JBUON 2019; 24(3): 1245-1251
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Astragalin induced selective kidney cancer cell death and these effects are mediated via mitochondrial mediated cell apoptosis, cell cycle arrest, and modulation of key tumor-suppressive miRNAs

Litong Zhu[1]*, Linfeng Zhu[2]*, Junzhe Chen[1], Tongxia Cui[1], Weitang Liao[1]

[1]Department of Nephrology, SunYat-Sen Memorial Hospital, SunYat-Sen University, Guangzhou, Guangdong 510120, China; [2]Department of General Surgery, the Central Hospital of MaAnShan, MaAnShan 243000, Anhui, China

*These two authors contributed equally to this work.

Summary

Purpose: *Kidney cancer is responsible for a significant number of deaths worldwide. This cancer is often diagnosed at advanced stages and there are frequent relapses following chemotherapy. Target therapies are used now for kidney cancer, while the use of chemotherapy declines. The currently used chemotherapeutic drugs have a number of adverse effects. Herein, we examined the anticancer effects of Astragalin against a panel of kidney cancer cells.*

Methods: *CellTiter-Glo Luminescent Cell Viability Assay Kit was used to examine the anti-proliferative effects of Astragalin. Acridine orange (AO)/ethidium bromide (EB), DAPI and annexin V/promidium iodide (PI) staining assays were used to examine the apoptotic cell death. Cell cycle analysis was performed by flow cytometry. The mRNA expression was checked by qRT-PCR and protein expression was examined by western blotting.*

Results: *Astragalin inhibited the growth of the all kidney cancer cell lines with IC_{50} ranging between 20 to 50 μM. Of note, Astragalin had low cytotoxic effects on the normal kidney cells with an IC_{50} of 110 μM. The experiments have shown that Astragalin exerts antiproliferative effects on the A498 kidney cancer cells by apoptotic cell death. This effect was concomitant with upregulation of apoptotic proteins such as caspase 3 and 9 and Bax. Astragalin also induced arrest of the A498 cells at the G2/M checkpoint of the cell cycle. Also, Astragalin could upregulate the expression of tumor-suppressive microRNAs.*

Conclusions: *These results suggest that Astragalin exerts potent anticancer effects on kidney cancer cells and could pave the way in the management of kidney cancer provided clinical studies are carried out.*

Key words: *Astragalin, renal cancer, autophagy, apoptosis, caspase 3, anticancer*

Introduction

Kidney cancer originates from the renal parenchyma and/or renal pelvis [1]. The majority of these cancers (around 90%) are adenocarcinomas originating from the renal parenchyma and the remaining 10% are pelvic cancers. Kidney cancer leads to considerable mortality and the incidence of this type of cancer has been reported to be increasing over the last few decades [2,3]. The major hurdles in the treatment of kidney cancer include frequent relapses and side effects accompanying the currently used chemotherapy [4].

Plants constitute an amazing and inexhaustible source of novel bioactive molecules. Over the years these molecules have been utilized in the

Corresponding author: Weitang Liao, PhD. Department of Nephrology, SunYat-Sen Memorial Hospital, SunYat-Sen University, no.107 Yanjiang West, Yuexiu District, Guangzhou, Guangdong 510120, China.
Tel: +86 18221227971, Email: AnneliesePisciw@yahoo.com
Received: 12/10/2018; Accepted: 30/10/2018

development of drugs for treating human disorders [5]. However, with the advancement made in the field of natural products'chemistry, the purely isolated plant metabolites are now being used in the alleviation of human diseases [7]. Flavonoids include a huge group of plant metabolites that have shown promising potential for drug development. Flavonoids are commonly found in edible plants and hence are essential components of food [8]. Studies have shown that intake of flavonoids lowers the risk of development of deadly diseases such as cancer [8]. Astragalin is an important flavone and is considered to exert substantial pharmacological potential [9]. It is commonly isolated from plants and has been reported to halt the growth of several types of cancer cells [10]. For example, Astragalin has been shown to inhibit the growth of human leukemia cells [11]. However, the anticancer effects of Astragalin on kidney cancer are mostly unknown as yet. The main aim of the present study was to investigate the anticancer effect of Astragalin including apoptosis, cell cycle arrest and key tumor-suppressive miRNs.

Methods

Cell viability and colony formation assay

The different human kidney cancer cell lines (Caki-1, A498, 786-O, 796-P and hTRT) were procured from Cell Bank of Chinese Academy of Science (Shanghai, China).

