Abstract. Pancreatic cancer is a common malignant tumour that affects individuals worldwide. In recent years, the incidence and mortality rates of pancreatic cancer have continuously increased. Currently, the primary clinical treatment methods for pancreatic cancer include surgical resection, chemotherapy and radiotherapy. However, these treatment methods rarely produce satisfactory therapeutic outcomes. Extensive research has also proven that the effective components of several traditional Chinese medicines, particularly flavonoids extracted from plants, have significant antitumour effects. Isoquercitrin, which is one of the flavonoids found in *Bidens pilosa* extracts, has a significant antitumour effect. However, the antitumour effect of isoquercitrin and its mechanism of action remain unclear. The objective of the present study was to investigate the effect of isoquercitrin on the progression of pancreatic cancer and to further understand the biological characteristics of the participation of isoquercitrin in the progression of pancreatic cancer.

In vitro, we found that a therapeutic dose of isoquercitrin significantly inhibited proliferation, promoted apoptosis and induced cell cycle arrest within the G1 phase in pancreatic cancer cells. Isoquercitrin activated caspase-3, -8 and -9 and reduced the mitochondrial membrane potential. In addition, isoquercitrin inhibited the expression level of the δ opioid receptor; however, isoquercitrin had no effect on the κ and µ opioid receptors. Furthermore, isoquercitrin inhibited extracellular signal-regulated kinase (ERK) phosphorylation and promoted c-Jun N-terminal kinase (JNK) phosphorylation. In vivo, we found that a therapeutic dose of isoquercitrin significantly inhibited xenograft growth in nude mice. In summary, the present study demonstrated that isoquercitrin inhibits human pancreatic cancer progression in vivo and in vitro and that its molecular mechanism may be closely related to opioid receptors and to the activation of the mitogen-activated protein kinase (MAPK) signalling pathway.

Introduction

Pancreatic cancer is a common malignant tumour of the digestive system. The incidence and mortality rates of pancreatic cancer are extremely high (1,2). In addition, pancreatic cancer is the fourth most common cause for cancer-related deaths, and the 5-year overall survival rate is less than 2% (3). In recent years, the incidence rate of pancreatic cancer has gradually increased; pancreatic cancer severely affects the lives and health of individuals. Currently, the primary treatment method for pancreatic cancer is surgical resection. However, only a small number of patients (10-20%) qualify for surgical treatment due to the difficulty in early diagnosis of this cancer (4-6). In addition, the results from chemotherapy and radiotherapy are often unsatisfactory due to the ability of tumour cells to evade cell death (7,8). Therefore, the search for new therapeutic drugs with effective anticancer effects is extremely urgent.

Traditional Chinese medicines have multi-target, multi-stage and multi-effect antitumour effects. Traditional Chinese medicines can act on various stages of tumourigenesis and tumour development. In addition, traditional Chinese medicines have minor toxic side-effects and can improve body immunity; tumour cells rarely become resistant to traditional Chinese medicines. Therefore, traditional Chinese medicines have become a focus of antitumour drug studies. Thus far, extensive research has proven that flavonoids achieve antitumour effects by inhibiting the proliferation of some tumour cells, inducing the apoptosis of tumour cells, and regulating the expression levels of related oncogenes and tumour-suppressor genes (9-14). Isoquercitrin is the effective monomer component of *Bidens pilosa* extracts. The molecular formula of isoquercitrin is C_{20}H_{21}O_{12}, and the molecular weight of isoquercitrin is 464.38 (15,16). Previous studies have shown...
that isoquercitrin can inhibit the proliferation of various types of human tumour cells, indicating that this compound has a potential anticancer effect. In addition, the anticancer effects of isoquercitrin on liver cancer and on glioblastoma have been proven (17-20). However, the antitumour effect of isoquercitrin on pancreatic cancer cells and the mechanisms of isoquercitrin antitumour action remain unclear.

