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A preliminary study of the anti-proliferative effect of Aronia Melanocarpa extract on human colon cancer cells and its relation with human TERT protein

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ABSTRACT

Aim: Aronia melanocarpa is a fruit exhibiting antioxidant, anti-inflammatory, antidiabetic, hypolipidemic, and anticarcinogenic properties. Telomerase is a critical factor in the development of colon cancer, and it is being looked at as a target for cancer treatments. In the present study, we aimed to examine the potential effect of Aronia melanocarpa extract on cell viability and protein concentration of the human telomerase reverse transcriptase (hTERT) in the human colon cancer cell line (HT-29) and the non-tumorigenic human umbilical vein endothelial cell line (HUVEC).

Methods: Cell lines were treated with different concentrations of Aronia extract (50-750 µg/ml) for 48 h. The cytotoxic activity of Aronia extract was determined using the MTT assay. hTERT protein concentration (pg/ml) was measured using a sandwich ELISA.

Results: The MTT assay test showed that Aronia extract induces 50% cell death (IC_{50}) at a concentration of 186 µg/mL at 48 h post-treatment in the HT-29 cell line. Cytotoxicity results showed a dose-dependent decrease in cell viability in the HT-29 cell line. However, increasing Aronia extract concentrations showed no similar effect on the HUVEC cell line. The hTERT protein concentration in HT-29 cells was 1.9-fold higher than that of HUVEC cells. Increasing concentrations of Aronia extract was linked with a significant decrease in hTERT protein level in HT-29 cells, whereas the hTERT protein concentration in HUVEC cells did not change significantly.

Conclusions: Our results suggest that A. melanocarpa may be a potential therapeutic agent for anticarcinogenic activity. Also, the anti-proliferative effects of Aronia extract may be attributed, at least partly, to the decreased hTERT protein level of HT-29 cells.

Key words: Aronia melanocarpa, hTERT, cell viability, telomerase, colon cancer.

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Introduction

Human colon cancer is one of the most common cancers in men and women and the fourth most common cause of cancer-related mortality worldwide [1]. Chemotherapy and radiotherapy are often used to treat colon cancer; however, they are associated with several adverse effects in cancer patients. While synthetic anticancer medications prolong survival, they often lead to systemic toxicity and have multiple side effects, including nausea, vomiting, anorexia, diarrhea, and bleeding. Such adverse effects often threaten the patients' quality of life, making it challenging to continue cancer therapy. Although numerous beneficial strategies have been developed to prevent or ameliorate these adverse effects, these strategies might be insufficient. Synthetic anticancer drugs induce normal cell toxicity and gradually increase resistance in cancer cells [2]. They are also high cost. In addition, restricted access to diagnostic tools limits the use of synthetic anticancer drugs in the treatment of cancer in developing countries, especially in rural areas. However, recent studies suggest the use of on-target drugs to overcome the limitations of cancer chemotherapy. Monoclonal antibodies (mAbs) are valuable tools because they can significantly target cancer cells and fight cancer in many ways [3]. mAbs may affect tumor cells by changing the activation status of membrane-bound receptors or neutralizing cytokines required for cellular growth and proliferation, disrupting the growth signaling pathways of malignancies [4]. Despite recent breakthroughs in targeted medicines, their clinical effectiveness has been limited. They are not effectively curative and prohibitively expensive. Unfortunately, on-target drugs may not cause long-lasting effects on tumor cells due to drug resistance and tumor cell plasticity [5]. Growing evidence suggests colon cancer is a multi-modal disease where variables in the tumor microenvironment play a role in its etiology. Long-term cancer prevention may save more lives than current chemotherapies and targeted medications. Given the toxicity and expensive cost of contemporary medications, there is a rising interest in researching natural products that are safe and economical for colon cancer prevention and adjunctive therapy along with conventional treatments. Therefore, identifying novel medications for prevention and using alternative cancer therapy regimens are desirable. In this sense, natural products derived from plant extracts are seen as promising since they are rich suppliers of chemicals with many medicinal applications [6, 7]. Today, the majority of anticancer medications are derived from plants. In addition, plant metabolites have demonstrated potent chemoprotective activity against colon cancer cells by inducing apoptosis and cell cycle arrest, modulating tumor-suppressing microRNA, and inhibiting oncogene and anti-apoptotic factors [8]. Therefore, it is crucial to discover novel anti-cancer agents derived from natural products for the treatment of colon cancer.

