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Basic Study

Extract of *Cycas revoluta* Thunb. enhances inhibitory effect of 5-fluorouracil on gastric cancer cells by AKT-mTOR pathway

Xing-Liang Cui, Ke-Ji Li, Hai-Xia Ren, Yong-Jian Zhang, Xiao-Dong Liu, Bao-Guo Bu, Lei Wang

Abstract

BACKGROUND

Gastric cancer is one of the most common and deadly malignancies around the world. Despite the recent progress in medical, the 5-year survival rate of gastric cancer is still unsatisfactory. During the chemotherapy for gastric cancer, 5-fluorouracil (5-Fu) is one of the first-line antineoplastic treatments, which can effectively induce apoptosis in cancer cells. However, the effect of 5-Fu is limited by the drug resistance of malignant tumor. The previous studies have reported Sotetsuflavone from *Cycas revolute* Thunb. could markedly suppress the proliferation of lung cancer cells by apoptosis, while its effect on gastric cancer remain unknown.

AIM

To investigate the inhibitory effect of *Cycas revolute* Thunb. and overcome drug resistance of gastric cancer cells to 5-Fu.

METHODS

Cell viability was carried out to determine the killing effect of natural extract of *Cycas revoluta* Thunb.on gastric cancer cells, and the half-maximal effective

concentration and the half-maximal lethal concentration were calculated for confirming it. Wound-healing and transwell assay were performed to its inhibitory effect on mobility of gastric cancer cells. Clonogenic assay was employed to investigate its synergistic effect with 5-Fu, and the apoptotic rate was detected by Hoechst staining. Western blotting was performed to examine the expression of related proteins and investigate the molecular mechanism. The expressions of proteins, like mammalian target of rapamycin (mTOR) and p-AKT, were detected in different combination of treatments for 48 h, and then analyzed by ECL detection system.

RESULTS

The gastric cancer cells were more sensitive to natural extract of *Cycas revolute* Thunb., and the extract could effectively inhibit the migration and invasion of gastric cancer cells. It could also be used with 5-Fu to improve its anti-cancer effect by enhancing the chemosensitization of gastric cancer cells. With the extract, a further reduction of drug-resistance-related proteins, p-AKT and mTOR, induced by 5-Fu was observed after 48 h by western blot. Compared to the sample with only 5-Fu, the expressions of mTOR and p-AKT were significanly reduced by about 50% and three quarters, respectively. It was also shown in this study that the natural extract of *Cycas revolute* Thunb. further increased the apoptotic rate of gastric cancer cells when using 5-Fu. The apoptosis-related protein X-linked inhibitor of apoptosis protein and apoptosis inducing factor were significantly reduced and increased, respectively, in 5-Fu-resistant gastric cancer line SGC-7901/R treated with the extract and 5-Fu, while the expression of survivin remained stable.

CONCLUSION

The natural extract of *Cycas revolute* Thunb. could effectively inhibit cell growth of gastric cancer and be used to enhance its anti-cancer effect of 5-Fu by AKT-mTOR pathway.

Key words: Gastric cancer; 5-fluorouracil; *Cycas revolute* Thunb.; Apoptosis

Core tip: 5-fluorouracil (5-Fu) is one of the effective treatments for gastric cancer, one of the most common and deadly malignancies around the world. However, the effect of 5-Fu is limited by the drug resistance of gastric cancer. Here we reported that natural extract of *Cycas revolute* Thunb. could effectively inhibit cell growth, migration and invasion in gastric cancer. Furthermore, it could be used with 5-Fu to enhance its anti-cancer effect by AKT-mTOR pathway.

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INTRODUCTION

Gastric cancer remains the fourth most common malignancy diagnosed around the world, especially in Eastern Asia, Eastern Europe as well as Middle and South America[1-3]. It also is the third main cause in the death related to malignancy, which just behind the lung and liver cancer[4]. In 2012, there was about 951600 new patients diagnosed with gastric cancer, and over 700000 deaths related to gastric cancer have been recorded[5].