The development of pancreatic cancer is related to not only the abnormal proliferation and differentiation of cells but also abnormal apoptosis. The proliferation and apoptosis of tumour cells are precise, genetically regulated processes. Previous studies have shown that the mitogen-activated protein kinase (MAPK) signal transduction pathway regulates pancreatic cancer cell proliferation and apoptosis (21,22). However, pancreatic cancer development and progression are also closely related to opioid receptor pathways (23,24). In the present study, we used the effective monomer component of Bidens pilosa extract, isoquercitrin, for research. We used isoquercitrin to interfere with pancreatic cancer cells, studied the regulating effect of isoquercitrin on the proliferation, apoptosis and cell cycle of pancreatic cancer cells, and determined the possible molecular mechanism of the antitumour effect of isoquercitrin.

Materials and methods

Cell culture, drugs and antibodies. The human pancreatic cancer cell lines BxPC-3 and AsPC-1 were both purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The aforementioned cells were cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% foetal bovine serum (FBS) in an incubator with saturated Park Memorial Institute (RPMI)-1640 medium containing 10% FBS was used for single cell suspensions. The cells were cultured in a 96-well cell culture plate containing 10% FBS was used for single cell suspensions.

The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was used for assessing cell viability. Pancreatic cancer cells in logarithmic growth phase were selected. Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS was used for single cell suspensions. The cells were cultured in a 96-well cell culture plate (1x10^4 cells/well). Then, the cell culture plate was placed in an incubator overnight. Different concentrations (0, 50, 100, 200 and 400 µM) of isoquercitrin were added, with 6 replicates in each group. The cells were cultured for 24, 48 and 72 h. MTT (20 µl) (0.5 mg/ml) was added to each well, and the cell culture plate was placed in an incubator at 37°C for another 4 h. Finally, 150 µl of dimethyl sulphoxide (DMSO) was added. A microplate reader was used to measure the optical density (OD) value (A490 nm) of each well at 490 nm.

Flow cytometry was used to analyse the cell cycle. The cells were cultured in a 6-well cell culture plate at a seeding density of 1x10^5 cells/well. After the cells were treated with different doses of isoquercitrin for 48 h, the cells were trypsinised, and a single cell suspension was prepared. Subsequently, 70% cold anhydrous ethanol was added, and the cells were fixed at 4°C overnight. After the cells were washed with phosphate-buffered saline (PBS), the cells were treated with 50 mg/l of ribonuclease A (RNase A) at 37°C for 30 min, after which the cells were placed in an ice bath for 2 min. Next, the cells were stained with 50 mg/l propidium iodide (PI) without light exposure for 30 min at 4°C. Then, the cells were examined using a flow cytometer, and CellQuest software was used to analyse the cell cycle distribution of cells in each group.

Flow cytometry was used for assessing cell apoptosis. The cell density was adjusted to 1x10^4 cells/well. After treatment with different doses of isoquercitrin for 48 h, the cells were trypsinised, and a single cell suspension was prepared. Five microlitres of Annexin V-fluorescein isothiocyanate (FITC) and 10 µl of PI were successively added to the cell suspension, mixed well and incubated for 20 min at room temperature without light exposure. Finally, a flow cytometer was used to assess cell apoptosis.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total ribonucleic acid (RNA) was extracted from the cells in each group. After purity and integrity testing, the total RNA was reverse transcribed. After the RNA concentration was calculated, an RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to conduct the RT-PCR according to the manufacturer’s instructions. The primers used in the experiment were synthesised by Invitrogen (Carlsbad, CA, USA). Table 1 lists the primer sequences. The PCR reaction volume was 50 µl. The reaction conditions were as follows: 94°C for 2 min; denaturing at 94°C for 2 min, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec with 32 cycles in

Table I. Primers sequences for DOR, MOR, KOR and β-actin.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>DOR</td>
<td>5'-ACCAAGATCTGGTGTCCTC-3'</td>
<td>5'-CGATGACGAAGATGTTGATG-3'</td>
</tr>
<tr>
<td>MOR</td>
<td>5'-TCTGGCTCCAAAGAAGAGA-3'</td>
<td>5'-CAATGCAGAAGTGCAGCAAGAA-3'</td>
</tr>
<tr>
<td>KOR</td>
<td>5'-CGTCTCAAGAGCTCGAGCTCCG-3'</td>
<td>5'-TATGTTGAAGGGAGATCCAGC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-AAGGAAGGGCTGGGAAGATGC-3'</td>
<td>5'-CTGGGACGACATGGAGAAAA-3'</td>
</tr>
</tbody>
</table>

DOR, δ opioid receptor; MOR, µ opioid receptor; KOR, κ opioid receptor.