Aronia melanocarpa (black chokeberry) is a plant that belongs to the family Rosaceae. A.melanocarpa contains anthocyanins (cyanidin 3-O-galactoside, cyanidin 3-O-arabinoside, cyanidin 3-O-xyloside, and cyanidin-3-O-glucoside,), flavonoids (quercetin 3-O-vicianoside, quercetin 3-O-robinobioside, and other quercetin glucosides), phenolic acids (chlorogenic acid, neochlorogenic acid, caffeic acid, and ferulic acid), and vitamin C and E [9]. Aronia berries have many beneficial effects on human health, including antioxidant, anti-inflammatory, anti-atherosclerotic, and antidiabetic activities. In addition, Aronia melanocarpa exhibits an inhibitory effect on breast, lung, and colon cancer cell proliferation,

which has been confirmed by in vitro and in vivo studies [10-13]. Phenolic and non-phenolic components of Aronia berries have potential to interfere with several pathways linked to cancer, such as inhibition of cell growth, cell cycle arrest, induction of apoptosis, and the regulation of cell metabolism. However, affected cancer-related pathways do not explain the molecular processes behind the anti-cancer efficacy of Aronia melanocarpa. So far, no adverse or toxic effects related to Aronia melanocarpa fruit, juice, or extracts have been reported in the literature [14]. Telomerase is an enzyme that maintains telomere length by synthesizing nucleotide repeats at the distal ends of chromosomes. Human telomerase consists of two components, human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR) subunits [15]. hTERT is the catalytic subunit of telomerase, which is considered the key determinant of its activity [16]. Even though telomerase activity is very low or undetectable in somatic cells, it is high in approximately 85% of human cancers [17]. The activation of telomerase is a critical step in the formation of malignant tumors. It has been reported that telomerase activity is very high in colorectal adenocarcinomas [18, 19].

Telomerase activity promotes cell proliferation and prevents apoptosis of cancer cells [20], whereas telomerase inhibition induces intense cell death in actively dividing tumor cells [21]. Therefore, targeting telomerase is a promising therapeutic intervention for cancer treatment. A strong correlation has been observed between hTERT mRNA expression, telomerase activity, and hTERT protein levels in colon cancer [22]. As a result, the hTERT protein level may be used as an indirect indicator of telomerase activity and as a potential biomarker for colorectal cancer [17].

However, there is currently limited information concerning A. melanocarpa inhibiting the proliferation of colon cancer cells and its effect on the hTERT protein level. In this study, we investigated for the first time the cytotoxic effect of Aronia extract on human colon cancer (HT-29) and human umbilical vein endothelial (HUVEC) cell lines, along with the concentration of the hTERT protein.

Materials and metods

Aronia Melanocarpa extract was provided as a dark purple-colored powder by Artemis International (Fort Wayne, IN, USA). It is a purified extract containing a number of anthocyanins, proanthocyanidins, phenolic acids, and flavonoids, which were standardized by the producer on the anthocyanin content (standardized to a minimum of 15%).

Cell culture and Aronia extract treatment

The HT-29 (passage number 12) and HUVEC cell (passage number 9) lines were obtained from the Department of Biochemistry, Istanbul University. The cells were cultured in T-25 flasks containing RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen, UK), 1% penicillin-streptomycin (Biological Industries) and 2 mM L-glutamine (Biological Industries) in a fully humidified atmosphere containing 5% CO_2 at 37° C. Polyphenolics from Aronia melanocarpa were dissolved in the solvent dimethyl sulfoxide (DMSO) (Sigma) prior to use. The cells were treated with Aronia extract at 0, 50, 100, 200, 400, 500, and 750 µg for 48 h after 24 h of culture.

MTT assay

Evaluation of cytotoxicity was assessed by the 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazoliumbromide (MTT, Sigma) method. The MTT test is based on the principle of converting yellow-color formazan crystals in MTT solution to a purple color by the

mitochondrial succinate dehydrogenases of living cells. The reduction activity depends on the intracellular concentrations of NADH and NADPH.