Cell viability assay was carried out using CellTiter-Glo Luminescent Cell Viability Assay Kit (G7571, Promega) according to the manufacturer's protocol. A graph was plotted using GraphPad Prism5 software. The effect of Astragalin on the formation of A498 colonies was investigated as described earlier [12].

Quantitative real-time PCR

The RNA was isolated from the kidney cancer tissues and cell lines by TRIzol reagent and then transcribed into cDNA using RevertAid cDNA synthesis kit. The expression of tumor-suppressive microRNAs was determined by qRT-PCR as described previously [13].

Apoptosis assay

For AO/EB staining, the kidney cancer A498 cells (0.6×10^6) were grown in 6-well plates. Following an incubation period of around 12 h, the A498 cells were subjected to Astragalin treatment for 24 h at 37°C. As the cells sloughed off, 25 µl cell cultures were put onto glass slides and subjected to staining with a solution (1 µl) of AO and EB. The slides were cover-slipped and examined with a fluorescent microscope. DAPI and annexin V/PI staining were performed as described previously [14].

Cell cycle analysis

After incubating the kidney A498 cells with varying concentrations of Astragalin (0, 10, 20 and 40 µM) for 24 h, the cells were subjected to washing with phosphate buffered saline (PBS). Afterward, the A498 cells were stained with PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Western blotting

The A498 cells were firstly subjected to washing with ice-cold PBS and then suspended in a lysis buffer at 4°C, which, afterwards, was shifted to 95°C. After that,

Table 1. Anticancer effects of Astragalin on different kidney cancer cell lines as depicted by cell viability assay and expressed as IC_{50}

No.	*Cell lines*	*IC_{50} (µM)*
1	Caki-1	30
2	A498	20
3	786-O	50
4	769-P	25
5	hTRET	110

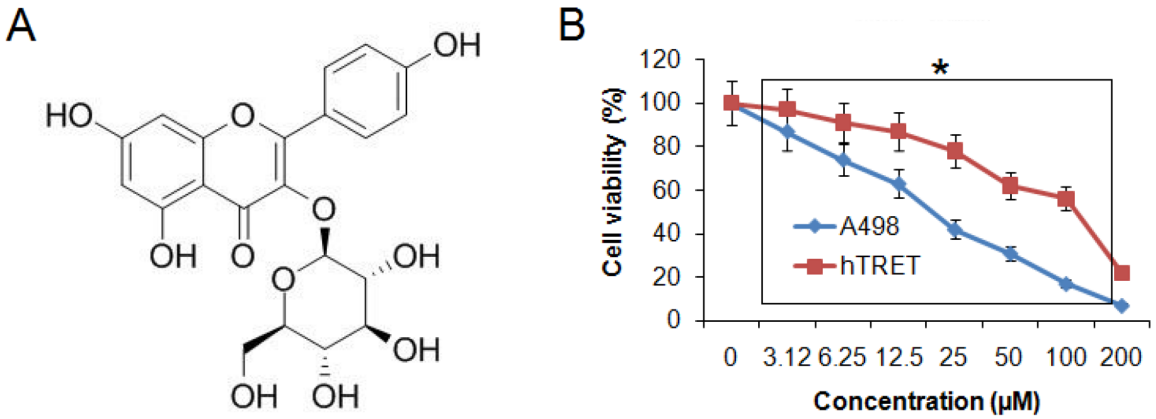

Figure 1. A: Chemical structure of Astragalin. **B:** Effect of Astragalin on the viability of the A498 kidney cancer cells and normal hTRET kidney cells. The values represent the mean of three experiments ± SD (*p< 0.05).

the protein content of each cell extract was checked by Bradford assay. About, 40 µg of protein were loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with tris-buffered saline (TBS) and exposed to primary antibodies at 4°C. Following this, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualized by enhanced chemiluminescence reagent.

Statistics

Data is expressed as mean±SD and was statistically analyzed using Student-Newman-Keuls test or t-test. P<0.05 was taken as significant difference.

