Original Article

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Quercetin Change the Exosome Secretion and Total miRNA Concentration in Primary (Colo320) and Metastatic (Colo741) Colon Cancer Cell Lines

Kersetin Primer (Colo320) ve Metastatik (Colo741) Kolon Kanseri Hücre Dizilerinde Eksozom Salgısını ve Total miRNA Konsantrasyonunu Değiştirir

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ABSTRACT

Objective: Quercetin, which is considered a potential anti-cancer agent in the prevention of colon cancer, is one of its natural polyphenolic compounds. Extracellular vesicles, such as exosomes, secreted from cells and their components contribute to cellular behavioral characteristics by transporting proteins or miRNAs. In this study, we aimed to determine the cytotoxicity of Dicer, Ago2, eIF2α CD9 and CD63 and their effects on exosomal miRNA secretion and expression in Colo320 and Colo741 colon cancer cell lines applied quercetin.

Methods: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to analyze the cytotoxicity of quercetin. MTT analysis is a colorimetric analysis method applied to measure the metabolic activity of cells. The absorbance was measured at 570 nm by a spectrophotometer. Besides that, the indirect immunoperoxidase staining was used for the distribution of Dicer, Ago2, eIF2α, CD9, and CD63 in Colo320 and Colo741. Total miRNA in exosome was determined with miRCURY™ Kit.

Results: The immunoreactivities of eIF2α and CD9 significantly differed compared to the Colo741 control group after quercetin application. In addition, exosomal miRNA concentrations were higher in both quercetin applied-Colo320 and Colo741 cells.

ÖZ

Amaç: Kolon kanserinin önlenmesinde potansiyel bir anti-kanser ajan olarak kabul edilen kersetin, doğal polifenolik bileşiklerinden biridir. Hücrelerden ve bileşenlerinden salgılanan eksozom gibi hücre dışı veziküller, proteinleri veya miRNA'ları taşıma yoluyla hücresel davranış özelliklerine katkıda bulunur. Bu çalışmada, kersetin uygulanan Colo320 ve Colo741 kolon kanseri hücre dizilerinde Dicer, Ago2, eIF2α CD9 ve CD63'ün sitotoksisitesini ve eksozomal miRNA salgılanması ve ekspresyonu üzerine etkilerini belirlemeyi amaçladık.

Yöntemler: Kersetin sitotoksisitesinin analizi için MTT testi kullanıldı. Colo320 ve Colo741'de Dicer, Ago2, eIF2a, CD9 ve CD63'ün dağıtımı için dolaylı immünoperoksidaz boyaması kullanıldı. Eksozomdaki toplam miRNA, miRCURY™ Kit ile belirlendi.

Bulgular: Kersetin uygulamasından sonra eIF2a ve CD9 immünoreaktiviteleri Colo741 kontrol grubuna kıyasla önemli ölçüde farklılık gösterdi. Ayrıca eksozomal miRNA konsantrasyonları hem kersetin uygulanan Colo320 hem de Colo741 hücrelerinde daha yüksekti.

Sonuç: Kersetin uygulamasının Colo320 ve Colo741 hücrelerinde eksozomal salgılamayı tetiklediği sonucuna vardık. Bununla birlikte, primer ve metastatik kolon adenokarsinom hücrelerinde kersetin

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ABSTRACT

Conclusion: We concluded that quercetin triggered exosomal secretion in Colo320 and Colo741 cells. However, exosomal component should be evaluated in future investigations to understand the quercetin role in primary and metastatic colon adenocarcinoma cells.

Keywords: Colon cancer, exosomes, miRNA, quercetin

ÖZ

rolünü anlamak için gelecekteki araştırmalarda eksozomal bileşen değerlendirilmelidir.

Anahtar Sözcükler: Kolon kanseri, eksozomlar, miRNA, kersetin

Introduction

Colorectal cancer (CRC) is a severe gastrointestinal malignancy of which frequency is rapidly increasing worldwide (1). The risk of CRC increases with smoking, unhealthy lifestyle habits, and poor diet (2). The tumor microenvironment (TME) is critical in the survival and metastatic properties of CRC cells. TME refers to the cellular environment in which the tumor interacts, and this cellular environment includes cancer cells, immune cells, fibroblasts, cytokines, vascular tissue, extracellular matrix and proteins that contribute to tumor growth. It is emphasized that the TME is an important factor contributing to the development of resistance to cancer therapy. Cellular and non-cellular components of TME are important in contributing to CRC progression and metastasis. TME helps cancer cells to communicate with stromal cells and arranges the secretion of different proteins or exosomes which play crucial roles in the features of CRC cells (3,4). The development of new biomarkers is a vital strategy for public health to decrease the mortality of CRC effectively. Recent experimental studies have shown that exosomes may be important biomarker sources in CRC (5).

