Quercetin Inhibits Colorectal Cancer by Enhancing Apoptosis via PI3K/Akt Pathway

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Abstract

CRC is the second leading cancer globally, with a high lethality rate. The primary treatment of CRC is still surgery and chemotherapy with limited therapeutic effects. Quercetin, a component of traditional Chinese medicine, has shown its ability to anti-tumor and induce cell apoptosis through PI3K/AKT signalling pathway. This study aims to figure out how concentrations of quercetin and different treatment durations will influence the effects of induction of apoptosis. The experiments will use CL40 cells. Cell apoptosis is measured by flow cytometry, and cell growth by MTT assay. The phospho-AKT level is measured by anti-p-AKT Western Blot. Our research will determine the relationship between therapeutic effects, drug concentrations, and treatment durations. Further studies on the side effects of quercetin and methods to mitigate the side effects in the systematic use of quercetin should be done. Moreover, a more specific mechanism for the function of quercetin is waiting to clarify.

Keywords: quercetin, colorectal cancer, apoptosis, phospho-AKT

1. Introduction

Colorectal cancer (CRC) refers to the development of malignancy from colon or rectum, which is the second leading cancer across the world and accounts for numerous deaths every year [1]. With the progress of an ageing society, the incidence of CRC has gradually increased, and thus the burden from CRC has also grown. The treatment of CRC still mainly relies on surgical resection and chemotherapy [2]. However, recurrence and metastasis of CRC are common after traditional treatments, which always predict poor prognosis [3]. It is of great importance to investigate novel therapeutic targets in treating CRC.

Quercetin, a natural compound which owns the potential to inhibit and regulate the proliferation of tumor cells, has attracted more and more attention in recent years. Emerging studies [4]. showed that quercetin, with lipophilic property, could easily cross the cell membranes and played a role in anti-tumor process, inducing apoptosis and blocking cell cycles, et al.

Apoptosis is the spontaneous and programmed death of cells, which is regulated by genes to maintain homeostasis. Resistance to apoptosis of tumor cells is one of the main reasons for poor tumor treatment response [5]. It is widely accepted that apoptosis stimulation has the potential for inhibiting the development of cancer and preventing its recurrence and metastasis. Local tumor microenvironment affects the division and growth of cancer cells in most cases [3]. In tumor microenvironment, apoptotic cells could directly release anti-inflammatory mediators or indirectly induce anti-inflammatory responses of neighboring cells to inhibit the anti-tumor immune response of the body. Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/Akt) signaling pathway, which relates to several cell regulations including cancer cell proliferation and apoptosis, was proved to play a role in the anti-tumor process of quercetin [6].

2. Hypothesis

We hypothesize that at increasing concentrations and for longer durations treatment with quercetin can induce apoptosis enhancement via PI3K/Akt pathway, and thus affect the tumor microenvironment and inhibit CRC growth.

3. Methods

CL40 cell line (CRC cell line) will be treated with quercetin at increasing concentrations or for various durations. Then measure phospho-AKT by western blotting, apoptosis by FACS, tumor growth by MTT assay and tumor size. The positive control is Taxol, negative control is PBS/DMSO.

4. Materials

This research will use CL40 cell line (CB06214670), a human colorectal cancer cell line. And quercetin (Tsbiochem, Shanghai, China) will be diluted in dimethyl sulfoxide (DMSO) and stored at -20°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO will be purchased from

Tsbiochem. And Phospho-AKT1-S473 Rabbit mAb (AP0637) will be purchased from ABclonal.

4.1 *In vitro cell culture*

The colon cancer cell line used in this study is CL40, which requires incubation at 37°C and 5% CO_2 and special cell culture medium under in vitro culture conditions [7]. In addition to the basic nutrients, cell culture media must also have fetal bovine serum, streptomycin, and penicillin to provide a suitable microenvironment. After the cells mature, the researchers will collect and pass the cells.

4.2 *Western blotting*

Cells treated with quercetin at different concentrations or for various durations will be collected, lysed using RIPA lysate on ice for 30 min and spines for 10 min at 12000 rpm. SDS-PAGE loading buffer will be added and heated in boiling water for 10 min. The samples will be separated with 15% SDS-PAGE and then transfected to a PVDF membrane. The PVDF membrane will be sealed with TBST containing 5% skimmed milk at room temperature for 1 h and incubated with rabbit anti-p-AKT (AP0637, abclonal) overnight at 4°C [8]. The Goat anti-Rabbit fluorescent secondary antibodies will be used as a tag. After washing by TBST, the membrane will be photographed using a GE AI600 imager.

