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Research Article

The Induction of Apoptosis by Resveratrol Through Regulatory Effect of miR-21 on the Gene Expression of Bcl2 and Bax in HCT-116 Cells

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Abstract

Background: Resveratrol (RezV) which is found in several plants including grapes and types of berries has a vital role in inducing apoptosis and suppressing cell proliferation. Although the role of Bcl-2 in the apoptosis has been known in several pathways, the role and mechanism of miR-21 in the regulation of apoptosis in colorectal cancer (CRC) cells are unclear.

Objectives: The main aim of this study was to evaluate the effects of RezV on the expression level of miR-21, Bax, and Bcl2 in colorectal tumor cells.

Methods: In this study, the effect of RezV on the viability of CRC cells was evaluated by MTT assay. Then, the expression level of miR-21 was evaluated by real-time polymerase chain reaction (PCR) method. For evaluating HCT-116 cells apoptosis, the expression level of Bax and Bcl2 that are involved in the apoptosis pathway was investigated by the same method.

Results: RezV inhibits the viability of HCT-116 cells. MiR-21 gene expression was decreased after 24 hours of treatment with RezV. The reduction of miR-21 expression leads to the reduction of the Bcl2 gene expression level. Moreover, increasing the Bax/Bcl2 ratio enhances HCT-116 cells apoptosis.

Conclusion: In summary, RezV might be used as a co-treatment agent for CRC. On the other hand, conducting the in vivo study to evaluate the effects of RezV was critical.

Keywords: Resveratrol, miR-21, Bcl2, Bax, Apoptosis, Colorectal neoplasm

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies and a major cause of mortality and morbidity throughout the world (1,2). On the other hand, the incidence rate of CRC for the age group 20-49 years has increased (2). In terms of drug resistance, CRC is ranked first among the drug-resistant cancers. However, relapse is seen in 50% of patients. Therefore, understanding the molecular events involved in CRC and identifying the target molecules in this process seem necessary to find new treatments for this cancer. Because of side effects of long-term use of common therapeutic methods such as chemotherapy and radiotherapy using natural compounds with high efficacy and few side effects will be critical (3). Fruits and vegetables are a great source of vitamins and natural antioxidants, which include plant compounds that are in the chemical groups of aldehydes, alkaloids, flavonoids, glycosides, phenolic compounds, and terpenes. The phenolic compounds have many antioxidant, anti-inflammatory, and free radical scavenging properties. Current problems in the use of chemotherapy in the treatment and their several side effects for the patients, as well as the resistance of cancer cells to conventional drugs, have led researchers to think of new components with more treatment effects, fewer side effects, and less toxicity (4). Resveratrol (3,4,5′-trihydroxy-trans-stilbene) is one of the polyphenolic compounds found in different species of plants. The most abundant natural source of resveratrol (RezV) is Polygonum cuspidatum root extract which is used in traditional medicine in the Middle East. There is a significant amount of this compound in other species of plants such as peanut, red grapes, and berries in addition to Polygonum cuspidatum (5). In recent years, the biological effects of RezV on the biological function of cells have been studied. RezV is known as a compound with anti-cancer and antioxidant properties. on the other hand, RezV has anti-inflammatory and cardiovascular protection properties (6). RezV affects different biological function such as apoptosis, metastasis, invasion, and proliferation, leading to cancer cell death (7). Recent studies indicate that RezV has an anti-cancer effect on colon, prostate, breast, melanoma, liver, and glioma cancers (8-11).

Micro RNAs (miRNAs) are a group of small non-coding RNAs with a length of about 22 nucleotides

