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Original Article

Enhancement of the Efficacy of 5-fluorouracil by Amygdalin in HT29 Colorectal Cancer Cells via Increased Expression of the p53 Protein

Bahman Moradi Poodeh [1*], Bahare Vahidianfar [1]

[1] Department of Laboratory Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran

*** Corresponding Author:** Bahman Moradi Poodeh, Department of Laboratory Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran. E-mail: bmoradipoodeh@yahoo.com

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Abstract

Introduction: Colorectal cancer is one of the most common cancers in the world and causes many deaths in the world every year. The first-line treatment for this type of cancer is the drug 5-fluorouracil. The most important weaknesses of this drug are numerous side effects and resistance to this drug. Amygdalin, abundantly found in the seeds of fruits such as apples, apricots, and bitter almonds, is a cyanide compound traditionally consumed by people suffering from cancer. Antitumor effects of amygdalin have been reported in different types of cancer cells. The present study aimed to investigate the cytotoxic effects of amygdalin in combination with 5-fluorouracil on cell death and the expression of P53 protein in the colorectal cancer cell line HT29.

Methods: The cell viability of HT29 cells after treatment with different concentrations of amygdalin and 5-fluorouracil individually and in combination was evaluated by the MTT test. The expression level of P53 protein was measured by the Western blot method.

Results: HT29 cell viability was inhibited by amygdalin and 5-fluorouracil in a dose-dependent manner. The concurrent use of the combination of amygdalin and 5-fluorouracil showed that amygdalin enhances the lethal effects of 5-fluorouracil. Furthermore Also, amygdalin at a concentration of 10 mg/ml intensified the effects of a 100 µM concentration of 5-fluorouracil on the expression level of P53 protein.

Conclusion: The concurrent use of amygdalin-5-fluorouracil may be a valuable candidate for colorectal cancer patients. However, more studies are needed to confirm these results.

Keywords: Colorectal Cancer, Amygdalin, 5-fluorouracil, HT29 Cell Line, P53 Protein

Introduction

Cancer is a multifaceted disease that is primarily characterized by uncontrolled and proliferative cell growth, leading to the development of tumors. Colorectal cancer (CRC) is the fourth most common cancer and the second cause of death worldwide, resulting in numerous fatalities annually.[1]

5-fluorouracil (5-FU)-based chemotherapy is used as a first-line treatment for colorectal cancer. 5-FU acts in several ways, but principally as a thymidylate synthase (TS) inhibitor. Interrupting the action of this enzyme blocks synthesis of the pyrimidine thymidylate (dTMP), which is a nucleotide required for DNA replication. However, the response rate to 5-FU is low in patients with progressive colorectal cancer. The numerous side effects and the development of drug resistance by cancer cells are other drawbacks of this drug.[2]

Natural compounds found in various parts of plants have been traditionally practiced in different societies. These compounds typically exhibit limited side effects, and numerous studies have explored their use in combination with conventional treatments.[3]

Amygdalin is a natural compound containing cyanide, found primarily in the seeds of fruits such as apricots, peaches, apples, and bitter almonds, and it has been traditionally consumed by cancer patients. There is conflicting evidence regarding the anti-tumor effects of amygdalin.[4,5] In recent years, research has indicated that amygdalin may affect the destruction of cancer cells through various mechanisms, such as the induction of apoptosis by changing the expression of genes involved

in apoptosis, such as caspases, Bcl-2 family, and P53; suppression of cellular proliferation; and inhibition of angiogenesis.[6] The simultaneous use of anticancer drugs and natural compounds has been shown to have stronger cell death effects. In a study, it was shown that amygdalin enhanced the effects of lapatinib on cell death by increasing the expression of the protein box. Also, the simultaneous use of lapatinib and metformin synergistically increased cancer cell death.[5,6]