With a broad spectrum of activity to inhibit malignant cell, 5-fluorouracil (5-Fu) is commonly employed to against gastric, liver and colorectal cancers[6-8]. As a prevalent chemotherapeutic drug in clinical practice, 5-Fu can disrupt proliferation and DNA replication of cancer cells, such as gastric, breast and colorectal cancer cells, by inhibiting thymidylate synthase (TS) from synthesizing thymine, which finally induces cell apoptosis[9-11].

Apoptosis is an important molecular process for the stable and orderly growth of human. It is strictly controlled inside cell and related to many diseases, like cancer[12,13]. This complex process is regulated by a series of genes involving some key proteins, such as X-linked inhibitor of apoptosis protein (XIAP), apoptosis inducing factor (AIF) and survivin. XIAP is identified as a strong apoptotic regulator[14-18] and inhibits caspase-3, -7, and -9 of the apoptotic signaling pathway in mammalian cells. AIF is released and promotes apoptosis by intrinsic signaling cascades[19,20] when mitochondria respond to apoptotic stimuli, like the translocation of BH3 interacting domain death agonist (Bid)[21]. Survivin is a unique inhibitor of apoptosis (IAP), which doesn't directly interact with caspases but with some adaptors or cofactors[22-26].

Although 5-Fu is widely used as an anticancer drug, there are some serious problems needed to be solved, such as low effective response rate or severe side effects. One of the most critical concerns is the increasing drug resistance of malignant tumor. Many drug-resistance-related proteins were identified when using 5-Fu. For example, P-glycoprotein (P-gp) functions as a molecular 'pump' to expel chemotherapy drugs from the inside of the cell, and the

resistance of 5-Fu could be reversed if P-gp expression was reduced[27]. AKT was considered as a key protein in phosphiotidylinositol-3-kinase (PI3K)/Akt signaling pathway, which is activated by the phosphorylation of Thr308 and Ser473 on the plasma membrane and can phosphorylate various downstream substrates related to drug sensitivity[28]. Mammalian target of rapamycin (mTOR), a serine/threonine kinase, is a main downstream effector of PI3K/AKT signaling pathway[29]. It is also reported that drug resistance of 5-Fu may be mediated by AKT-mTOR pathway[30,31].

Fortunately, the drug resistance could be reduced when combining other compounds in practical use. Many previous studies reported that chemosensitization of cancer cells to 5-Fu can be achieved by using dietary fats particularly n-3 polyunsaturated fatty acids (PUFAs), puerarin, iRGD, troxerutin, *etc*[32-35].

Some Chinese medicines, like *Cycas revolute* Thunb., exhibit their potential for chemosensitization, which provides some novel insights for anti-cancer treatments. According to ancient records, *Cycas revolute* Thunb. is an evergreen palm woody plant[36] with useful medicinal value, such as reducing fever and dispersing congestion. The compound, Sotetsuflavone, with strongest anti-tumor activity among its main components was identified in lung cancer cell[37,38]. Since it can effectively induce apoptosis in lung cancer cell, we studied its effect on inhibiting growth, migration and invasion of malignant cell in gastric cancer. Furthermore, we evaluated its potential for chemosensitization with 5-Fu and investigated its potential molecular mechanism.

MATERIALS AND METHODS

Materials, reagents and antibodies

The leaf of Cycas Revolute Thunb. was collected from AnGuo herbal medicine market in HeBei Province of China and identified by our laboratory. DMEM (12800017) and trypsin (25300054) were purchased from Life Technologies (CA, United States). MTT (M2128) and 5-Fu (F6627) were

purchased from Sigma (Saint Louis, MO, United States). Anti-bodies against p-gp, XIAP, p-Akt, AIF, mTOR, survivin, GAPDH were purchased from Abcam (Shanghai, China).

The extraction of Cycas Revolute Thunb.