Cultured cells were collected by trypsinization at ~80% confluent and seeded in 96-well plates at a density of 1 x 10^4 cells per 100 µl of complete medium. The cells were incubated for 24 h at 37°C in a 5% CO_2 incubator. After 24 hours of incubation, the culture medium was removed and replaced with 100 µl of new complete medium for the control group. Cells were incubated with 100 µl of Aronia extract at different concentrations (50-750 µg/ml) for 48 h. At the end of the treatment, the culture medium was discarded. Cells were washed with phosphate-buffered saline (PBS) to remove non-adherent cells. Then, 10 µl of MTT solution (diluted in culture medium, 0.25 mg/ml) was added to each well before being incubated at 37 °C for 4 h. Following the incubation period, the MTT solution was removed, and 200 µl of DMSO was added to dissolve the formazan crystals, and incubated in a dark place for 2 h at room temperature. The absorbance of the colored solution was measured spectrophotometrically at an absorbance of 570 nm using an ELISA microplate reader. All experiments were performed at least three times independently, and their average values were calculated. The percentage of cell viability was calculated as follows:

$$\text{Cell viability (\%)} = 100x\,\frac{\textit{Absorbance of treated cells}}{\textit{Absorbance of untreated cells}}$$

Measurement of hTERT concentration by ELISA assay

The hTERT concentrations in cell lines were measured by a commercial sandwich ELISA kit, the Human Telomerase Reverse Transcriptase (hTERT) (cat. no. SEC241Hu; Cloud-Clone Corp., Houston, USA) according to the manufacturer's protocol.

Statistical analysis

Three independent experiments reported results as mean (M) ± standard deviation (SD). The One-way analysis of variance (ANOVA) test followed by the Bonferroni post hoc test was performed to compare the results. The concentration of Aronia extract, resulting in a 50% reduction of cell viability and inhibitory concentration (IC_{50} value), was derived from the curve fitting of the dose-response data using Graphpad Prism software, version 8.0.1 (GraphPad Software, San Diego, CA, USA). Values less than 0.05 were considered to be statistically significant.

Results

Effects of Aronia extract on HT-29 and HUVEC cells proliferation

HT-29 and HUVEC cells were treated with different concentrations of Aronia extract. The dose-response curve is illustrated in Figure 1. The viability of HT-29 cells treated with up to 50 µg/ml of Aronia extract showed no significant reduction. A significant loss of viability was detectable at 100, 200, 400, 500, and 750 µg/ml concentrations during the 48 h of treatment. Aronia extract inhibited growth in a concentration-dependent manner. The IC_{50} of Aronia extract is 186 µg/ml after 48 h on HT-29 cells (Figure 2). Aronia extract does not significantly affect the cell viability of HUVEC cells at concentrations up to 400 µg/mL in 48 h. Cell viability was significantly decreased following treatment with 500 and 750 µg/ml concentrations of Aronia extract compared to the control. The IC_{50} value of Aronia extract was not calculated on HUVEC cells because it yielded >50% inhibition of proliferation relative to untreated cells.

