RCC samples showed higher HIF-1α expression than samples identified with lower grades. Intensive VEGF expression in tumor cells was observed in low-grade (Grade 1 and 2) RCC samples and in high-grade (Grade 3) RCC samples as well.

Regarding the expression of MMPs and TIMPs, the quantitative RT-PCR results were mostly consistent with the results of the Human Angiogenesis Array. The results of gene expression analysis demonstrated a slight decrease in the expression of MMP-9 and an increase in the expression of TIMP-1 in tumorous samples compared to normal adjacent tissues, which correlates with an increase in the expression of these proteins. We also analyzed the expression of MMP-2 and TIMP-2 in the samples of the study. Both genes showed lower expression in tumorous tissues than in the adjacent healthy kidney tissue samples.

4.8. Shikonin Inhibits Cell Proliferation in a Dose- and Time-Dependent Manner

Shikonin exhibited an antiproliferative effect on the A-498 and CAKI-2 cells and significantly suppressed cell proliferation in a dose-dependent manner. To compare the effectiveness of shikonin with conventional drugs, the cells were treated with sunitinib. The results showed that shikonin significantly suppressed the cell proliferation of both the A-498 and CAKI-2 cells as early as 24 h at a 2.5 μM concentration. However, the induced significant

inhibition of cell proliferation in the A-498 cells by sunitinib was only observed at 20 μM, and a detectable inhibition of CAKI-2 cell proliferation was observed at 40 μM only 48 h after the drug administration. The inhibitory rate of shikonin gradually increased with time, indicating a time-dependent effect of shikonin on renal cell carcinoma cells. Morphological observation using an inverted microscope (Nikon Eclipse TS 100, Melvile, NY, USA) also showed an extremely pronounced effect of shikonin on both cell lines.

Next, we performed clonogenic cell survival assays to examine the inhibitory effect of shikonin on the clone formation ability of the chosen kidney cancer cells. The cells were treated with increasing doses of shikonin (1–20 μM) for two weeks for colony formation. Shikonin's ability to inhibit colony formation proved to be more effective on the A-498 cells than on CAKI-2. There was a decrease of about three-fold in the number of colonies if 1 μM of shikonin was used in comparison to the control group in the A-498 cells and about eight-fold at the 2.5 μM concentration of the drug. In the CAKI-2 cells, the effect was pronounced at 2.5 μM (~six-fold) only. In accordance with the results revealed by the cell proliferation assay, shikonin suppressed the colony formation of kidney cancer cells at a much lower concentration (1 μM) than sunitinib, which expressed the effect of a similar intensive inhibition on colony formation only at 20 μM.

4.9. Shikonin-Induced Apoptosis in Kidney Cancer Cells

The activation of caspase-3 and -7 proteases is crucial in apoptotic cell death; therefore, the caspase-3 and -7 activity in shikonin-treated CAKI-2 and A-498 cells was determined. After 48 h, a ~six-fold increase in the caspase-3 and -7 activity over the control cells was ob-served in these cell lines. Caspase-3 and caspase-7 were significantly increased after treatment with shikonin, with the maximum activity peaking at 10 μM concentration.

Since caspase-dependent apoptosis generally affects the cleavage of other proteins (e.g., poly (ADP-ribose) polymerase (PARP)), the expression of PARP after the shikonin treatment was investigated by immunoblotting and showed a significantly enhanced expression level of this protein 24 h after the treatment, and a further increasing expression at 48 h.

The immunoblotting data proved that enhanced levels of caspase-3 and caspase-7 were accompanied by decreased levels of antiapoptotic protein, B-cell Lymphoma 2 (Bcl-2); however, for the expression level of the proapoptotic protein, Bcl-2 Associated X-protein (Bax), shikonin did not have a detectable effect in comparison to the untreated control cells.

4.10. The Effect of Shikonin on the Expression of Apoptotic and Tumorsuppressor Genes

In the human CAKI-2 and A-498 cell lines for PI3K and p-AKT, the protein expressions were also analyzed. According to Western blot analyses, in the CAKI-2 cells, an increase in the expression level of PI3K and p-AKT was observed, reaching the highest at 48 h after treatment. The A-498 cells showed an increasing expression throughout the experiment for PI3K, but a clear decrease was revealed in the p-AKT protein.

Phosphatase and Tensin Homolog (PTEN) as a multifunctional tumor suppressor, and it has been found to play an important role in shikonin-induced apoptosis. According to the results presented for both cell lines, we observed a significant decrease in the expression of PTEN at the earlier stage, and then a slight increase over the treatment time.