The powder of the leaf of *Cycas Revolute* Thunb. was extracted by reflux extraction with 80% ethanol. The extracts are collected and concentrate under reduced pressure until there is no irritating odor. The product was dissolved in water and filtered. The filtrate was then extracted with dichloromethane, concentrated under reduced pressure and dried.

Cell culture

Cell lines MGC-803, SGC-790, HGC-27 were obtained from the ATCC (Manassas, VA, United States). SNU-5 was obtained from the cell resource center of Shanghai institute of life sciences, Chinese academy of sciences. GES-1 was obtained from genetics department, Beijing cancer research institute. SGC-7901/R was obtained from Shanghai institute of medicine, Chinese academy of sciences. Cell lines were cultured at 5% CO_2 and 37 ℃ with DMEM medium containing fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 U/mL). Cells were used and analyzed at logarithmic growth phase.

Cell viability and clonogenic assay

Ninty-six-well plates were emploed for cell culture in cell viability test. Gastric cell lines were treated with the extraction of *Cycas Revolute* Thunb. or 5-Fu after 24 h. The viability rate was measured by ATPlite assay (Perkin Elmer, Waltham, MA, United States)[39]. One thousand cells with different treatments were seeded into culture dish in clonogenic assays. The numbers of colonies were measured after 9 d.

Wound-healing migration assay

The MGC803 and HGC27 cells were cultured in six-well tissue culture plates and tested when the confluence is 80%. The wounds were created by sterile pipette tips (10-μL), and the loosely attached cells were washed out with phosphate-buffered saline. Light microscope was employed to photograph the progression of cell migration at different times, and in the scratched region, the number of migrated cells was calculated.

Transwell invasion assay

Twenty-four-well Boyden chambers (BD Falcon, Corning-Costar, New York, NY, United States), which have 8-mm pore size filter, was used for this assay. Samples were suspended and seeded in the insert chamber with DMEM/F12 media. It was incubated at 37 ℃ in 5% CO_2 for 24 h to allow cells migrate into the bottom, which has DMEM/F12 media and 10% FBS. Migrated cells were counted after staining with DAPI.

Western blotting analysis

Total protein was extracted with NP40 lysis buffer. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and polyvinylidene difluoride membrane were used for separating and transferring samples, in which they were closed by tris buffered saline tween-20 (TBST) solution containing 5% skim milk powder for 1 h. Primary antibodies were added overnight at 4 ℃, and then they were rinsed three times for 10 min in TBST. Secondary antibodies and the membranes were incubated for 1.5 h before they were washed. The results were analyzed by ECL detection system.

Cell apoptosis and Hoechst 33258 staining

The samples were collected after treated with the extract and washed with precooled phosphate-buffered saline (PBS). They were resuspended in 300 μL of a binding buffer diluted with PBS. After incubation for 10 min, 5 μL Annexin V-FITC was added and 5 μL PI was added later in incubation for 5 min. Then, they were rinsed three times with precooled PBS and fixed with 4%

paraformaldehyde for 30 min. After washing with PBS for three times, the Hoechst was added to the plate dropwise and incubated at room temperature for 15 min. The results were observed under a fluorescence microscope and photographed after final PBS washed.

Statistical analysis

The statistical methods used in this study were reviewed by Zhimin Shi from the College of Hebei University of Engineering. At least three times of repeats in the experiment were performed, and the data were processed by SPSS 20.0 statistical software. The standard deviation and Least Significant Difference were calculated by Student-Newman-Keuls test or Dunnett T3 test, in which $^{a}P < 0.05$ and $^{b}P < 0.01$.

RESULTS

The growth of gastric cancer cells was inhibited by the extract of Cycas revoluta Thunb.