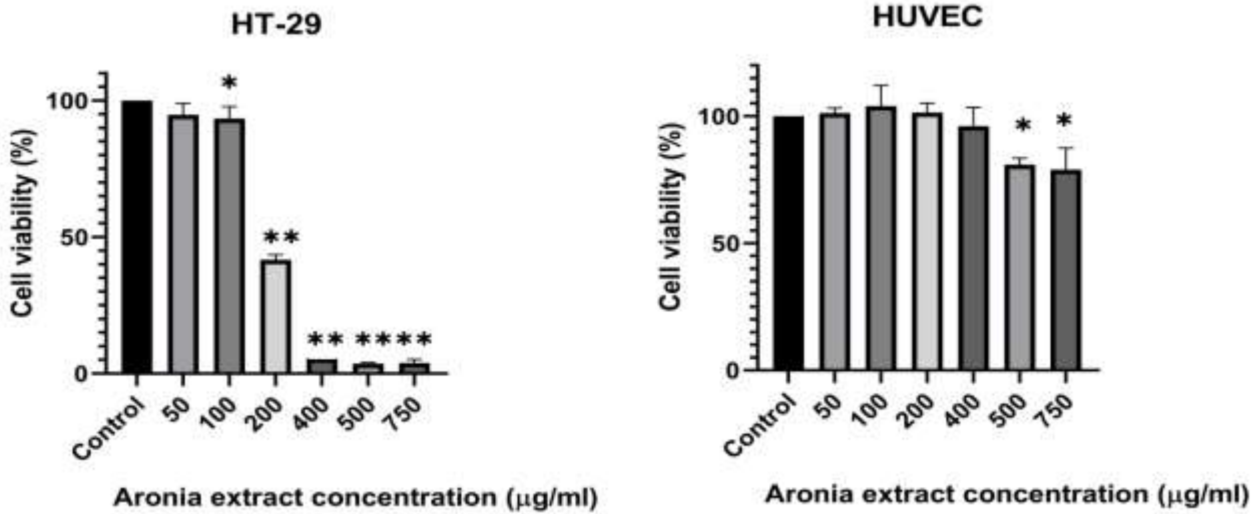

Figure 1. Effect of Aronia melanocarpa extract on cell viability of HT-29 and HUVEC cells as determined by the MTT assay. Cells were treated with the indicated concentrations of Aronia melanocarpa extract for 48 h. Data are expressed as cell viability (%) relative to controls not incubated with Aronia extract. Changes occurred in a dose-dependent manner. Each column represents the mean ± standard deviation for each group from at least three experiments. *P<0.05 **P<0.001 show significant differences compared to the control group.

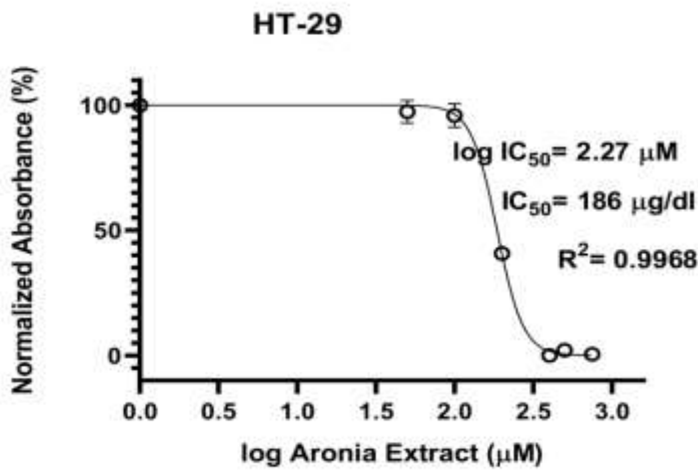

Figure 2. MTT assay results. The diagram shows that the IC_{50} dose of Aronia extract after 48 h was 186 µg/mL.

The MTT activity is proportional to the number of cells; hence, the observed absorbance correlates favorably with cell proliferation. As anticipated, aronia extract-treated cells exhibited a concentration-dependent inhibition in cell proliferation (Figure 3).

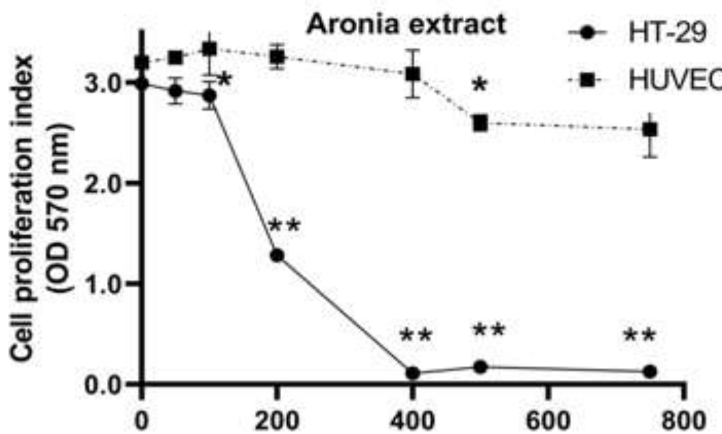

Figure 3. Cell proliferation was determined by the MTT assay. HUVEC cells and HT-29 colon cancer cells were treated with different Aronia extract concentrations for 48 h. Values indicate the mean ± S.D of triplicate experiments. *P<0.05 **P<0.001 show significant differences compared to the control group.

Table 1. Effect of different aronia extract concentrations on the hTERT protein level of HT-29 and HUVEC cells.