Numerous genes have been identified that play a role in tumorigenesis in humans. Generally, a mutation in a single gene does not cause cancer; rather, cancer develops when mutations occur in critical genes. These critical genes are divided into three major categories: proto-oncogenes, tumor suppressor genes, and DNA repair genes. Considering the role of numerous genes in tumorigenesis, it is essential to identify specific target genes for effective cancer treatment.[7,8] The P53 gene is one of the most critical genes, as its alterations significantly contribute to the tumorigenesis of many cancer cells, attracting the attention of numerous researchers. Studies indicate that mutations in the P53 protein-coding gene are present in about 20 to 25 percent of breast cancers, as well as in over 50 percent of bladder, colon, and lung cancers.[9] Due to its critical role, P53 is often referred to as the "guardian of the genome," as it protects the cell from damage during the G1-S phase of the cell cycle. Therefore, the P53 protein can apply its tumour-suppressive effects by inhibiting the cell cycle and inducing apoptosis in response to various intracellular and extracellular stimulants such as DNA damage.[10,11] Considering the antitumor effects that have been reported for amygdalin in various cancer cell lines, the present study aims to investigate the cytotoxic effects of amygdalin in combination with 5-fluorouracil on cell viability and P53 expression in the human colorectal cancer cell line HT29.

Materials and Methods

Amygdalin powder (A6005), MTT powder, and the antibiotics penicillin and streptomycin were purchased from Sigma-Aldrich, USA. DMEM medium and FBS were purchased from Gibco. The rabbit monoclonal anti-P53 antibody (9282s) was purchased from Abcam, the rabbit monoclonal anti-β-actin antibody (ab32503) and the HRP-conjugated anti-rabbit IgG antibody (7074s) were purchased from Cell Signaling, the ECL kit was purchased from Bio-Rad, and the HT29 cell line was purchased from the Pasteur Institute of Iran.

Cell Culture Conditions

The HT29 colorectal cancer cell line was cultured in DMEM (Dulbecco's Modified Eagle Medium) with high glucose, 10% fetal bovine serum, and 0.5% penicillin-streptomycin at 37 °C with 95% humidity and 5% CO_2.

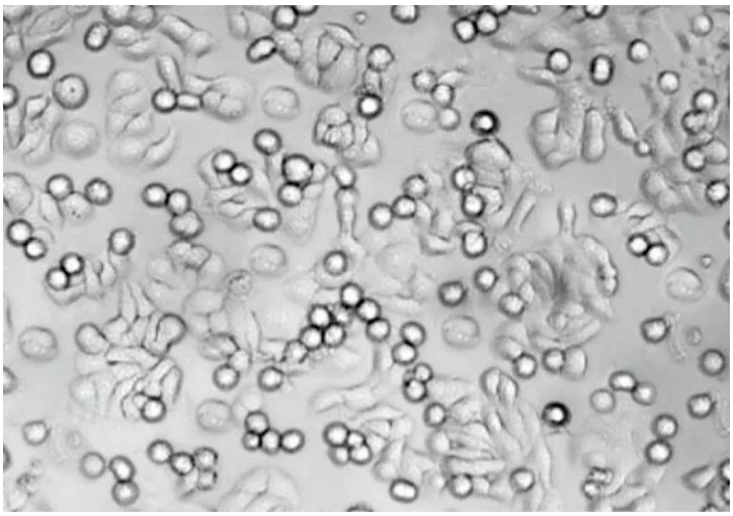

Figure 1. Morphology of HT29 Colon Cancer Cells.

Cell Culture and Cytotoxicity Assay

Approximately 6×10^3 HT29 cells were seeded in 96-well plates after the third passage. To allow the cells to attach to the bottom of the wells, the cells were incubated overnight. To evaluate the cytotoxicity of amygdalin and 5-FU, cells were treated with different

concentrations of amygdalin (2.5, 5, 10, 20, 40, and 80 mg/ml) and 5-FU (5, 25, 50, 100, and 200 µM) individually and in combination for 48 hours. Control group cells in each group remained untreated. After the end of the treatment time, the culture medium was removed from the cells using a micropipette. After removing the culture medium, 20 µl of MTT solution with a final concentration of 1 mg/ml was added to each well. The added solution was incubated with the cells for approximately 4 hours at 37 °C until purple crystals formed. After crystal formation, the yellow supernatant was carefully removed using a micropipette. To dissolve the crystals, 100 µl of dimethyl sulfoxide (DMSO) solvent was added to each well and placed in a shaking incubator for 30 minutes. Finally, the absorbance of the cells was read at 570 nm using an ELISA reader (BioTek 800 Winooski, USA).