that play essential roles in various biological processes. These small RNAs bind to the 3' untranslated region (3'UTR) of mRNAs and decrease expression of target mRNA by affecting RNA translation or degradation at post-transcriptional step (12). Studies have shown that miRNAs have an important role in cell proliferation and apoptosis. Moreover, miRNAs are involved directly in the development of various cancers such as lung, breast, colon, liver, and leukemia in humans (13). Some of the miRNAs are tumor suppressors, and some of them promote cancer (OncomiR), so they act as cancer inhibitor or cancer inducers. On the other hand, miRNAs have a vital role in the regulation of essential cellular functions such as physiological and pathophysiological actives. The miR-21 expression has a strong correlation with a large number of human diseases. The upregulation of MiR-21 has been found in epithelial-derived cancers such as pancreas, stomach, breast, prostate, lung, and colon cancer. This miRNA regulates several genes involved in cell cycle and apoptosis signaling pathway, introducing this small molecule as the right choice for gene therapy of cancers and effective biomarker in diagnosis of cancers, including CRC (14-16). Studies have shown that miR-21 regulates the expression level of Bax and Bcl2 (15,17). Bax and Bcl2 proteins are essential members of Bcl2 family which are involved in cell apoptosis. The upregulation of Bcl2 is common in several cancers. Bcl2 binds to the Bax and Bak pro-apoptotic proteins and inactivates them so it has a direct effect on the apoptosis pathway (18). Studies show that miR-21 downregulated Bax gene expression and upregulated Bcl2 gene expression and can be involved in apoptosis inhibition (19). Recent studies indicate that miR-21 is a target for RezV; on the other hand, RezV reduces miR-21 expression (20,21). By considering the beneficial effects of herbal compounds and their effects on the treatment of cancers (22), we studied the effect of RezV on the HCT-116 cells.

In this research, we tried to find the relationship between miR-21 and the main genes involved in cellular apoptosis in the RezV-treated cells. Moreover, there is a beginning for more complete research on whether the Bcl2 gene is a direct target of miR-21. The main objective of this study was to evaluate the effect of RezV on the gene expression of miR-21, Bax, and Bcl2 in CRC cells (HCT-116).

Materials and Methods

Cell Culture

CRC cell line (HCT-116) was purchased from Pasteur Institute (Tehran, Iran). The cells were cultured in DMEM culture medium (Gibco, Invitrogen, USA), supplemented with 10% FBS and 1% Penicillin/Streptomycin (Gibco, Invitrogen) and incubated at 37°C with 5% carbon dioxide and high humidity conditions. RezV powder (Alfa Aesar) was dissolved in ethanol, and intermediate concentration of 10 mM was prepared. Then, the cells were treated with different concentrations of RezV.

MTT Assay

Cytotoxic effect of RezV was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. About 6000 cells were seeded in each well of 96-well plate and incubated overnight. Afterwards, the cells were treated with different doses of RezV including 25, 50, 100, 200, and 400 mM, which were made from a stock solution of 10 mM (Alfa Aesar). The stock solution was prepared by dissolving RezV powder in ethanol. After 24 and 48 hours, 10 mL of MTT was added to each well, and the cells were incubated for 3 hours. Then, cell culture medium was removed, and 100 mL of DMSO was added to each well. Then, the optical density (OD) was measured at 570 nm wavelength by ELISA plate reader (23). The IC50 of RezV was determined by modified Karber's method according to the formula: IC50=log-1 [Xk-i(Σp-0.50)], in which Xk represents the logarithm of the highest drug concentration, *i* is that of the ratio of adjacent concentration and ΣP is the sum of the percentage of growth inhibition at various concentrations (24).

The Quantitative Gene Expression of Bax, Bcl2, miR-21 by Real-Time PCR

A predetermined number of cells were seeded in the 6-well plate and incubated overnight. The cells were treated with different doses of RezV, including 50, 100 and 150 µM. Afterwards, total RNA was extracted by RNX-plus (Cinnagen, Iran) according to the manufactured protocol. The RNA concentration and absorption (280/260 and 230/260) were confirmed by Nanodrop (Germany). The quality of RNA was evaluated by electrophoresis on 1% agarose gel. For the synthesis of first-strand cDNA, Thermo Fisher cDNA synthesis kit was used according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA of miRNAs was synthesized using miRNA amplification kit (ParsGenome Co., Iran) according to the manufacturer's instructions. Briefly, in the first step, an appropriate amount of RNA (calculated with a nanodrop) was incubated with 2 µL buffer (10x), 2 µL ATP, 0.5 µL polyA polymerase enzyme, and diethyl pyrocarbonate-treated water at 37°C for 10 minutes. Then, in the second step, 5 µL polyadenylated RNA was mixed with 2 µL buffer (5x), 0.5 µL reverse transcriptase enzyme, 1 µL primer, and 1 µL dNTPs. At the final point, the mixture was incubated in a thermal cycler at 42°C for 60 minutes, followed by 5 seconds at 85°C. The quantitative real-time PCR was done in CFX96 instrument (BioRad, USA). We used SYBR Green (Takara, Japan), primer pairs of Bax, Bcl2, and miR-21 as well as tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) and U6 (U6 Spliceosomal RNA) as reference genes (25). The