Aronia extract concentration (µg/ml)	hTERT concentration (pg/ml)	
	HT-29	**HUVEC**
0	198.90 ± 2.85	105.10 ± 2.29
200	95.64 ± 2.17 **	103.10 ± 1.74
400	85.01 ± 1.68 **	101.70 ± 1.57
500	64.27 ± 2.68 **	100.80 ± 0.43 *

*The data were represented as mean ± standard deviation. *P<0.05, **P<0.001 vs. untreated control groups.*

Effects of Aronia extract on hTERT concentrations

hTERT concentrations were presented in Table 1. The hTERT concentration after treatment of HT-29 cells with 200, 400, and 500 µg/ml of Aronia extract for 48 h was significantly decreased compared to the control. However, there were no significant differences in the hTERT concentration of HUVEC cells concerning treatment with 200 and 400 µg/ml concentrations of Aronia extract. There was a significant difference between hTERT levels following treatment with 500 µg/ml concentration of Aronia extract compared to the control.

Discussion

Most current anti-cancer drugs are derived from plants [23, 24]. Plants continue to hold much promise as a source of natural compounds that can be used to make new medications. Finding new and powerful natural products with chemopreventive potential and fewer side effects is essential to making cancer treatment more effective. Normal cells that do not express telomerase cannot lengthen their telomeres, resulting in a limited proliferative lifetime, while telomerase-expressing cancer cells (85% of cancer cells) can lengthen their telomeres using telomerase (containing hTERT and hTR subunits). This crucial distinction between normal and malignant cells makes telomerase a universal therapeutic target. Recent research has shown a correlation between telomerase activity and hTERT mRNA expression. These findings provide convincing evidence that the expression of hTERT was a rate-limiting factor in the enzymatic activity of human telomerase and that the upregulation of hTERT expression may play an essential role in human carcinogenesis [25]. Therefore, chemopreventive and chemotherapeutic agents that interfere with the regulation of telomerase activity are suggested to be crucial for triggering apoptosis in cancer cells.

Aronia melanocorpa exerts anti-proliferative effects on human colon cancer cells by suppressing cell growth, inducing apoptosis and cell cycle arrest, and upregulating the expression of numerous cell cycle-related genes [10, 26-28]. Normal colon and human HT-29 colon cancer cell lines were exposed to Aronia melanocarpa extract for 24 hours. At a dose of 50 mg monomeric anthocyanin/ml, Aronia extract suppressed the growth of human HT-29 colon cancer cells by 60% without affecting the growth of normal cells [10]. Zhao et al. [11] demonstrated that chokeberry (Aronia melanocarpa E.) anthocyanin-rich extract showed strong inhibition against HT-29 cell growth with an IC_{50} value of 25 µg/mL chokeberry anthocyanin-rich extract. In another study, the viability of Caco-2 colon cancer cells was examined after exposure to a non-toxic dose

of Aronia melanocarpa juice (2 hours per day for 4 days). It was discovered that this treatment inhibited Caco-2 cell proliferation and viability compared to untreated cells [13]. Aronia melanocarpa Elliot anthocyanins also showed anti-cancer activity by inhibiting Caco-2 cell proliferation, and the IC_{50} value was reported to be 148.3 μg/ml [29]. Based on in vitro and in vivo investigations, it is hypothesized that the majority of chokeberry extracts' anti-cancer properties result from the antioxidative activity of polyphenolics, particularly anthocyanins and chlorogenic acids. It has been reported that the antioxidant activity of Aronia berries was shown to be associated with their total procyanidin and anthocyanin content, and the cyanidin glycosides reduced HeLa human cervical cancer cell growth and enhanced reactive oxygen species (ROS) production [30]. The phenolic components of Aronia berries demonstrated strong antioxidant activity and cytotoxicity against HepG2 human liver cancer cells [31]. The black chokeberry extract is effective against human HT-29 colon cancer cells, and this activity was associated with its total phenolic content and caffeic and chlorogenic acids levels [32]. These results were validated by further research demonstrating the anthocyanin-rich blackberry extract's antioxidant, anti-inflammatory, and anti-proliferative properties against HT-29 cells [33]. Anthocyanins in Aronia berries decrease inflammatory cytokines, SLC1A5 (solute carrier family 1 member 5) expression, and mTOR phosphorylation in Caco-2 cells. SLC1A5 is a cancer-specific amino acid carrier that regulates cell proliferation and invasion. Targeting SLC1A5 may lead to identifying new anti-cancer drugs derived from Aronia berries [34]. Furthermore, it was discovered that the anthocyanins found in Aronia berries inhibit Caco-2 cell development via the Wnt/-catenin signaling pathway. Cyanidin-3-O-galactoside is one of the phenolic constituents of Aronia berries that has antitumor potential. Berry extracts containing cyanidin-3-O-galactoside inhibited BGC-803 human gastric cancer cell growth by inducing cell death via gene alterations, including increases in Bax and Bak expression and reductions in Bcl-2 and Bcl-xl expression [35]. Chlorogenic acid inhibits cancer cell proliferation, migration, and invasion by acting on p53, p38 MAPK, c-Jun amino-terminal kinase (JNK), c-Myc, ROS, and other targets. Ursolic acid, a pentacyclic triterpene derived from Aronia berries [36, 37], inhibits NF-κB activation, and angiogenesis, and metastasis. Ursolic acid displayed an anti-inflammatory effect via targeting histamine, lipoxygenase, cyclooxygenase, phospholipase, nitric oxide, and ROS, all of which mediate its anti-cancer activity [38, 39]. Therefore, future research on Aronia berries might concentrate more on optimizing the dosages of phenolic and other ingredients present, creating novel formulations, and isolating new active compounds and their synthetic modifications.