4.11. MAPK/PI3K Pathways Might Be Associated with Shikonin-Induced Cell Apoptosis

To evaluate shikonin's capability of modulating the Extracellular signal-Regulated Kinases (ERK) signaling pathways, the effects of shikonin on the phosphorylation of p44/42 MAPK (tErk) were detected. The treatment of the A-498 and CAKI-2 cells with 2.5 μM shikonin for 24 h led to a significant inhibition of the Phospho - p44/42 MAPK (pErk) - expression level, in comparison to the expression levels of the total p44/42 MAPK protein. Collectively, the results indicate that shikonin inhibited the proliferation of kidney cancer cells through the induction of apoptotic cell death, and that the shikonin-induced apoptosis in kidney cancer cells could be mediated via a mitochondria-dependent pathway.

Gene expression analyses of the NFkB gene in the CAKI-2 and A-498 cells after the treatment with increasing doses of shikonin (2.5–10 μM) within 24, 48, and 72 h incubation periods were performed by qRT-PCR. We observed a ~five-fold decrease in the NF-κβ messenger RNA (mRNA) expression in shikonin-treated A-498 and CAKI-2 cells compared to the nontreated control group. In accordance with the qRT-PCR data, a significantly reduced level of the NF-κβ protein was detectable at 48 h (~two-fold) by Western blot analyses.

Another aim of this study was to look for the relation between the NF-κB and the factors of apoptosis-like p53, which may affect tumor development and has a function in cell cycle control. We observed a significant decrease in the expression of p53 in the CAKI-2 cells ($p < 0.05$) immediately after 24 h of shikonin treatment. However, the A-498 cells revealed a slight decrease in the expression of p53 after 24 h of incubation with shikonin, while, after 72 h of

treatment, we could observe a ~three-fold increase in the expression of the p53 gene, in comparison to the control cells ($p < 0.05$).

4.12. The Effect of Shikonin on the Expression of Multidrug Transporter Genes

The overexpression of multidrug resistance genes (MDRs) can confer cancer cells with multidrug resistance, and the inhibition of tumor cell efflux can effectively increase the sensitivity of tumor cells to chemotherapeutic drugs.

We aimed to investigate the expression of the most common multidrug transporter genes (BCRP1, ABCC6, ABCB1, ABCB5) of kidney cancer in the shikonin-treated A-498 and CAKI-2 cells. After 24 h of shikonin treatment, we observed a ~two-fold increase in the expression of the BCRP1 gene, both in the A-498 and CAKI-2 cells, which previously displayed a decreased expression in the control level after 72 h of shikonin treatment. Significant differences ($p < 0.05$) in the expression of the BCRP1 gene were observed after 24 h of treatment in both cell lines. The expression of ABCC6 revealed a significant ($p < 0.05$) decrease after 24 h of incubation with shikonin in the A-498 cells, which increased to the control level after 72 h. There is no expression of the ABCC6 transporter gene in the CAKI-2 cells, in either the control or the shikonin-treated cells. However, the ABCB1 expression significantly ($p < 0.05$) decreased in both of the cell lines, even directly after 24 h after treatment. The expression of ABCB5 almost completely decreased in the shikonin-treated CAKI-2 cells. The ABCB5 expression in the A-498 cells showed a 50% decrease after 24 h, which almost peaked up to a three-fold increase compared to the control untreated A-498 cells after 48 incubation hours with shikonin.

4.13. The Effect of Shikonin on the Expression of the Extracellular Matrix Proteins

The RT-qPCR results revealed that the shikonin treatment significantly ($p < 0.05$) decreased the expression of CXCR4, MMP-2, MMP-9, and E-cadherin in the CAKI-2 cells. Also, a decrease in CXCR4, MMP-2, and E-cadherin in the A-498 cells was observed. However, in the expression of CXCR4, a ~two-fold increase can be seen after 72 h of treatment, compared to the untreated control cells. MMP-9 revealed a slight increase immediately after 24 h of treatment and then decreased after 48 h of incubation with shikonin.

The expressions of E-cadherin and CXCR4 were detected at the protein level as well. For the CAKI-2 cells, we observed a significant decrease in the expression of E-cadherin and a slight decrease in the expression of CXCR4. After 72 h of the treatment, we could see a sharp increase in the expression of both proteins compared to the 24 and 48 h samples. In the A-498

cells, E-cadherin showed a significant increase in its expression after 24 h of shikonin treatment. However, after 48–72 h of incubation with shikonin, the expression of E-cadherin was decreased below the level measured in the control cells. The expression of CXCR4 significantly decreased after only 24 h following treatment, and there was no increase after the 48 and 72 h treatments.