Results

Astragalin inhibited the growth of kidney cancer cells

The antiproliferative effects of Astragalin (Figure 1A) were assessed on a panel of kidney cancer and normal cell lines by CellTiter-Glo Luminescent Cell Viability Assay Kit. It was found that Astragalin triggered antiproliferative effects on all the kidney cancer cell lines (Table 1). The maximum antiproliferative effects were observed against the A498 cells with an IC_{50} of 20 µM (Figure 1B). Nonetheless, the IC_{50} of Astragalin was found to be

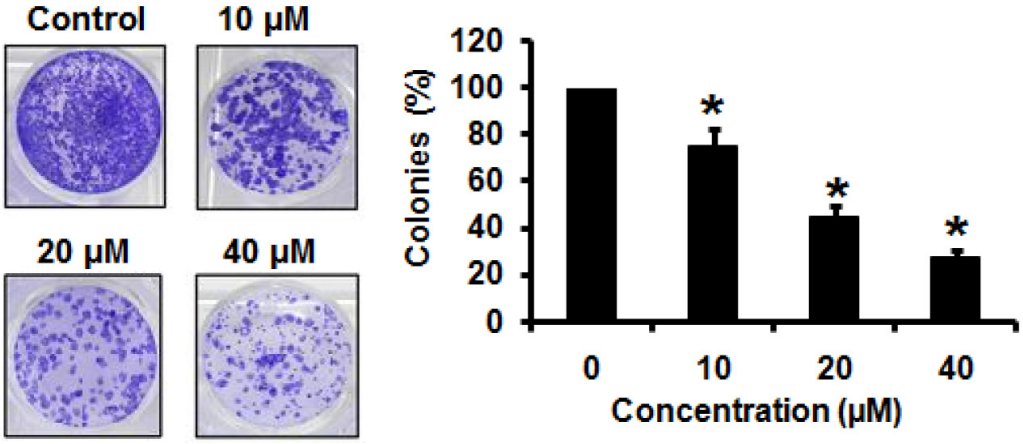

Figure 2. Effect of Astragalin on the colony formation of the A498 kidney cancer cells. The experiments were performed in triplicate and show inhibition of colony formation concentration-dependently (*p<0.05).

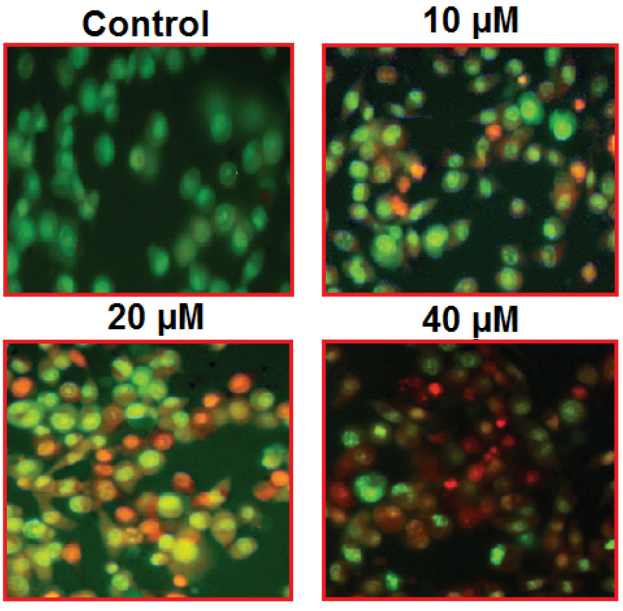

Figure 3. Astragalin triggers apoptosis in a concentration-dependent manner in A498 kidney cancer cells as depicted by AO/EB staining. Orange and red colored cells represent apoptotic cells. The experiments were performed in triplicate.

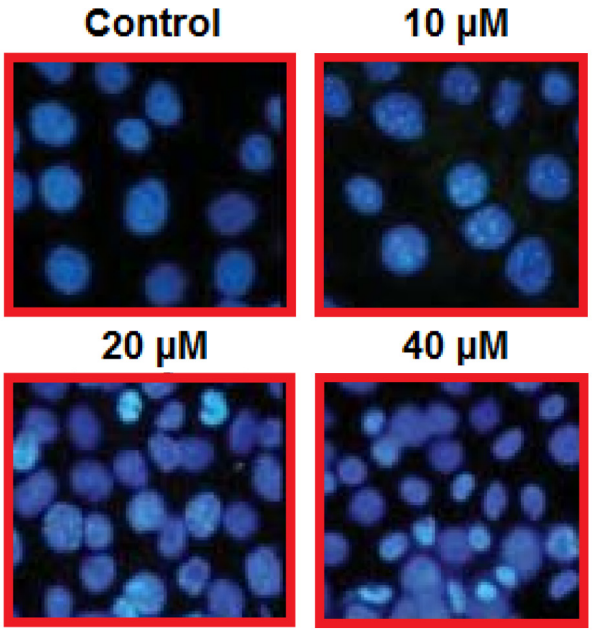

Figure 4. Astragalin induces apoptosis in a concentration-dependent manner in A498 kidney cancer cells as depicted by DAPI staining. The experiments were performed in triplicate.