4.3 *Flow cytometry to detect apoptosis*

CL40 cells will be subcultured into 6-well plates and treated with quercetin at 20, 40, 80,120 and 160 μmol/L or 40 μmol/L taxol or 40 μmol/L PBS/DMSO for 12 h. Or CL40 cells will be treated with 40 μmol/L quercetin respectively for 4 h, 8 h or 16 h. Then digest cells with trypsin and collect cell suspension. Subsequently, the cells will be centrifuged, washed, resuspended, labeled with propidium iodide (PI). After 30-min incubation in the dark, cells will be uploaded on the flow cytometer to test cell apoptosis.

4.4 *MTT assay*

CL40 cells will be plated in 96-wells plates for 24 h. Then cells will be treated with quercetin at 20, 40, 80 ,120 and 160 μmol/L or 40 μmol/L taxol or 40 μmol/L PBS/DMSO. Or CL40 cells will be treated with 40 μmol/L quercetin for 4 h, 8 h or 16 h. Subsequently, MTT will be added to the each well at concentration of 5 mg/ml and incubated with the cells for 4 h [9]. The supernatant will be discarded and DMSO will be used to dissolve the crystals. Then use a Multiskan GO scanner (Thermo Fisher Scientific) to measure the absorbance of each well at 570nm. The measured absorbance of DMSO-treated cells will serve as a normalized reference. Each MTT experiment will be repeated for three times, for which the average value is represented.

4.5 *Mouse model*

Six-week-old BALB/C nude mice will be purchased from Animal Center of Shanghai Institute of Materia Medica Chinese Academy of Sciences. They will be raised at 26-28°C in a 12h light-night cycle. After six days of standard laboratory diet, isolated 200ul colon cancer cell suspension will be inoculated into the same position in the neck of nude mice. Then xenograft mice will be divided into groups with treatments at different drug concentrations (20, 40, 80,120 and 160 μmol/L) or for different durations (1d, 2d and 3d). The injection will be done once a day, with Taxol serving as positive control and PBS/DMSO negative [6]. Tumor size will be calculated as $(L \times W^2)/2$ to evaluate the therapeutic effects.

5. Results

Table 1. Combination of Possible Results (CR)

Treatments	Possible Results	CR 1	CR 2	CR 3	CR 4	CR 5	CR 6	CR 7	CR 8	CR 9	CR 10	CR 11	CR 12	CR 13	CR 14	CR 15	CR 16
Concentration or time increases	p-AKT increases	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	MTT death increases	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	AnnexinV/ PI apoptosis increases	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
	tumor size of xenografts decreases	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-

Treatments	Possible Results	CR 1	CR 2	CR 3	CR 4	CR 5	CR 6	CR 7	CR 8	CR 9	CR 10	CR 11	CR 12	CR 13	CR 14	CR 15	CR 16
	Supporting hypothesis	YES	Partially	Partially	Partially	Partially	Partially	Partially	Partially	Partially	Partially	Partially	Partially	Partially	Partially	NO	NO

Note: "+" represents a positive result, "-" represents a negative result.

Combination of Possible Result 1: Quercetin increases the level of phospho-AKT, MTT death, AnnexinV/PI apoptosis and decreases tumor size of xenografts in a concentration- and time-dependent manner (See Table 1).

Combination of Possible Results 2-4: Quercetin increases the level of phospho-AKT and decreases tumor size of xenografts in a concentration- and time-dependent manner. But the MTT death and/or AnnexinV/PI apoptosis are not (See Table 1).

Combination of Possible Results 5-6: Quercetin increases the level of phospho-AKT and AnnexinV/PI apoptosis in a concentration- and time-dependent manner. But tumor size of xenografts is not (See Table 1).

Combination of Possible Results 7-8: Quercetin increases the level of phospho-AKT in a concentration- and time-dependent manner. But AnnexinV/PI apoptosis and tumor size of xenografts are not (See Table 1).

Combination of Possible Results 9-12: Quercetin does not increase the level of phospho-AKT in a concentration- and time-dependent manner, but tumor size of xenografts decreases in a concentration- and time-dependent manner (See Table 1).

Combination of Possible Results 13-14: Quercetin increases AnnexinV/PI apoptosis in a concentration- and time-dependent manner, but the level of phospho-AKT and tumor size of xenografts are not influenced by concentration and time (See Table 1).

Combination of Possible Results 15-16: Quercetin neither increase the level of phospho-AKT or AnnexinV/PI apoptosis nor decrease tumor size of xenografts the in a concentration- and time-dependent manner (See Table 1).