Exosomes are small membrane vesicles (50-150 nm) excreted from different cells including cancer cells. They contain protein, RNAs (microRNAs, mRNAs, long noncodingRNAs) organels etc (6,7). Also, the composition of exosomes alters according to cell type and includes specific membranous markers such as CD9, CD81, CD63 etc. (8). Many studies suggested that proteins and/or miRNAs carrying with exosomes might effectively control the tumorigenesis, surveillance and resistance of CRC (9,10). Moreover, the exosomal miRNAs can be used to recognize and track the cancer cells (11,12).

The genes encoding miRNAs are much longer than the processed mature miRNA molecule. Many miRNAs are known to reside in introns of their pre-mRNA host genes and share their regulatory elements, primary transcript, and have a similar expression profile. For the remainder of miRNA genes that are transcribed from their own promoters, few primary transcripts have been fully identified. MicroRNAs are transcribed by RNA polymerase II as large RNA precursors called pri-miRNAs and comprise of a 5' cap and poly-A tail (13). The pri-miRNAs are processed in the nucleus by the microprocessor complex, consisting of the RNase III enzyme Drosha, and the double-stranded-RNA-binding protein, Pasha/DGCR8. The resulting pre-miRNAs are approximately 70-nucleotides in length and are folded into imperfect stem-loop structures. The pre-miRNAs are then exported into the cytoplasm by the karyopherin exportin 5 (Exp5) and Ran-GTP complex. Ran (ras-related nuclear protein) is a small GTP binding protein belonging to the RAS superfamily that is essential for the translocation of RNA and proteins through the nuclear pore complex. The Ran GTPase binds Exp5 and forms a nuclear heterotrimer with pre-miRNAs. Once in the cytoplasm, the pre-miRNAs undergo an additional processing step by the RNAse III enzyme Dicer generating the miRNA, a double-stranded RNA approximately 22 nucleotides in length. Dicer also initiates the formation of the RNA-induced silencing complex (RISC) (14). RISC is responsible for the gene silencing observed due to miRNA expression and RNA interference (15-19).

Therefore, miRNAs and factors of miRNAs biosynthesis are significant post-transcriptional modulators that have vital and critical roles in both health and disease. For this reason, miRNAs and their biogenesis factors, such as Drosha, Dicer, and Ago2, are being extensively studied for the target treatment of cancers (20,21).

In treating CRC, multi-targets and various action mechanisms with decreased toxicity have improved clinical outcomes. In the last decade, use of polyphenolic compounds with chemotherapeutic drugs demonstrated their synergistic effects on cancer cells. Dietary polyphenolic compounds affect different molecular procedures by acting as chemopreventive blockers in CRC (22). One of the polyphenolic compounds, quercetin, is being investigated with relevance to its anticancer activity on CRC. Experimental studies showed that different concentration and application period for quercetin were impressive in inhibiting cancer formation and cell death, viability and mitosis (23,24). Moreover, it is thought that the anti-cancer properties of quercetin may be related to its structure and exosomal secretions including miRNAs levels in CRC (20,21).

Recent evidence indicated that the polyphenolic compound, quercetin could be potential as new therapeutic tool in cancer treatment because of its anti-oxidant, anti-inflammatory and anti-cancer effects. In contrast to that, the role of quercetin on secretion of exosome and its related components and also miRNA levelshas not been elucidated in colon cancer cells and also a comparison between primary CRC cancer cells and metastatic cells has not been performed. Additionally, dysregulation of eukaryotic translation initiation factor 2α (eIF2α) which is a

critical factor during protein synthesis is related with metabolic disorders including cancer (25). The limited experimental studies have addressed the effects of quercetin on the expression of eIF2α in CRC. In this study, we aimed to search the role of quercetin on secretion of exosome, its related components and also miRNA levels in Colo320 and Colo741 CRC cell lines.

Methods

Cell Culture

Primary (Colo320, ATCC: CCL-220.1) and metastatic (Colo741, ECACC: 93052621) CRC cell lines were cultured in RPMI 1640 (Biochrom; FG- 1215) medium containing Fetal Bovine Serum (10%, FBS, Capricorn Scientific), 1% penicillin-streptomycin [1%, (Biochrom; A- 2213)], and L- glutamine (1%, EMD Millipore; K- 0282) at a 37 ˚C and 5% CO_2 in atmosphere.

Cytotoxicity Analyze

The cell cytotoxicity of quercetin was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide assay (MTT, Fisher, 158990050). The MTT assay was performed according to the principles we reported previously (26). Colo320 and Colo741 cells were administered with different quercetin (Sigma; Q- 4951) concentrations (5, 10, 25, 50 and 100 µg/mL) for 24 h or 48 h.

