的目的：肾癌是导致全球死亡的主要原因之一。这种癌症通常在晚期被诊断出来，化疗后有频繁的复发。靶向疗法现在用于肾癌的治疗，而化疗的使用正在减少。目前使用的化疗药物具有许多副作用。因此，我们研究了欧芹酰胺对肾癌细胞的抗肿瘤作用。

材料与方法：使用细胞Titer-Glo荧光细胞活力检测试剂盒来检查欧芹酰胺对细胞生长的抑制作用。使用AO/EB、DAPI和Annexin V/PI染色来检查细胞凋亡。使用流式细胞术进行细胞周期分析。通过qRT-PCR检查mRNA表达，通过Western blotting检查蛋白表达。

结果：欧芹酰胺抑制了所有肾癌细胞系的生长，IC₅₀在20到50 µM之间。值得注意的是，欧芹酰胺对正常肾细胞的毒性作用较低，IC₅₀为110 µM。实验表明，欧芹酰胺对A498肾癌细胞具有抗增殖作用，通过凋亡导致细胞死亡。这种作用伴随着 caspase 3和9和Bax的上调。欧芹酰胺也能诱导A498细胞在G2/M期的细胞周期阻滞。此外，欧芹酰胺可以上调肿瘤抑制microRNAs的表达。

结论：这些结果表明，欧芹酰胺对肾癌细胞具有强大的抗肿瘤作用，并为肾癌的管理提供了可能。这些结果表明，欧芹酰胺对肾癌细胞具有强大的抗肿瘤作用，并为肾癌的管理提供了可能。这些结果表明，欧芹酰胺对肾癌细胞具有强大的抗肿瘤作用，并为肾癌的管理提供了可能。
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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 miRNAs.

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}

<table>
<thead>
<tr>
<th>No.</th>
<th>Cell lines</th>
<th>IC_{50} (μM)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Caki-1</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>A498</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>786-O</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>769-P</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>hTRT</td>
<td>110</td>
</tr>
</tbody>
</table>

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

![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.](image)

![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.](image)
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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.
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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 IC₅₀ 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,
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