The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was used for assessing cell viability. Pancreatic cancer cells in logarithmic growth phase were selected. Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS was used for single cell suspensions. The cells were cultured in a 96-well cell culture plate (1x10^4 cells/well). Then, the cell culture plate was placed in an incubator overnight. Different concentrations (0, 50, 100, 200 and 400 µM) of isoquercitrin were added, with 6 replicates in each group. The cells were cultured for 24, 48 and 72 h. MTT (20 µl) (0.5 mg/ml) was added to each well, and the cell culture plate was placed in an incubator at 37°C for another 4 h. Finally, 150 µl of dimethyl sulphoxide (DMSO) was added. A microplate reader was used to measure the optical density (OD) value (A490 nm) of each well at 490 nm.
total. The obtained PCR products were verified using 1.0% agarose gel electrophoresis. In addition, a gel imaging system was used for scanning analysis.

Western blot analysis. Each group of tumour cells was collected, and protein was extracted. The bicinchoninic acid assay (BCA) was used for protein quantification. After the protein sample of each group was loaded, electrophoresis was conducted until the bromophenol blue reached the bottom of the separation gel. Then, the machine was powered off. The proteins were transferred to a membrane at 100 V for 2 h. The membrane was blocked with 5% non-fat powdered milk at room temperature with shaking for 1 h. The primary antibody, which was diluted with Tris-buffered saline with Tween-20 (TBST) solution, was added to the membrane and incubated at 4˚C overnight. The dilution ratios for the primary antibodies were as follows: phosphorylated ERK1/2 (1:800), phosphorylated JNK (1:800), phosphorylated p38 (1:800), DOR (1:800), MOR (1:800) and KOR (1:800). The secondary antibody was added to the membrane and incubated at 37˚C for 1 h. The membrane was shaken and washed 3 times (15 min each time) with a TBST solution. Images were acquired using darkroom development techniques for chemiluminescence. The results were analysed after the films were developed. The grey level ratio of the target protein to β-actin was used to represent the protein expression level.

Inoculation of nude mice. The protocol for the animal experiment was approved by the Medical Ethics Committee of Guilin Medical University. Nude mice were purchased from the Animal Experiment Center of Guilin Medical University, Guilin, Guangxi, China. In total, 40 male nude mice (6-8 weeks old, ~20 g) were used. The nude mice were randomly divided into the control and the isoquercitrin group, with 20 mice in each group. After tumour formation, isoquercitrin was administered to the mice in the isoquercitrin group by intragastric administration every day, and tumour growth was observed and measured on the 7, 14, 21 and 28 days. Four weeks later, the nude mice were sacrificed by cervical vertebra dislocation. Under aseptic conditions, subcutaneous xenograft tissue was excised for index determination.

Statistical analysis. Statistical Package for the Social Sciences (SPSS) 18.0 software was used for the statistical analysis. The measurement data are presented as the means ± standard deviation, and the enumeration data are expressed as percentages. One-way analysis of variance was used for the comparison between the groups. The Q-test was used for multiple comparisons. The results were considered to indicate a statistically significant result when p<0.05.

Results

Isoquercitrin inhibits pancreatic cancer cell proliferation. Different doses (0, 50, 100, 200 and 400 µM) of isoquercitrin were used to treat BxPC-3 and AsPC-1 cells for 24, 48 and 72 h. The MTT assay was used to assess cell viability. We found that isoquercitrin had varying levels of inhibition on the proliferation of BxPC-3 and AsPC-1 cells and that this inhibitory effect was time- and dose-dependent. As the concentration of isoquercitrin increased, the A490 nm values of the BxPC-3 and AsPC-1 cells gradually decreased; the decrease in BxPC-3 and AsPC-1 cells was the most prominent when the isoquercitrin concentration was 200 µM (Fig. 1).