Table 1. Specification of Primer Pairs

Primer name	Primer sequences	Annealing Temperature	Product size (bp)	Accession (Gene id)
Bax- F	CGCCGTGGACACAGACTC	53	129	NM-138761.3
Bax-R	GCCTTGAGCACCAGTTTG			
Bcl-2- F	TGGAGAGTGCTGAAGATTGA	50	121	NM-000657.2
Bcl-2- R	GTCTACTTCCTCTGTGATGTTGTAT			
YWHAZ- F	AACAGCTTTCGATGAAGCCAT	52	120	NM-003406
YWHAZ- R	TGGGTATCCGATGTCCACAAT			

Primer pairs used in this study were shown in Table 1. Real-time PCR primer pairs for miR-21 and U6 were obtained commercially from ParsGenome Co. (Iran)

The thermal cycling conditions for real-time PCR involved initial denaturation at 95°C for 5 seconds, 40 cycles of denaturing at 95°C for 30 seconds, 30 seconds of annealing temperature according to Table 1, and 30 seconds of extension at 72°C. For miRNA, the extension temperature was changed to 62°C for 20 seconds. The levels of mRNA and miRNA expression were normalized to YWHAZ, U6, and 5S ribosomal RNA (rRNA), respectively. Relative expression levels were reported by $2^{-\Delta\Delta CT}$ method (26).

Statistical Analysis

The Mann-Whitney and Kruskal-Wallis tests were used for evaluation of the mean difference between 2 groups and more than 2 groups. The Pearson test was used for evaluating the correlation of Bcl2 and miR-21 expression levels. *P* values of less than 0.05 were considered significant.

Results

Inhibitory Effect of RezV on the Viability HCT-116 Cells

We investigated the effect of various concentrations of RezV (25, 50, 100, 200, and 400 µM) on HCT-116 cells viability by MTT assay. As shown in Figure 1, RezV affected viability of the CRC cells. RezV had a significant cytotoxic effect at 100 mM concentration at 24 hours and 50 mM at 48 hours. To calculate the IC50 of RezV, the graph of cell viability was plotted versus the Log (concentration of RezV). By considering the equation of linear model fitted with data, the IC50 can be calculated. The IC50 values of RezV at 24 and 48 hours were calculated to be 100 mM and 50 mM, respectively.

Suppression of miR-21 Expression by RezV

The first step in the study of the mechanism of the inhibitory effect of RezV on the expression of miR-21 was the determination of expression level of this miRNA in the control group and 3 groups treated with different concentrations of RezV. To find the best internal control gene, we evaluated the expression level of U6 and 5S rRNA as a candidate reference gene for normalization of miRNA expression level. The results of the Kruskal-Wallis test indicated that U6 expression was not significantly different between the treatment groups (*P* value=0.08). Therefore, U6 was used as a reference gene for normalization of miRNA expression level. The expression level of miR-21

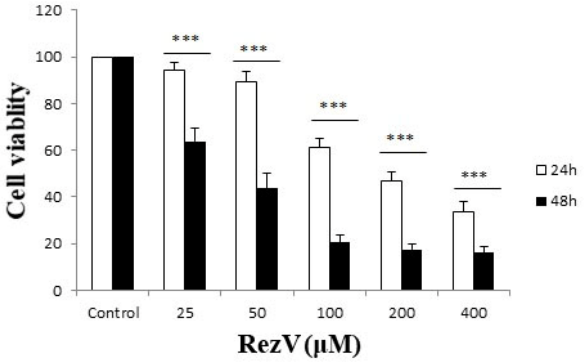

Figure 1. Effect of Resveratrol on HCT116 Cell Viability.
The HCT-116 cells were treated with different concentrations of RezV for 24 and 48 hours. There is a significant difference in viable cells at 100and 50 µM dose of RezV at 24 and 48 hours, respectively (*** $P<0.001$).