Yet, to date, no research has investigated how Aronia extract effects hTERT protein concentration in cancer cells. However, some studies explore the influence of natural compounds derived from plants on the expression, activity, and concentration of telomerase in colon cancer cell lines. Resveratrol (stilbene) has been reported to show antitumor activity by inhibiting the expression of the hTERT protein. It substantially down-regulated telomerase activity in colon cancer cell lines [40]. Morus rubra, often known as red mulberry, exhibited telomerase-inhibiting activity owing to the presence of polyphenols. Demir et al. [41] have found that the dimethyl sulfoxide extract of M. rubra induces apoptosis in human colon cancer cell line WiDr through downregulation of hTERT mRNA expression. Several studies have

shown that the level of hTERT protein is strongly correlated to the expression of the hTERT gene and the activity of telomerase. The decrease in hTERT protein level was often coupled with downregulation of hTERT mRNA [42] and telomerase activity [43]. Samad et al. [22] reported that berberine treatment might downregulate hTERT gene expression and reduce telomerase activity and hTERT protein levels in colorectal cancer cell line (HCT 116), thereby inhibiting cell proliferation.

In this study, we examined how the hTERT protein level was effected by Aronia melanocarpa extract in HT-29 colon cancer and HUVEC cell lines. As shown in Figure 1, the current investigation revealed that Aronia melanocarpa extract was non-toxic to the control HUVEC cell line. This data reinforces previous findings that Aronia melanocarpa is safe to take, even at larger quantities, since it does not have any side effects on normal cells. Additionally, our results are compatible with previous findings in research on malignant colon cancer cells.

The results confirmed that Aronia extract has a direct, dose-dependent, inhibitory effect on cell proliferation in the HT-29 colon cancer cell line. Following a 48-hour treatment with Aronia extract, the IC_{50} for cell viability was determined at 186 µg/mL. In addition, for the first time, relatively high concentrations of this compound were found to be able to substantially decrease hTERT protein concentration. No statistically significant differences in cell viability and hTERT protein concentration were observed in HUVEC cells following a 48-h treatment with Aronia extract except of 50 and 100 µg/ml. However, the interference of Aronia melanocarpa with the hTERT protein level shows that polyphenols may play an unknown role in developing innovative anti-cancer strategies. These results further support the potential role of Aronia melanocarpa in the chemoprevention/chemotherapy of human colon cancer cells.

Conclusions

In conclusion, Aronia extract inhibits the proliferation of HT-29 human colon cancer cells and this accompanied with decreasing hTERT protein level. The mechanism may be associated with the downregulation of hTERT mRNA gene expression and telomerase activity, resulting in a decrease in protein concentration. Further data is required to prove that hTERT protein concentration significantly correlates with telomerase activity and hTERT mRNA expression. These preliminary findings suggest that hTERT protein level may function as a prognostic indicator irrespective of kinetic parameters; nevertheless, this hypothesis needs to be verified using large-scale studies, including more detailed mechanistic and clinical data.

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Ethical statement: *Ethics committee decision was not taken as it was a laboratory study.*

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