Isoquercitrin induces pancreatic cancer cell apoptosis. To understand the mechanism of inhibition of pancreatic cancer cells by isoquercitrin, we used a therapeutic dose of isoquercitrin to treat BxPC-3 and AsPC-1 cells. Forty-eight hours later, Annexin V-FITC/PI staining flow cytometry was used to assess cell apoptosis. Compared with the control group, we found that the number of apoptotic cells increased significantly in the isoquercitrin group (p<0.05) (Fig. 2A). In addition, we also found that a therapeutic dose of isoquercitrin could significantly increase the activities of caspase-3, -8 and -9 (Fig. 2B). The above results indicated that isoquercitrin induces caspase family-dependent apoptosis of pancreatic cancer cells.

Isoquercitrin induces cell cycle arrest in pancreatic cancer cells. To further investigate the mechanism of inhibition of pancreatic cancer cell proliferation by isoquercitrin, we used a therapeutic dose of isoquercitrin to treat BxPC-3 and AsPC-1 cells. Forty-eight hours later, flow cytometry was used to determine the cell cycle changes. We found that a therapeutic dose of isoquercitrin could reduce the numbers of BxPC-3 and AsPC-1 cells that entered the S and the G2/M phase; the majority of the cells were arrested within the G1 phase (Fig. 3A). In addition, we also found that the levels of
cyclin-dependent kinase (CDK)4, CDK6 and cyclin D1, which regulate the cell cycle, were significantly decreased (Fig. 3B), indicating that the antiproliferation effect of isoquercitrin on pancreatic cancer cells may be due to cell cycle arrest.

Isoquercitrin inhibits the proliferation of pancreatic cancer cells via the MAPK signalling pathway. To elucidate the molecular mechanism of inhibition of pancreatic cancer cell proliferation by isoquercitrin, we used different doses of isoquercitrin to treat the BxPC-3 cells. Forty-eight hours later, western blot analysis was conducted to analyse the protein and phosphorylation levels of ERK, JNK and p38MAPK. With increasing doses of isoquercitrin, we found that the ERK phosphorylation level was gradually decreased, whereas the
JNK phosphorylation level was gradually increased; however, no significant change in the p38MAPK phosphorylation level was observed (Fig. 4). Our results indicate that isoquercitrin could inhibit ERK phosphorylation and promote JNK phosphorylation, which in turn, induced pancreatic cancer cell apoptosis.

Isoquercitrin inhibits pancreatic cancer cell proliferation via regulation of the opioid receptor signaling pathway. To further elucidate the molecular mechanism of the inhibition of pancreatic cancer cell proliferation by isoquercitrin, we used different doses of isoquercitrin to treat the BxPC-3 and AsPC-1 cells. Forty-eight hours later, RT-PCR and western blot
analysis were used to evaluate the messenger RNA (mRNA) and protein levels, respectively, of the µ, δ and κ opioid receptors. First, we found that the δ opioid receptor was expressed in pancreatic cancer cells, whereas the µ and κ opioid receptors were not expressed (Fig. 5A). After the cells were treated with a therapeutic dose of isoquercitrin, the gene and protein expression levels of the δ opioid receptor both significantly decreased compared with the control group (p<0.05) (Fig. 5B). However, after we added the specific active δ opioid receptor, the inhibitory effect of isoquercitrin on tumour cell proliferation was significantly reduced (p<0.05) (Fig. 5C). The above results demonstrated that isoquercitrin may inhibit pancreatic cancer cell proliferation via downregulation of the δ opioid receptor.

Isoquercitrin inhibits the growth of xenografts in nude mice. The nude mice were routinely fed, and xenografts were successfully transplanted. At all of the time points, we found that the mean tumour volume of the isoquercitrin group was significantly smaller than that of the control group (p<0.05). In addition, we also found that the mean weight of the surgically removed tumours from the mice in the isoquercitrin group was significantly less than that of the surgically removed tumours from the mice in the control group (p<0.05) (Fig. 6). The above results indicated that isoquercitrin could also significantly inhibit pancreatic cancer progression in vivo.