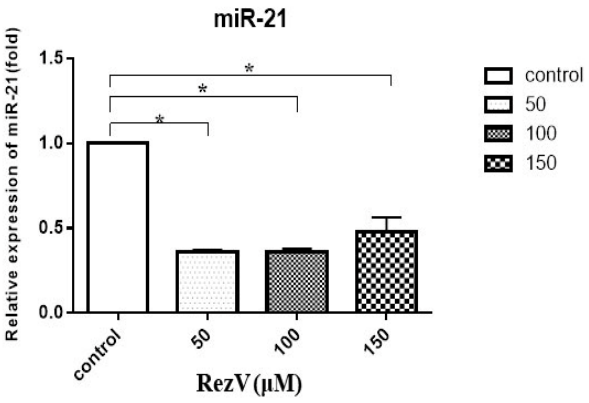

Figure 2. Effect of Resveratrol on the miR-21 Expression.
Resveratrol leads to downregulation of miR-21 after 24 hours of treatment with RezV at different doses (* $P<0.05$).

after 24 hours of treatment with RezV at 50, 100, and 150 mM concentrations was measured using real-time PCR. As shown in Figure 2, with an average decrease of 2.7 fold, the mean ± SD CT value of miR-21 at 50, 100, and 150 doses was 0.36±0.01, 0.36±0.02, and 0.47±0.09, respectively, so miR-21 expression levels significantly have decreased.

Downregulation of Bcl2 Expression Followed by miR-21 Suppression

In this study, for mRNA normalization, the following genes were evaluated: 18s (18s Ribosomal RNA), Glyceraldehyde-3P Dehydrogenase (GAPDH), Beta Actin (ACTB), and YWHAZ. The results of the Kruskal-Wallis test indicated that YWHAZ showed the least and acceptable variability (P =0.431) in treatment groups, therefore, we use that as the reference gene. The Pearson correlation coefficient between Bcl2 and miR-21 expression levels was -0.711 (P <0.05). In the present study, in addition to reducing miR-21 gene expression, RezV decreased Bcl2 gene expression in HCT-116 cells. As shown in Figure 3, on average, there has been a three-fold decrease in the expression of this gene. The mean ± SD CT value of Bcl2 at 50, 100, and 150 doses was 1.36+0.29, 0.76+0.05, and 0.33+0.02 with P-values of 0.032, 0.252, and 0, respectively, (P <0.05) as compared with control.

Downregulation of Bcl2 Gene and Upregulation of Bax Gene

The results of this study showed that the expression of the Bax gene increased approximately one-fold at all three doses of treatment with RezV compared to the control. This increase shows a significant difference (0.019) with control using the Mann-Whitney test. The mean ± SD CT value of Bax at 50, 100, and 150 doses was 1.36 + 0.09, 1.39 + 0.36, and 1.23 + 0.18, respectively. The increase was slightly reduced in the group treated with 150 mM RezV compared to the 2 doses of 100 and 50. However, all 3 groups showed a significant difference compared to the control group (Figure 4).

Change in Bax/Bcl2 Ratio After Treatment With RezV

Bax and Bcl2 genes are Bcl2 family members and are the main regulators of apoptotic pathways. The ratio of the expressions of these genes determines the fate of the cell in the direction of death or cell survival. Bcl2 is an anti-apoptotic protein, and Bax is the homological structure of Bcl2 and promotes apoptosis. Therefore, the relation between the expressions of Bax and Bcl2 is an important factor in apoptosis. As shown in Figure 5, Bax/Bcl2 ratio increased significantly after RezV treatment.