To investigate the tumor inhibition effect of extract of Cycas revoluta Thunb., we carried out cell viability assays with different doses (from 0 μg/mL to 350 μg/mL). The results of dose-response assays showed the anti-cancer effect of extract of Cycas revoluta Thunb. was significant in gastric cancer after 24 h, especially in low and medium doses treatments (from 0 μg/mL to 250 μg/mL) (Figure 1A). For treatments under 250 μg/mL of the extract, the rate of viability of gastric cancer cells (MGC-803, SGC-790, HGC-27 and SUN-5) were drastically decreased with increasing concentrations of the extract, while that of normal human gastric epithelium GES-1 cells remained stable, which suggested that the gastric cancer cells were more sensitive towards the killing effect of *Cycas revoluta* Thunb. natural extract than normal gastric cells. Next, we analyzed the half-maximal effective concentration (EC50) and the half-maximal lethal concentration (LC50) of all cell lines (Figure 1B). It is shown that the EC50 values of gastric cancer cells ranged from 176.44 μg/mL to 194.88 μg/mL, and the LC50 values of them ranged from 135.23 μg/mL to

152.20 μg/mL. Compared to EC50 (291.32 μg/mL) and LC50 (280.27 μg/mL) values of GES-1, the extract of *Cycas revoluta* Thunb. was obviously more effective to the gastric cancer cells. In addition, under a high concentration of the extract (from 250 μg/mL to 350 μg/mL), the viability rate of gastric cancer cells showed an increase, which may be due to a screening effect for resistant cells or other adaptive mechanism.

The extract of Cycas revoluta Thunb. reduced migration and invasion ability of gastric cancer cells

To determine the effect of *Cycas revoluta* Thunb. natural extract on migration ability of gastric cancer cells, we carried out wound-healing assays. Gastric cancer cells MGC-803 and HGC-27 were selected for the test, and low-dose the extract (60 μg/mL) was employed for subsequent functional analyses, which reduced the cell viability by about a fifth. Our results showed that the extract of *Cycas revoluta* Thunb. significantly reduced the migration ability of gastric cancer cells after 24 h of extract treatment, especially for MGC-803, whose width of wound was over twice that of control (Figure 2A). To further investigate its effect on invasion ability of gastric cancer cells, we performed transwell invasion assays. It is shown that the *Cycas revoluta* Thunb. natural extract could markedly reduce cell invasion ability in both MGC-803 and HGC-27 (Figure 2B). All those results above demonstrated that the *Cycas revoluta* Thunb. natural extract could effectively inhibit the malignant cell migration and invasion in gastric cancer.

The extract of Cycas revoluta Thunb. enhanced anti-cancer effect of 5-Fu by chemosensitization

To determine whether *Cycas revoluta* Thunb. natural extract can be used with other anti-cancer drugs, we chose 5-Fu, one of the most widely used chemotherapy drugs, for the clonogenic assays.

By assessing colony formation ability, we could conclude that although the inhibitory effect of 5-Fu was stronger than that of the extract, and combining

two kinds of drugs could further enhance the inhibitory effect of 5-Fu (Figure 3A).

To investigate whether the increase of inhibitory effect is due to chemosensitization, cell viability assays were carried out with low-dose the extract (60 μg/mL) and increasing dose of 5-Fu for 24 h. It is demonstrated that the presence of *Cycas revoluta* Thunb. natural extract significantly sensitized gastric cancer cells to 5-Fu (Figure 3B). The EC50 and LC50 values dramatically reduced both in MGC-803 (1.6 times and 2.8 times) and HGC-27 (1.8 times and 3.5 times), suggesting the *Cycas revoluta* Thunb. natural extract had additive and synergistic effect with 5-Fu in inhibiting gastric cancer cells. To further confirm this result, we detected the expression of three key drug-resistance-related proteins including p-gp, p-AKT and mTOR by western blot. As a result, all of three proteins significantly reduced when 5-Fu was used with the *Cycas revoluta* Thunb. natural extract (Figure 3C), which suggested the reduced drug-resistance of gastric cancer cells.