Discussion

Since the discovery of flavonoids from *Bidens pilosa*, studies have focused on using these flavonoids to treat hypertension, hyperlipidaemia, diabetes and hepatic fibrosis. *Bidens pilosa* is the dry whole grass of composite *Bidens pilosa*. *Bidens pilosa* primarily grows in the warm and moist environment of southern China and has a relatively long history of being used for treating diseases, such as malaria, diarrhoea, dysentery and hepatitis (25-28). Recently, extensive studies have found that flavonoids from *Bidens pilosa* have antitumour effects (29-32). This discovery indicates that in addition to the aforementioned functions, flavonoids from *Bidens pilosa* also play an important role in antitumour activity.

Isoquercitrin is one of the flavonoids present in *Bidens pilosa* extracts (33). In recent years, studies have shown that isoquercitrin has anti-inflammatory, anti-allergic, antioxidant and anti-injury effects (34-37); furthermore, studies on flavonoids have entered a new stage of research. Research has shown that isoquercitrin can inhibit the proliferation of some tumour cells, such as liver cancer and glioblastoma cells (17-20). However, few reports have examined the effect of isoquercitrin on pancreatic cancer and its mechanism. The development and progression of pancreatic cancer are closely related to abnormal cellular proliferation and apoptosis. To elucidate the effect of isoquercitrin on pancreatic cancer, *in vivo* and *in vitro* experiments were conducted in the present study to determine the inhibitory effect of isoquercitrin on pancreatic cancer progression. In addition, the expression levels of related proteins and genes were examined to analyse the possible signalling pathways through which isoquercitrin inhibited the growth of pancreatic cancer cells. The results from the present study provide experimental bases for the utilisation of isoquercitrin.

Numerous previous studies have shown that the monomers of many plant extracts can inhibit the proliferation of various types of tumour cells. This phenomenon is more common among flavonoid extracts (38-40). In the present study, we found that the proliferative ability of tumour cells was
significantly affected when different doses of isoquercitrin were used to treat pancreatic cancer cells. With increasing doses of isoquercitrin, the proliferative ability of the pancreatic cancer cells was gradually decreased. The proliferative ability of pancreatic cancer cells was decreased the most when the isoquercitrin dose was 200 μM. The above result indicates that isoquercitrin had a significant, dose-dependent, inhibitory effect on pancreatic cancer progression in vitro. In addition, we also conducted in vivo experiments in which isoquercitrin was used to inhibit pancreatic cancer progression. After treatment with a therapeutic dose of isoquercitrin, we found that the tumour formation rate of xenografts in nude mice was decreased significantly and that tumour growth was inhibited, indicating that isoquercitrin also inhibits pancreatic cancer progression in vivo. The abovementioned experimental results are consistent with the tumour-inhibiting effects of the monomer components of most flavonoids.

Previous studies have shown that the carcinogenesis and progression of pancreatic cancer are closely related to the maladjustment of the regulatory mechanism of cancer cell proliferation and apoptosis (41). In addition, tumour development and progression are closely related to cell cycle progression. Research has shown that tumour cell arrest at the G1 or S phase is an extremely important molecular mechanism for antagonising tumour progression (42). Our results indicated that the number of apoptotic pancreatic cancer cells increased significantly and in a dose-dependent manner after treatment with a therapeutic dose of isoquercitrin for 48 h. In addition, we also found that the protein expression levels of caspase-3, -8 and -9 were significantly increased in the tumour cells. Extensive research has proven that the occurrence of apoptosis is primarily dependent on the death receptor pathway and on the mitochondrial pathway (43). Caspase family members participate throughout the initiation and execution processes of apoptosis. After upstream caspase-9 is activated, caspase-9 then activates downstream caspase-3 and -8 to further initiate the caspase cascade reaction, which then begins apoptosis (44). Our above mentioned research results demonstrated that isoquercitrin-induced pancreatic cancer cell apoptosis may occur by activating the caspase family. Furthermore, the G1 phase percentage of pancreatic cancer cells was increased significantly, and cell proliferation was inhibited after treatment with a therapeutic dose of isoquercitrin for 48 h in the present study, indicating that isoquercitrin had an inhibitory effect on pancreatic cancer cells transitioning from G1 to S phase, which is consistent with the results from a study conducted by Huang et al (17).