Discussion

There are many problems in the chemotherapy of cancerous patients. They have several side effects during treatment. Additionally, cancer cells have resistance to the common drugs, especially in CRC (27). Such matters encouraged the researchers to find new component with more effective treatment and minimal toxicity (28). In general, the apoptosis decreased and proliferation increased in the cancer cell population (29-31). On the other hand, one of the interesting strategies for the interactions of plant compounds is their ability to induce apoptosis (32). Therefore, apoptosis can play an important role in the treatment of cancer. RezV is a plant compound that is considered by researchers. Many studies have been conducted on RezV and the induction of apoptosis by this compound. Studies have also shown that this combination, while having a low cytotoxic effect for normal cells, plays a significant role in apoptosis of cancer cells (9,11). Therefore, in the present study, we selected RezV as an effective combination for inhibiting cell proliferation and apoptosis. The present study attempts to investigate the molecular mechanism of the effects of RezV on growth inhibition and apoptosis induction in colorectal

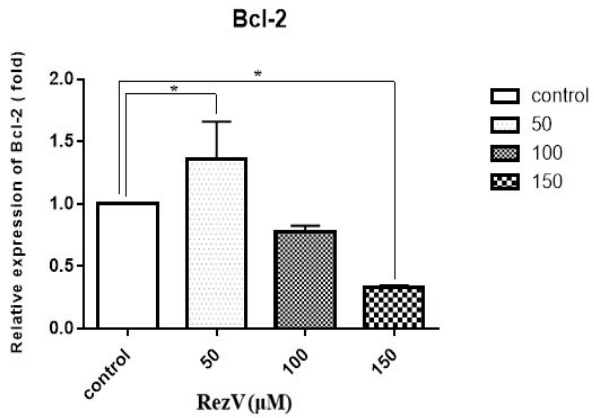

Figure 3. Effect of Resveratrol on the Bcl2 Expression.
The expression level of Bcl2 was increased after 24 hours of treatment with RezV at the dose of 50 µM and decreased after treatment with RezV at the dose of 150 µM (*P<0.05).

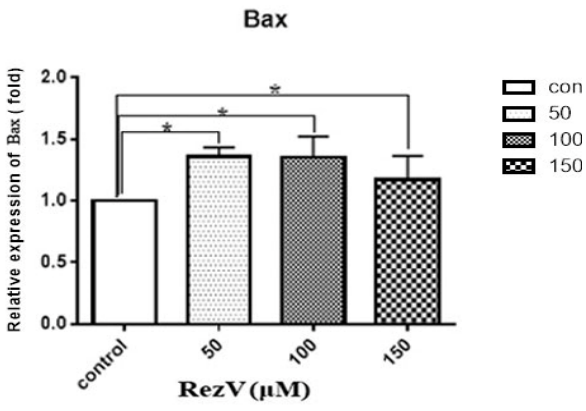

Figure 4. Effect of Resveratrol on the Bax Expression.
Bax expression level after 24 hours of treatment with different doses of RezV significantly increased compared to the control group. However, this increase was less at the dose of 150 µM (*P<0.05).

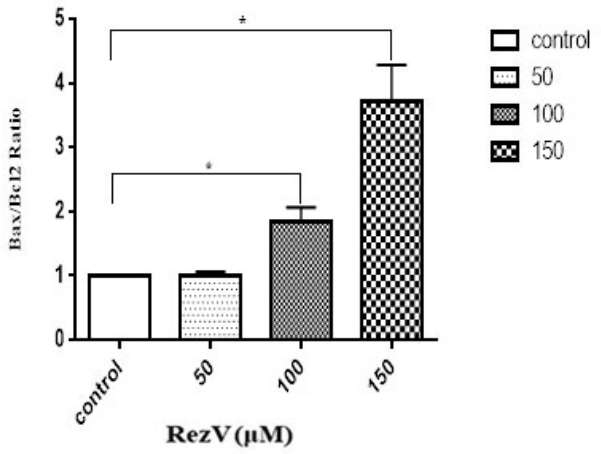

Figure 5. Effect of Resveratrol on the Bax/Bcl2 Ratio.
The Bax/Bcl2 ratio after 24 hours of treatment with RezV at doses of 100 µM and 150 µM was significantly increased compared to the control (*$P<0.05$).