The chemosensitization of 5-Fu with extract of Cycas revoluta Thunb. was mediated by apoptosis

Since the Sotetsuflavone from *Cycas revolute* Thunb. would induced apoptosis in lung cancer cells, we performed Hoechst 33258 staining in 5-Fu-resistant gastric cancer line SGC-7901/R after treated with the extract, 5-Fu and both of them. It is observed that both the extract and 5-Fu could induce apoptosis in SGC-7901/R, which demonstrated the extract of *Cycas revoluta* Thunb. could similarly induce apoptosis in gastric cancer cells (Figure 4A). Moreover, the combination of 5-Fu and the extract exhibited an obvious increase in apoptotic effect (Figure 4A), which suggested the chemosensitization of 5-Fu may be mediated *via* the apoptosis that was induced by the extract of *Cycas revoluta* Thunb. To confirm this hypothesis, we examined the expressional level of three important proteins which involved the apoptosis pathway. It is shown that XIAP and AIF was significantly reduced and increased, respectively, while the expression of survivin remained stable (Figure 4B).

This result demonstrated the chemosensitive enhancement of 5-Fu and the extract of *Cycas revoluta* Thunb. may due to the further activation of apoptosis.

DISCUSSION

As one of the most common cancers, gastric cancer was frequently diagnosed and leaded to thousands of deaths all around the world. Around 500000 people suffered from it died in China just in 2015[3]. Although 5-Fu was employed in chemotherapy against gastric cancer, its effect varies, which may be mainly due to the drug resistance of gastric tumor[10,11,27]. To overcome the problem of increasing drug resistance, 5-Fu usually is used with other compounds to enhance the sensitivity of cancer cells. According to previous studies, *Cycas revolute* Thunb., one of the Chinese traditional medicines, exhibited this potential on synergistic effect[37,38]. Therefore, we investigated its inhibitory effect and the effect of chemosensitive enhancement with 5-Fu in gastric cancer.

In this study, it was shown that the extract of *Cycas revoluta* Thunb. could effectively inhibit the growth of gastric cancer cell with little killing effect on the normal gastric cell in low and medium dose treatments (from 0 μg/mL to 250 μg/mL). Combining the significantly decreased EC50 and LC50 values in gastric cancer cells, we can conclude that the gastric cancer cells are more sensitive to the extract of *Cycas revoluta* Thunb. than normal gastric cells. However, the inhibitory effect on normal cells was dramatically increased when the concentration of the extract is over 250 μg/mL. On the contrary, the viability rate of cancer cells rose. It may be because of the strong screening effect of high concentration of *Cycas revoluta* Thunb. natural extract, in which drug-resistant cancer cells proliferate rapidly, or other adaptive mechanism of gastric cancer cells induced by the high concentration. This result also suggests that the dose of *Cycas revoluta* Thunb. natural extract should be strictly controlled during practical application. Besides, it is also demonstrated that *Cycas revoluta* Thunb. natural extract could significantly reduce the migration and invasion ability of gastric cancer cells, which further

confirm its inhibitory effect on gastric cancer cells.

To determine if *Cycas revoluta* Thunb. natural extract can be used with 5-Fu, we carried out clonogenic assays. The results showed that 5-Fu exhibited a stronger inhibitory effect than the extract did, and combining two drugs further inhibited the colony formation of cancer cells. By analyzing EC50 and LC50 values, we can conclude that the sensitivity of cancer cells to 5-Fu was increased when there is *Cycas revoluta* Thunb. natural extract added. This result was further confirmed by detecting the expression of drug-resistance-related proteins p-gp, p-AKT and mTOR. The expressions of p-AKT and mTOR were even further reduced in the treatments of 5-Fu when combining with *Cycas revoluta* Thunb. natural extract, which suggested that they are highly related to *Cycas revoluta* Thunb-mediated enhancement of sensitivity in gastric cancer cells. The mTOR was involved in the phosphorylation of AKT, which activate this enzyme. The activation of AKT-mTOR pathway was widely observed in various cancers, such as bladder Cancer, breast Cancer and non-small cell of lung cancer[40-45]. This pathway plays an important role in regulating the proliferation, survival, metastasis, as well as drug resistance of tumors, such as paclitaxel or endocrine therapy[44,45]. Some preclinical and clinical evidence also suggested that NEAT1, BAG-1 and XPC were involved in the enhanced drug resistance of cancer cells mediated by AKT-mTOR pathway[41,42,44,45], which may provide some clues for us to explore the mechanism that the extract sensitize gastric cancer cells to 5-Fu by AKT-mTOR pathwayfurther.