Western Blotting

To examine the expression level of the p53 protein, six-well plates were used for cell culture. Approximately 3x10^5 HT29 cells were seeded in the plates. The culture medium was removed after overnight incubation to allow the cells to attach to the bottom of the wells. After cell attachment, the cells were treated with specific concentrations of amygdalin and 5-FU, both individually and in combination.

At the end of the treatment period, the plates containing the cells were transferred from the incubator to ice, and the culture medium was immediately removed completely using a micropipette. The cells were then gently washed with cold, sterile PBS. 1000 microliters of lysis buffer containing protease inhibitors were added to each well for protein extraction. After protein extraction, the Lowry method was used to determine the protein concentration.

After determining the protein concentration, protein samples (50 micrograms) were subjected to electrophoresis on a 12% polyacrylamide gel (SDS-PAGE). After the completion of electrophoresis, the protein samples were transferred from the gel to a PVDF membrane. After the completion of the transfer process, the membrane containing the proteins was incubated in a blocking buffer (skim milk) of 5% for 1 hour at room temperature to block non-specific binding sites.

After the blocking step, the PVDF membrane was washed three times with TBST1X for 10 minutes each to prepare it for binding to the antibody. After the completion of the washing step, the PVDF membrane was incubated overnight at 4 °C with shaking in a solution of the primary antibody anti-p53 antibody at a dilution of (1:3000) and anti-β-actin antibody as an internal control at a dilution of (1:6000). After the completion of the overnight incubation, the membrane was washed three times with TBST (15 minutes each) and then incubated with a secondary antibody conjugated with HRP enzyme at room temperature for 1 hour. Finally, the membrane was washed again with TBST (3 times, 15 minutes each), and after the completion of the washing, the membrane was exposed to ECL kit solutions for 2 minutes, and then by placing it in a ChemiDoc imaging system, the desired protein bands appeared.

Statistical Analysis

To ensure accuracy and reliability, all experiments were repeated three times. Data were analyzed using SPSS version 24 (Chicago, IL, USA) for statistical analysis and Prism software for graphing. One-way ANOVA followed by Tukey's post hoc test was used to compare mean values between groups. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was set at $P<0.05$.

Results

Cytotoxic Effect of 5-FU on HT29 Cells

As shown in Figure 2, 5-FU exhibited a dose-dependent inhibitory effect on the viability of HT29 cancer cells. At concentrations of 100 µM and above, and after a 48-hour treatment period, the drug significantly induced cell death compared to the control group ($P<0.001$).

Effect of Amygdalin on Cell Viability in the HT29 Cell Line

As illustrated in Figure 3, amygdalin exhibited a dose-dependent inhibitory effect on the viability of HT29 cancer cells. At concentrations of 10 mg/ml and above, and after a 48-hour treatment period, the compound significantly induced cell death compared to the control group ($P<0.001$).

Effect of Combined Treatment with 5-FU and Amygdalin on the Viability of HT29 Cells

In this phase of the experiment, the effects of amygdalin in potentiating the effects of low doses of

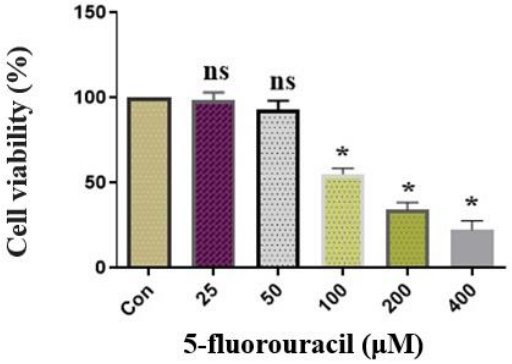

Figure 2. *In vitro* Cytotoxic Effect of 5-fluorouracil on Cell Viability of HT29 Cells. Cells were treated with indicated concentrations of 5-fluorouracil for 48 hours. Cell viability was determined by the MTT method. Data are obtained from three independent experiments and are expressed as mean ± standard error of the mean. (ns: non-significant, *P<0.001).