comparatively higher against the normal hTRET kidney cells (IC_{50}; 110 μM) (Figure 1B). Moreover, it was found that the anticancer effects of Astragalin on kidney cancer cells were concentration-dependent. Additionally, Astragalin exerted inhibitory effects on the colony development of A498 cells concentration-dependently (Figure 2).

Astragalin induced apoptosis in kidney cancer cells

To find out if Astragalin prompts apoptosis in the A498 cells, AO/EB staining was performed which showed significant alterations in the nuclear morphology and membrane blebbing of the A498 cells (Figure 3). Furthermore, DAPI staining also showed an increased number of DAPI-positive

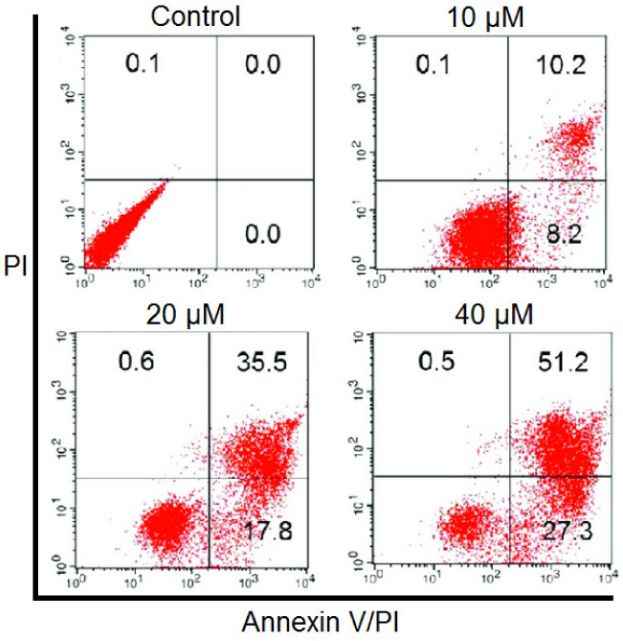

Figure 5. Estimation of apoptotic cell populations of Astragalin-treated A498 kidney cancer cells as depicted by Annexin V/PI staining. The experiments were repeated three times and the values represent the mean±SD (*p< 0.05).

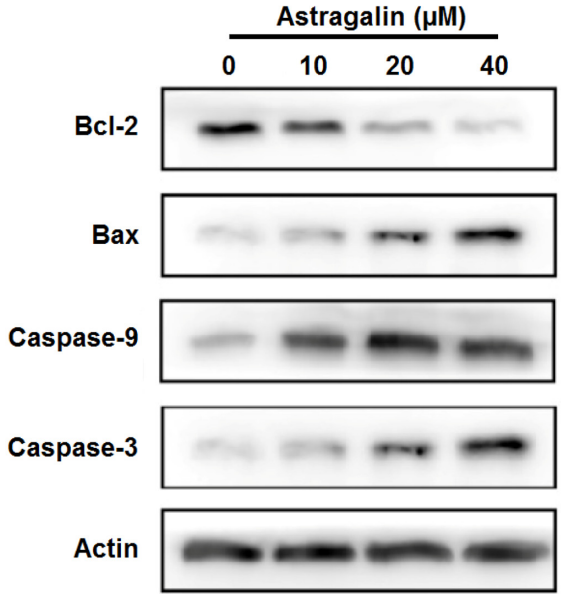

Figure 6. Astragalin alters the apoptosis-related protein expression as depicted by western blotting. The experiments were performed in triplicate. The Figure shows increased expression of Caspases 3, 9 and Bax and decreased expression of Bcl-2 in A498 cells after Astragalin treatment.