Our results of Hoechst 33258 staining proved that using two compounds will obviously increase the rate of apoptosis in cancer cells, which suggests *Cycas revoluta* Thunb. natural extract further induces apoptosis in 5-Fu treatments. This hypothesis was further determined by examining the expression of apoptosis-related proteins XIAP, AIF and survivin. It is observed that activator AIF increased and inhibitor XIAP decreased, that further explain the enhancement of apoptosis in gastric cancer cells. However, the expression of survivin remained stable, which suggest this enhancement

of apoptosis may not be mediated by survivin.

In conclusion, this study suggests that the *Cycas revoluta* Thunb. natural extract can inhibit the growth, migration and invasion of gastric cancer cells. Moreover, it can be used with 5-Fu to enhance its effect by AKT-mTOR pathway, which provides a promising strategy in chemotherapy against gastric cancer.

ARTICLE HIGHLIGHTS

Research background

As one of the most frequent cancers, gastric cancer caused more than 700000 deaths in just 2012 around the world. Although 5-fluorouracil (5-Fu) was employed in the treatment against gastric cancer, its effect was severely affected by the drug resistance of gastric cancer cells. *Cycas revoluta* Thunb. was a kind of promising drug for this purpose, while its effect on gastric cancer remain unknown.

Research motivation

To find new ways for chemical sensitization of cancer cells and improve the effect of 5-Fu during chemotherapy against malignancies.

Research objectives

To explore the anti-cancer effect of *Cycas revoluta* Thunb. in gastric cancer and investigate its chemical sensitization effect to gastric cancer cells during the treatment of 5-Fu.

Research methods

The half-maximal effective concentration and the half-maximal lethal concentration of drugs were determined by cell viability test. Then the influence of *Cycas revoluta* Thunb. on movability of gastric cells was investigated by wound-healing and transwell assay. The synergistic effect between *Cycas revoluta* Thunb. and 5-Fu was confirmed by clonogenic assay

and cell apoptotic detection. The expressions of crucial proteins were measured by western blotting.

Research results

It was shown in our study that natural extract of *Cycas revolute* Thunb. tends to kill gastric cancer cells rather than normal gastric cells. Besides, the growth, migration and invasion of gastric cancer cells were significantly inhibited by the extract. *Cycas revolute* Thunb. can also improve the inhibitory effect of 5-Fu and effectively induce cell apoptosis during the treatments. Western blotting analysis showed that the expression of P-glycoprotein, p-AKT and mammalian target of rapamycin (mTOR) were markedly reduced, which may suggest AKT-mTOR pathway playes an important role in chemical sensitization effect induced by *Cycas revolute* Thunb.

Research conclusions

Our study demonstrated the natural extract of *Cycas revolute* Thunb. can significantly inhibit the growth, migration and invasion of gastric cancer cells. Furthermore, it was also shown in our study that it could improve the effect of 5-Fu and promote cell apoptosis during chemotherapy. Therefore, our study provides a new drug for improving the clinical effect of chemotherapy in gastric cancer. Besides, it was shown in our study that the enhancement of 5-Fu's effect induced by *Cycas revolute* Thunb. may mediated by AKT-mTOR pathway, which offers a novel mechanism of the chemical sensitization effect of *Cycas revolute* Thunb.

Research perspectives

In the future, the research may reveal the main component of *Cycas revolute* Thunb. that enhance the sensitivity of cancer cells and further develop it application in anti-cancer treatments. And the molecular pathway related to AKT-mTOR may further detected for explain this mechanism.

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