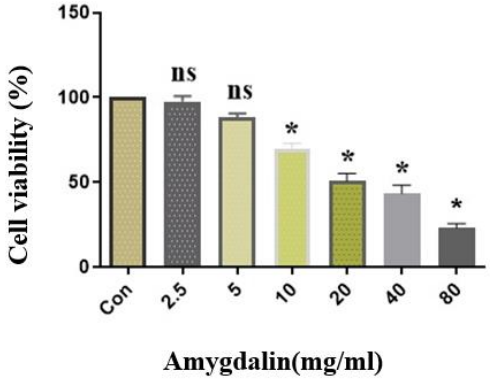

Figure 3. *In vitro* Cytotoxic Effect of Amygdalin on Cell Viability of HT29 Cells. Cells were treated with indicated concentrations of amygdalin for 48 hours. Cell viability was determined by the MTT method. Data are obtained from three independent experiments and are expressed as mean ± standard error of the mean. (ns: non-significant, *P<0.001).

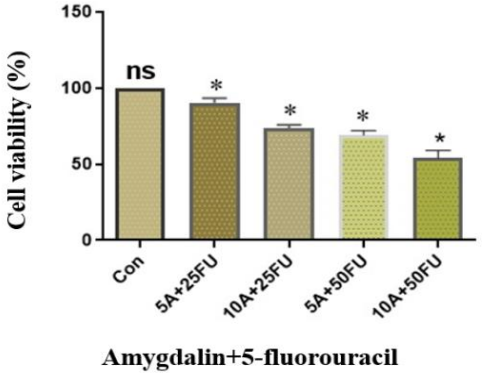

Figure 4. Cytotoxic Effect *In vitro* of Co-treatment of 5-fluorouracil with Amygdalin on Cell Viability of HT29 Cells. Cells were treated with specified concentrations of 5-fluorouracil and amygdalin for 48 hours. Cell viability was determined by the MTT method. The data are obtained from three independent experiments and are shown as mean ± standard error of the mean. (ns: non-significant, *P<0.001).

5-FU on the viability of HT29 cells were investigated. HT29 cells were simultaneously treated with amygdalin and 5-FU for 48 hours, and cell viability was assessed using the MTT assay. The results of the combined treatment of amygdalin and 5-FU on HT29 cell viability are presented in Figure 4. The results showed a significant decrease in the viability of HT29 cells in the group treated simultaneously with 5 mg/ml amygdalin and 25 µM 5-FU compared to the control group (P<0.001). However, the viability of this cell line when treated with 25 µM 5-FU and 5 mg/ml amygdalin individually did not exhibit a significant decrease compared to the control group (P>0.05). These results confirm that amygdalin potentiates the killing effects of 5-FU.

Effect of Silibinin and 5-FU on P53 Protein Expression

HT29 cells were treated with 10 mg/ml amygdalin and 100 µM 5-FU individually and in combination (10 mg/ml amygdalin and 100 µM 5-FU) for 48 hours. After the treatment period, P53 protein expression was evaluated using Western blotting. The results are shown in Figure 5. The results indicated that neither

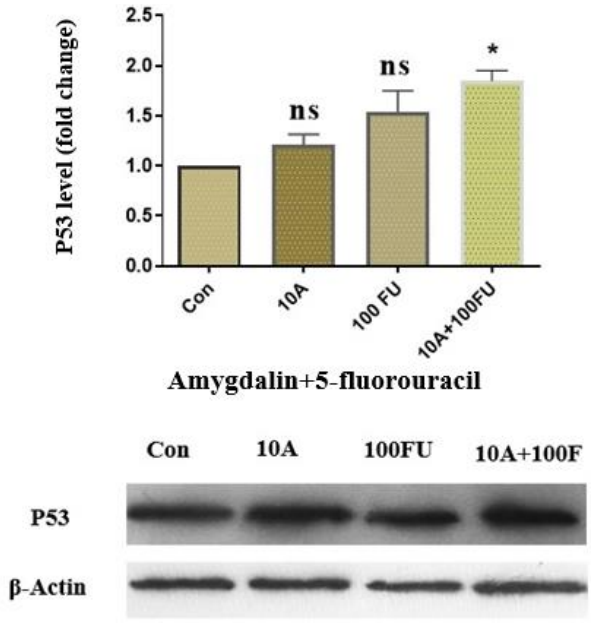

Figure 5. *In vitro* Effect of Simultaneous Treatment of 5-fluorouracil with Amygdalin on P53 Protein Expression in HT29 Cells. Cells were treated with specified concentrations of 5-fluorouracil and amygdalin for 48 hours. P53 protein expression was determined by the western blotting method. The data are obtained from three independent experiments and are expressed as mean ± standard error of the mean. (ns: non-significant, *P<0.05).