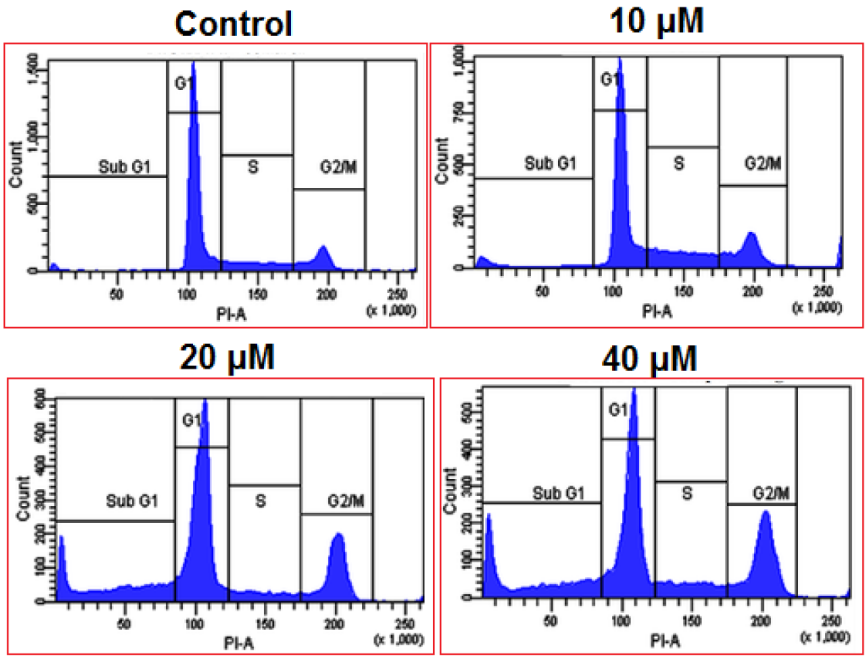

Figure 7. Astragalin causes G2/M cell cycle arrest in the A498 cells as depicted by flow cytometry. The Figure shows that Astragalin treatment led to increase of cells in the G2/M phase. The experiments were performed in triplicate.

cells, indicative of apoptosis (Figure 4). Annexin V/PI staining showed that the apoptotic cell percentage increased up to 27.3% at 40 µM Astragalin (Figure 5). The apoptosis was further confirmed by the increased expression of Caspase 3, 9 and Bax and decreased expression of the Bcl-2 in A498 cells (Figure 6).

Astragalin caused G2/M arrest of kidney cancer cells

Astragalin effects on the distribution of A498 kidney cancer cells was assessed by flow cytometry which showed that Astragalin caused a significant increase in the percentage of the A498 cells in the G2/M phase of the cell cycle. The percentage of A498 cells in the G2/M phase increased from 12.55 to 33.16% upon treatment with Astragalin (Figure 7). These results indicate that Astragalin induces G2/M cell cycle arrest of the kidney cancer cells.

Astragalin upregulated the expression of the tumor suppressive microRNAs

The effect of Astragalin was also investigated on the expression of tumor-suppressive microRNAs including microRNA-124, microRNA-203, microRNA-34a and microRNA-145. The results showed that Astragalin at IC_{50} concentration (i.e, 20 µM) caused a significant increase in the expression of all the microRNAs investigated in the present study. The fold increase of microRNA expression ranged between 3.5 to 6.2. The highest upregulation was observed in the case of microRNA-203 and the lowest upregulation was observed in the microRNA-34a (Figure 8A-D).

Discussion

The incidence of kidney cancer is increasing at an alarming rate world over [14]. The clinical outcome is dismal due to its diagnosis at advanced stage, frequent relapses and flaws connected with the currently used treatment strategies. Besides, the emergence of chemoresistance in cancer cells further makes it difficult to treat kidney cancer [3]. Herein, we report that a natural flavonoid Astragalin exerts growth inhibitory effects on human kidney cancer cells. The anticancer activities were dose-dependent and these results were also complemented by the clonogenic assay wherein dose-dependent inhibition of the colony formation was observed. Previous studies have also reported the anticancer potential of Astragalin. Astragalin has been reported to inhibit the growth of lung cancer and hepatocellular carcinoma [15,16]. Furthermore,