The δ opioid receptor is the primary member of the opioid receptor superfamily. It has been reported that the δ opioid receptor is widely existent in various types of malignant tumour tissues and is closely related to the survival and proliferation of tumour cells (45). However, no valid evidence exists to determine whether the δ opioid receptor acts in the same manner on pancreatic cancer. We found that the δ opioid receptor was expressed in pancreatic cancer cells. However, we found that the μ and κ opioid receptors were not expressed in pancreatic cancer cells. In addition, 48 h after treatment with a therapeutic dose of isoquercitrin, the expression level of the δ opioid receptor in pancreatic cancer cells was significantly decreased, and cell proliferation was inhibited. However, when the δ opioid receptor was activated simultaneously when isoquercitrin was used to treat the pancreatic cancer cells, the inhibitory effect of isoquercitrin on tumour cell proliferation was significantly weakened or disappeared. The above result strongly indicates that the functional status of the δ opioid receptor plays a key role in the inhibition of pancreatic cancer development and progression by isoquercitrin. Further studies are required to understand its molecular mechanism.

The MAPK signalling pathway is one of the important signal transduction systems in organisms. Extensive research has proven that the MAPK signalling pathway is an important information transmission pathway from the cell surface to the cell nucleus and is the converging point of multiple signalling pathways (cell proliferation, cell differentiation) (46,47). The MAPK signalling pathway primarily includes 3 classical signalling pathways, ERK, p38MAPK and JNK. Thus far, research has shown that these 3 signalling pathways, ERK, p38MAPK and JNK, all participate in regulating the development and progression of various types of malignant tumours. A study conducted by Robbs et al demonstrated that the ERK signalling pathway participates in regulating the proliferation and differentiation of various types of tumour cells (48). The present study demonstrated that a therapeutic dose of isoquercitrin significantly inhibits ERK phosphorylation, which in turn, affected pancreatic cancer progression, which is consistent with the results from a study conducted by Robbs et al (48).

Extensive research has proven that the JNK and p38MAPK signalling pathways primarily participate in mediating cellular apoptosis; after cytoplasmic JNK is phosphorylated, this protein regulates the downstream target protein or the activity of the target protein to mediate cellular apoptosis (49). In addition, downregulation or knockout of the c-Jun gene or altering JNK phosphorylation sites can significantly inhibit tumour progression and extend survival time (50). The results of the present study demonstrated that the phosphorylation level of JNK inside of tumour cells increased significantly and that the apoptosis of tumour cells occurred after treatment with a therapeutic dose of isoquercitrin, which further proves the aforementioned theory. Previous studies have proven that p38MAPK is generally continuously activated in many types of tumours; abnormal activation of the p38MAPK pathway is closely related to the development and progression of these tumours. Existing research has shown that inhibiting the p38MAPK pathway can have an inhibitory effect on the development of various types of malignant tumours (51,52). However, we found that a therapeutic dose of isoquercitrin could not alter the p38MAPK phosphorylation level, which may be related to the fact that pancreatic cancer and other organ tumours have specific differences and that p38MAPK expression may have multiple subtypes in pancreatic cancer. The above mentioned issues warrant further study.

In summary, we found that isoquercitrin had an inhibitory effect on pancreatic cancer progression in vivo and in vitro. In addition, the molecular mechanism of the inhibitory effect of isoquercitrin may be closely related to opioid receptors and to the activation of the MAPK signalling pathway. Therefore, isoquercitrin may soon become a new drug target in the clinical treatment of pancreatic cancer, and the present study provides important theoretical bases for searching for new types of antitumour drugs.
Acknowledgements

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References