amygdalin at a concentration of 10 mg/ml nor the drug at a concentration of 100 µM had a significant effect on P53 protein expression (P>0.05). However, when cells were treated simultaneously with both drugs, there was a significant increase in P53 protein expression compared to the control group (P<0.05).

Discussion

Colorectal cancer is one of the leading causes of cancer-related deaths in both developed and developing countries.[12] Despite advancements in colorectal cancer treatment, there remains a requirement for more effective and less toxic therapeutic and preventive measures.[2] Natural compounds have been traditionally used for cancer treatment due to their low toxicity and wide availability, but the molecular mechanisms underlying their effects are not completely understood.

In recent years, numerous studies have investigated the mechanisms of function of these compounds in various cancer cell lines. Natural compounds can induce cancer cell death through various mechanisms, such as regulating proliferation, inducing cell cycle arrest, and inducing programmed cell death.[13,14]

5-FU is commonly used as a first-line treatment for colorectal cancer patients. However, despite its beneficial effects, this drug has been associated with numerous side effects and the development of drug resistance, restricting its clinical application.[13] In recent years, therapeutic approaches combining chemotherapy drugs with natural compounds have obtained significant attention to enhance treatment efficacy and reduce the side effects of chemotherapy. Several studies have shown that amygdalin can induce death in various cancer cells through different mechanisms.[15] Our study demonstrated that increasing the concentrations of 5-FU and amygdalin resulted in a dose-dependent increase in cell death. Furthermore, we illustrated that the simultaneous treatment of HT29 cells with amygdalin and 5-FU exhibited a stronger cytotoxic effect compared to treatment with either drug individually. When HT29 cells were treated with 25 µM 5-FU and 5 mg/ml amygdalin individually, no significant effect on cell death was observed. However, simultaneous treatment with 25 µM 5-FU and 5 mg/ml amygdalin resulted in significant cell death of HT29 cells. Numerous studies have indicated that amygdalin can induce cancer cell death in various cell lines. In a study conducted by Moradi Poodeh and colleagues, it was shown that amygdalin enhances the effects of lapatinib in inducing death of SKBR3 cells by increasing the expression of the pro-apoptotic protein Bax.[4] Our results demonstrated that in the HT29 colorectal cancer cell line, neither 10 mg/ml amygdalin nor 100 µM 5-FU individually had a significant effect on P53 protein expression. However, when cells were treated simul taneously with both drugs at the same concentrations, a significant increase in P53 protein expression was observed. The P53 protein normally induces cell cycle arrest to allow for DNA repair or apoptosis, preventing the proliferation of cells with severely damaged DNA. One of the key target genes of P53 is the pro-apoptotic protein Bax, which induces apoptosis in cancer cells.[16,17] Amygdalin appears to induce cell death through its effect on p53 protein and its effect on p53 target genes such as Bax gene expression. Therefore, based on our results, we can conclude that amygdalin enhances the cytotoxic effects of 5-FU by increasing P53 expression and possibly affecting Bax expression, thereby inducing apoptosis or cell cycle arrest and leading to the death of HT29 colorectal cancer cells.

Conclusion

This study demonstrated that the combination of 5-FU and amygdalin exhibited greater effects on the viability of HT29 colorectal cancer cells compared to treatment with either drug individually. These effects were associated with increased P53 protein levels in the cell line. These results suggest the potential benefit of combining these drugs through the induction of apoptosis and cell cycle arrest, leading to the death of HT29 colorectal cancer cells.

These results confirm the possible advantage of the combination of 5-FU and amygdalin drugs for intervention in patients with colorectal cancer, although more comprehensive studies are needed for a general conclusion in this case, such as in vivo studies.

Conflict of Interest

The authors declare no conflicts of interest.

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