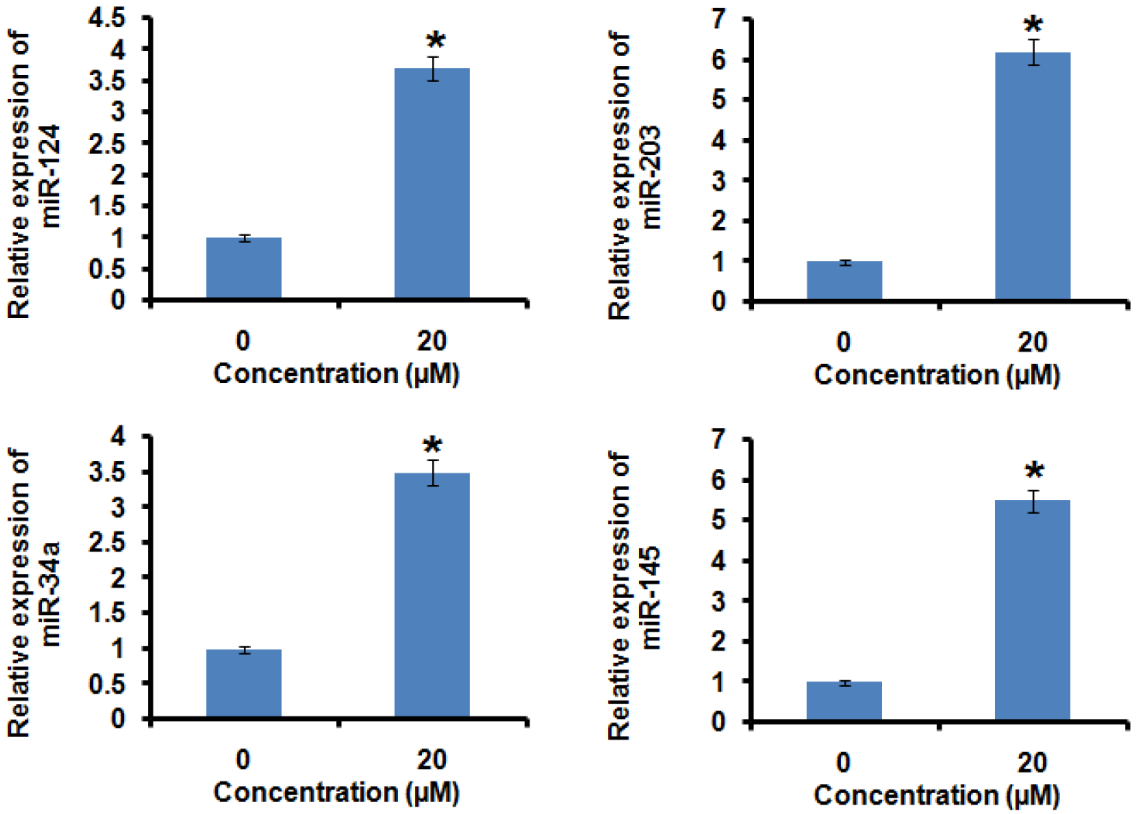

Figure 8. Astragalin upregulates the expression of tumor-suppressive microRNAs (signaling as depicted by qRT-PCR). The experiments were repeated thrice and the values represent the mean of three experiments ± SD (*p< 0.05).

it was observed that Astragalin exhibits limited cytotoxicity on the normal kidney cells indicating that this molecule specifically targets the cancer cells. To decipher the underlying mechanism, we investigated if Astragalin triggers apoptotic cell death in the A498 cells. The AO/EB staining revealed that Astragalin induces membrane blebbing and apoptosis. DAPI staining further confirmed the induction of the apoptosis and annexin V/PI staining demonstrated that the percentage of the apoptotic cells increased with increasing concentrations of Astragalin. Apoptosis in A498 cells was further confirmed by examining the expression of the apoptosis-related proteins. Astragalin increased the expression of Caspase 3 and 9 and Bax and decreased the expression of Bcl-2. Apoptosis removes the defective or cancer cells and maintains the tissues homeostasis. Apoptosis also prevents the development of drug resistance in cancer cells [17]. Previous studies have also shown that Astragalin induces apoptotic cell death in cutaneous melanoma cells [18]. Cell cycle arrest also causes halt of cancer cell growth [19]. Herein, we found that Astragalin treatment caused arrest of the A498 kidney cancer cells in the G2/M checkpoint. MicroRNAs are often dysregulated in cancer cells and have been considered to be important therapeutic targets for the treatment of a wide array of cancers. In this study, the expression of four tumor-suppressive microRNAs was investigated in the A498 kidney cancer cells and it was found that Astragalin caused significant upregulation of all these microRNAs ultimately preventing tumorigenesis. To sum up, Astragalin could inhibit the growth of A498 kidney cancer cells and may be used as a lead compound for the development of new systemic cancer therapy.