6. Discussion

Considering the high incidence and morality of colorectal cancer, it is urgent to develop an effective method of treatment due to the limitation of current therapies. Previous studies report that quercetin promotes apoptosis of colorectal cancer cells through the inhibition of the activation of PI3K/AKT signaling pathway. And it has been proved that the therapeutic effects of quercetin increase as the concentration (20, 40, 80 μmol/L) increases (Na 1584) or the length of treatment time is prolongs [10]. To confirm whether quercetin enhances apoptosis via PI3K/AKT signaling pathway in a concentration- and time-dependent manner, concentrations of five gradients will be performed and cells will be treated for various action time. CL40, a colorectal cancer cell line will be used in this research. Consequently, measurements of the levels of phospho-AKT and cancer cell apoptosis will be done by western blot, flow cytometry and MTT assay.

Combination of Possible Result 1 supports the hypothesis that quercetin enhances apoptosis of colorectal cancer cell in a dose- and time-dependent manner, as shown in Table 1, also, the increasing concentrations and durations promote cell apoptosis through the increasing inhibition of the phosphorylation and activation of AKT. Given that higher concentrations of quercetin own better therapeutic effects, the best dose for clinical use depends on the balance of therapeutic effects and side effects. More studies should be done to clear the mechanism of side effects of quercetin to develop approaches to overcome the toxic effects of systemic quercetin treatment. Or quercetin can be loaded in some nanoparticles that target to the tumor, thus increasing the local concentration of quercetin. And quercetin should be used continuously to maintain a certain blood concentration to prolong the length of treatment time.

A combination of Possible Results 2-8 partially support the hypothesis, which shows that the level of phospho-AKT increases as the time or concentration increases. Table 1 shows in CR3, 4, 7 and 8 that increasing levels of phospho-AKT do not follow increasing AnnexinV/PI apoptosis indicates that the downstream of PI3K/AKT may be inhibited by quercetin. So clearer function of quercetin should be clarified. And the failure of in vivo experiment in CR5-8 may be related to the inadequate distribution in tumor locations or the catabolism of quercetin in vivo. So effective delivery helps to enhance the therapeutic effects. And keeping the active formation of quercetin in vivo also plays a role.

Besides, the contradictory observations of MTT death and AnnexinV/PI apoptosis in CR3, 7, 11 and 15 indicate that 1. quercetin may kill cells via other methods without the procedure of apoptosis; 2. quercetin may also inhibit the proliferation of colorectal cells instead of only promoting apoptosis (See Table 1). But the contradictory

observations of MTT death and AnnexinV/PI apoptosis in CR2, 6, 10 and 14 will be rarely happened, indicating that quercetin may simultaneously promote the proliferation of colorectal cells while promoting apoptosis. So, more research about the relationship between quercetin and proliferation or cell death should be done.

CR4 implies that the increasing level of phospho-AKT may affect the tumor size through the change in cancer cell size. So how quercetin induces atrophy should be investigated.

CR9-12 show that tumor size decreases as the concentration or time increases which partially supports the hypothesis. The results indicate that quercetin may promote the apoptosis through other pathways or inhibit tumor growth via other types of cell death. So further studies should be done to figure out the more specific mechanisms of the function of quercetin. Clearer comprehension of the pathway helps us to choose other drugs that target to complementary pathways.

As the observations in CR13-14 show, quercetin enhances the apoptosis in a dose- and time-dependent way without the engagement of AKT. So more specific mechanisms of the pathway through which quercetin works are needed to study.

CR15-16 suggests that quercetin does not influence the level of phospho-AKT, apoptosis and tumor size in a concentration- and time-dependent manner, which completely contradicts our hypothesis. The observations imply that quercetin may not be capable of inducing cell apoptosis thus inhibiting tumor growth at all.

7. Conclusion

Generally, this research explores the relationship between the therapeutic effects of quercetin and drug concentrations and treatment durations. The results of our study will confirm whether quercetin promotes apoptosis through inhibition of the activation of AKT in a concentration- and time-dependent manner. The most possible results on the relationship could provide reference for clinical use of quercetin and drive explorations on the mitigation of toxic effects. Given that a high concentration of quercetin performs better therapeutic effects, advanced delivery of quercetin could be a promising strategy for the treatment of colorectal cancer. And the relationship between therapeutic effects and treatment durations can guide the use frequency of quercetin clinically. Also, our study may provide evidence on the significance of keeping the blood drug concentration. Besides, other results suggest that quercetin may work through the extra pathway in addition to PI3K/AKT or induce other types of cell death. So, the mechanism of the function of quercetin on cancer is waiting for clarification.

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