tumor cells. The miR21 is a specific miRNA which was upregulated in many epithelial-derived cancers including breast, pancreas, lung, gastric, prostate, and colon cancers (33-35). This oncogene miRNA plays a significant role in regulating the proliferation, apoptosis, and metastasis of cancerous cells (35). Therefore, it can be used as an important diagnostic biomarker in cancers, especially in CRC (14). The previous study showed that miR-21 was the target for RezV and RezV can have an effective role in reducing the expression of this miRNA (16). In this study, the effects of RezV on the viability of HCT-116 cells were investigated using MTT method. The results of this study showed that RezV, depending on the concentration at different times, has a significant effect on inhibiting the growth of HCT-116 cells. Furthermore, RezV treatment leads to downregulation of miR-21 which is similar to the results obtained by Stiegelbauer et al in 2014 (16). The overexpression of miR-21 is common in cancerous patients and reducing the expression level of this miRNA leads to apoptosis induction; these evidences confirm the interference of this microRNA in the cell apoptosis pathway (34). According to the results of recent studies, the upregulation of miR-21 suppresses the expression of the Bax gene and enhances the expression of the Bcl2 gene, leading to the inhibition of apoptosis. Based on the results of studies by Boise et al and Hockenbery et al, Bcl2 is involved in the apoptosis signaling pathway. The results of their finding showed that Bcl2 was upregulated in various cancers, and its reduction was associated with increased cell apoptosis (36,37). We found that RezV (150 µM) leads to the downregulation of Bcl2 in HCT-116 cells. Our results indicate that downregulation of miR-21 was associated with the Bcl2 gene expression. Consistent with the results of studies conducted by Xu et al and Seca et al, our results indicated that down-regulation of miR-21 was associated with Bcl2 downregulation (38,39). Although the results of studies are similar to those of the present study, Wickramasinghe et al has reported that miR-21 expression is reduced by estradiol in breast cancer cells (MCF-7) and this decrease has caused an increase in the expression of the target miR-21 genes, PDCD4, PTEN, and Bcl2. However, they continue to state that this is not a definite result and that the increase in Bcl2 gene expression can not be attributed solely to the decrease in expression of estradiol-induced miR-21 (40). According to our study and similar studies, it seems that Bcl2 is not the direct target of miR-21 (38,39,41). Based on the results of recent studies, RezV regulates Bcl2 expression through the PTEN/AKT axis. On the other hand, downregulation of miR-21 by RezV decreases the AKT activity, leading to the downregulation of BCL2 (10,42).

The anti-apoptotic proteins of the Bcl2 family, as previously described, act against pro-apoptotic proteins. In fact, Bcl2 is one of the key proteins in the family in the apoptosis pathway that bind to and inactivate BH3-domain pro-apoptotic proteins (Bax and Bak) (18). It means that the ratio of anti- to pro-apoptotic dimers determines the fate of the cell in the direction of apoptosis. We observed that Bax gene expression increased in HCT-116 cells treated with RezV. Although the Bax expression was not very high at the dose of 150 mM RezV, it had a significant difference in comparison with control. By examining the Bax/Bcl2 ratio, studies have shown that the role of Bcl2 in the development of CRC is more important compared to Bax (43,44).

Similar results obtained in this study also show that the effect of downregulation of Bcl2 gene on apoptosis process is more significant than the effect of upregulation of Bax gene. According to the description, in the present study, the Bax/Bcl2 ratio as an apoptosis index, which determines the apoptosis or cell death due to exposure to cytotoxic materials, was also investigated.

Conclusion

Briefly, in our study, we found the relationship between the inhibitory effect of RezV on miR-21 and the effect of RezV on increasing the Bax/Bcl2 ratio in HCT-116 cells. After 24 hours of RezV treatment, miR-21 gene Expression level and following that Bcl2 gene expression level was decreased. Increasing the expression ratio of Bax/Bcl2 enhances HCT-116 cells apoptosis. Therefore, it seems that RezV by affecting miR-21 (onco-miR) in crucial genes involved in cell apoptosis pathway can be considered a novel co-treatment method in CRC.

Conflict of Interest Disclosures

None.

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