4.14. The Effect of Shikonin on the Expression of miR-21 and miR-155

Both miR-155 and miR-21 serve as oncogenic miRNAs widely expressed in different human tissues and have been identified to be upregulated in numerous cancer types. They possibly control cell proliferation, migration, and invasion, as well as inhibit apoptosis.

The CAKI-2 and A-498 cells were incubated with shikonin for 48 h, the RNA was extracted, and an examination of the expression level of the miRNAs by qRT-PCR was completed. According to the data shown, there were no significant changes in the expression level of the examined miRs of the treated cells.

5. Summary

In the course of our work, we investigated the expression pattern of certain epigenetic factors (miRNAs) that might play a role in the development of kidney tumors in the samples of patients diagnosed with kidney tumors at the Urology Clinic of the University of Debrecen. Examining the expression levels of hsa-miR-15b, hsa-miR-99b and hsa-miR-181a, we not only observed that the tumorous tissue samples express the mentioned miRNAs at a significantly lower level, but we also could see that the level of expression is related to the pathological grade of the tumorous samples. Several miRNAs are related to the regulation of molecular signaling pathways involved in RCC tumorigenesis, which miRNAs can provide a basis for RCC therapy, as they can balance pro- and antiangiogenic processes and regulate individual steps of angiogenesis. Targets of hsa-miR-15b, hsa-miR-99b and hsa-miR-181a potentially involved in angiogenesis were identified by in silico database analysis. Examining the expression levels of the targets by qRT-PCR, we observed an increase in the expression of ANG, VEGF and HIF-1α in the tumorous samples, and among the VEGF receptors, VEGFR-1 and VEGFR-3 also showed increased expression. We also found that VEGF and HIF-1α show stronger expression in tumorous tissues as the pathological grade progresses. Among matrix metalloproteinases and their inhibitors, we observed the co-expression of MMP-9/MMP-2 and TIMP-1/TIMP-2, which may play a role in tumor vascularization.

Regarding the therapy of metastatic kidney tumors, we investigated the effect of a natural active ingredient, shikonin, on cell proliferation and apoptosis in human kidney tumor cell lines. Similarly to sunitinib, which is already used in therapy, shikonin exerts its effect as a tyrosine kinase inhibitor, and as it turned out from our studies, unlike sunitinib, it is also effective at a much lower concentration. It exerts its effect on apoptosis through the regulation of the PI3K/AKT and MAPK pathways, and by reducing the expression of specific extracellular matrix proteins (CXCR4, MMP-2/-9, E-cadherin) that may inhibit cell invasion. We have observed that shikonin treatment effectively reduces the expression of some multidrug transporters, thus it can play a significant role against existing drug resistance.

It is hoped that our results will contribute to the identification of biomarkers necessary for the early diagnosis of kidney tumors, and shikonin, which has proven to be an effective active ingredient based on our in vitro experiments, may play an important role as a therapeutic application in the future (whether used as a monotherapeutic or combination therapy agent).

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List of publications related to the dissertation

1. **Király, J.**, Szabó, E., Fodor, P., Vass, A., Choudhury, M., Gesztelyi, R., Szász, C., Flaskó, T., Dobos, N., Zsebik, B., Steli, Á. J., Halmos, G., Szabó, Z.: Expression of hsa-miRNA-15b, -99b, -181a and Their Relationship to Angiogenesis in Renal Cell Carcinoma.
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2. **Király, J.**, Szabó, E., Fodor, P., Fejes, Z., Nagy, B. J., Juhász, É., Vass, A., Choudhury, M., Kónya, G., Halmos, G., Szabó, Z.: Shikonin Causes an Apoptotic Effect on Human Kidney Cancer Cells through Ras/MAPK and PI3K/AKT Pathways.
Molecules. 28 (18), 1-22, 2023.
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List of other publications

3. Farkasinszky, G., Péli-Szabó, J., Károlyi, P. K., Rácz, S., Dénes, N., Papp, T., **Király, J.**, Szabó, Z., Kertész, I., Mező, G., Halmos, G., Képes, Z., Trencsényi, G.: In Vivo Imaging of Acute Hindlimb Ischaemia in Rat Model: a Pre-Clinical PET Study.
   Pharmaceutics. 16 (4), 1-14, 2024.
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4. Szegedi, K., Szabó, Z., Kállai, J., **Király, J.**, Szabó, E., Bereczky, Z., Juhász, É., Dezső, B., Szász, C., Zsebik, B., Flaskó, T., Halmos, G.: Potential Role of VHL, PTEN, and BAP1 Mutations in Renal Tumors.
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