Conclusion

Astragalin exerts considerable anticancer effects on the human kidney cancer cells by induction of apoptosis. Besides, Astragalin also triggers G2/M cell cycle arrest of the kidney cancer cells. Hence, Astragalin may prove a potential lead molecule and warrants further investigations.

Conflict of interests

The authors declare no conflict of interests.

References

1. Alsaiari A, Joury A, Aljuaid A, Wazzan M, Pines JM. The content and quality of health information on the internet for patients and families on adult kidney cancer. J Cancer Edu 2017;32:878-84.
2. Nayan M, Punjani N, Juurlink DN et al. Statin use and kidney cancer survival outcomes: a systematic review and meta-analysis. Cancer Treat Rev 2017;52:105-16.
3. Motzer RJ, Jonasch E, Agarwal N et al. Kidney cancer, version 3.2015. J Nat Compre Cancer Net 2015;13:151-59.
4. Gallardo E, Méndez-Vidal MJ, Pérez-Gracia JL et al. SEOM clinical guideline for treatment of kidney cancer. Clin Transl Oncol 2018;20:47-56.
5. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Natural Prod 2016;79:629-61.
6. Patridge E, Gareiss P, Kinch MS, Hoyer D. An analysis of FDA-approved drugs: natural products and their derivatives. Drug Discov Today 2016;21:204-7.
7. Harvey AL, Edrada-Ebel R, Quinn RJ. The re-emergence of natural products for drug discovery in the genomics era. Nat Rev Drug Discov 2015;14:111.
8. Seleem DI, Pardi VI, Murata RM. Review of flavonoids: A diverse group of natural compounds with anti-Candida albicans activity in vitro. Arch Oral Biol 2017;76:76-83.
9. Nakabayashi T. Isolation of astragalin and isoquercitrin from bracken, Pteridium aquilinum. Bull Agricult Chem Soc Japan 1955;19:104-9.
10. Ni Z, Zhang Q, Qian J, Wang L. Effect of Astragalin on matrix secretion and beta 1 integrin mRNA expression in human mesangial cells. Chin Med J 1999;112:1063-7.
11. Chen M, Cai F, Zha D et al. Astragalin-induced cell death is caspase-dependent and enhances the susceptibility of lung cancer cells to tumor necrosis factor by inhibiting the NF-κB pathway. Oncotarget 2017;8:26941.
12. Nakamura N, Kusunoki Y, Akiyama M. Radiosensitivity of CD4 or CD8 positive human T-lymphocytes by an in vitro colony formation assay. Radiation Res 1990;123:224-7.
13. Xu X, Cai N, Zhi T et al. MicroRNA-1179 inhibits glioblastoma cell proliferation and cell cycle progression via directly targeting E2F transcription factor 5. Am J Cancer Res 2017;7:1680.
14. Macleod LC, Odisho AY, Tykodi SS, Holt SK, Harper JD, Gore JL. Comparative Effectiveness of Initial Surgery vs Initial Systemic Therapy for Metastatic Kidney Cancer in the Targeted Therapy Era: Analysis of a Population-based Cohort. Urology 2018;113:146-52.
15. Chen M, Cai F, Zha D et al. Astragalin-induced cell death is caspase-dependent and enhances the susceptibility of lung cancer cells to tumor necrosis factor by inhibiting the NF-κB pathway. Oncotarget 2017;8:26941.

16. Li W, Hao J, Zhang L, Cheng Z, Deng X, Shu G. Astragalin reduces hexokinase 2 through increasing miR-125b to inhibit the proliferation of hepatocellular carcinoma cells in vitro and in vivo. J Agricult Food Chem 2017;65:5961-72.
17. Koff JL, Ramachandiran S, Bernal-Mizrachi L. A time to kill: targeting apoptosis in cancer. Int J Molec Sci 2015;16:2942-55.
18. You OH, Shin EA, Lee H et al. Apoptotic effect of Astragalin in melanoma skin cancers via activation of caspases and inhibition of Sry-related HMg-box gene 10. Phytother Res 2017;31:1614-20.
19. Saleem M, Asif J, Asif M, Saleem U. Amygdalin, from Apricot Kernels, Induces Apoptosis and Causes Cell Cycle Arrest in Cancer Cells: An Updated Review. Anticancer Agents Medic Chem 2018;18:1650-5.
20. Garzon R, Calin, GA, Croce CM. MicroRNAs in cancer. Annu Rev Med 2009;60:167-79.