Immunocytochemical (IHC) Analyses

The both types of cells were cultured in other culture medium with or without quercetin for 48 h. Dicer, eIF2c (Ago2), eIF2α, CD63, and CD9 distributions were evaluated with indirect immunoperoxidase staining protocols in Colo320 and Colo741 cells as we previously described (26). Fixation was performed with 4% paraformaldehyde on all cells from all groups for 30 min. They were washed with phosphate buffer saline (PBS) and then incubated with 3% of H_2O_2, then with blocking agent solution (ready to use, Thermo scientific, TP-125-HL). Primary antibodies against Dicer (Santa Cruz sc- 136981), eIF2α (Santa Cruz sc- 133132), Ago2 (Santa Cruz sc- 376692), CD9 (Santa Cruz sc- 13118) and CD63 (Santa Cruz sc- 5275) were added and incubated overnight at 4 °C. The cells from all groups were washed with PBS and biotinylated secondary antibody and streptavidin-peroxidase complex were added (ready to use, Thermo scientific, TP-125-HL), respectively. After washing with PBS, diaminobenzidine was added for 5 min. The counterstain was performed with Mayer's hematoxylin solution (Bio Optica; 1213). The mounting medium was applied and H-SCORE semi-quantitative grading evaluation was used for antibodies intensity (24).

miRNA Analyzes

The culture medium from all groups were collected and exosome and total miRNA levels were measured according to the manufacturer's of miRCURY™ RNA isolation kit (cat no/ ID 76743).

Data Analysis

Mean ± standard deviation was used for data expression in the assay. All data were analyzed statistically with GraphPad software. The differences were evaluated using Mann-Whitney U tests and a $p<0.05$ was considered statistically significant. All experiments were done in triplicate.

Results

After cytotoxicity analyses, 25 µg/mL of quercetin administration for 48 hours was suitable for both Colo320 and Colo741 cells (Figure 1).

The intensity of dicer was weak in Colo320 cells after quercetin administration, but, it was not significant when compared with control Colo320 cells (Figure 2A, B, Table 1). The vigorous intensity of eIF2α in both control (Figure 2C) and quercetin administrated (Figure 2D) Colo320 cells was observed; however, this immunoreactivity was not statistically significant (Table 1). Similar and weak Ago2 and CD9 immunoreactivities were detected in both groups of Colo320 cells (Figure 2E-2H, Table 1). The immunoreactivity of CD63 was moderate and not significant in both groups of Colo320 cells (Figure 2I, 2J, Table 1).

In Colo741, metastatic colon adenocarcinoma cells, Dicer immunoreactivity was strong and not significant in both groups (Figure 3A, 3B, Table 1). Decreased and statistically significant immunoreactivity of eIF2α after quercetin application was detected in Colo741 cells (Figure 3C, 3D, Table 1). The intensity of Ago2 was weak and similar in both groups of Colo741 cells (Figure 3E, Figure 3F). The CD9 immunoreactivity was vigorous after quercetin application on Colo741 cells (Figure 3H), and it was statistically significant when compared with control Colo741 cells (Figure 3G, Table 1). Weak and similar CD63 immunoreactivity was detected in both groups of Colo741 cells (Figure 3I, and Figure 3J).

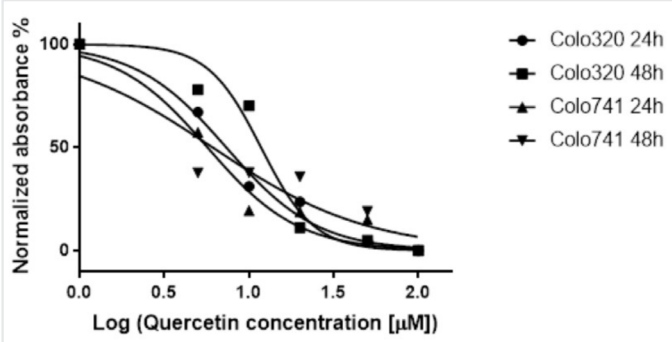

Figure 1. MTT assay results and calculated IC_{50} values for Colo320 and Colo741 cancer cells after quercetin application. Colo320 and Colo741 cells were applied at five different concentrations (5-100 µM) for 24 and 48 h. Cell viability was affected by dose and incubation time. In the experiments, 25 µg/mL of quercetin for 48 hours was used as the effective dose and incubation time

After quercetin application on both Colo320 and Colo741 cell lines, total exosomal miRNA concentrations were detected as 12.13 ng/μL and 15.15 ng/μL, respectively. Higher but not significant the total miRNA concentration was calculated in quercetin-applied both Colo320 and Colo741 colon adenocarcinoma cell lines than in control cell lines.

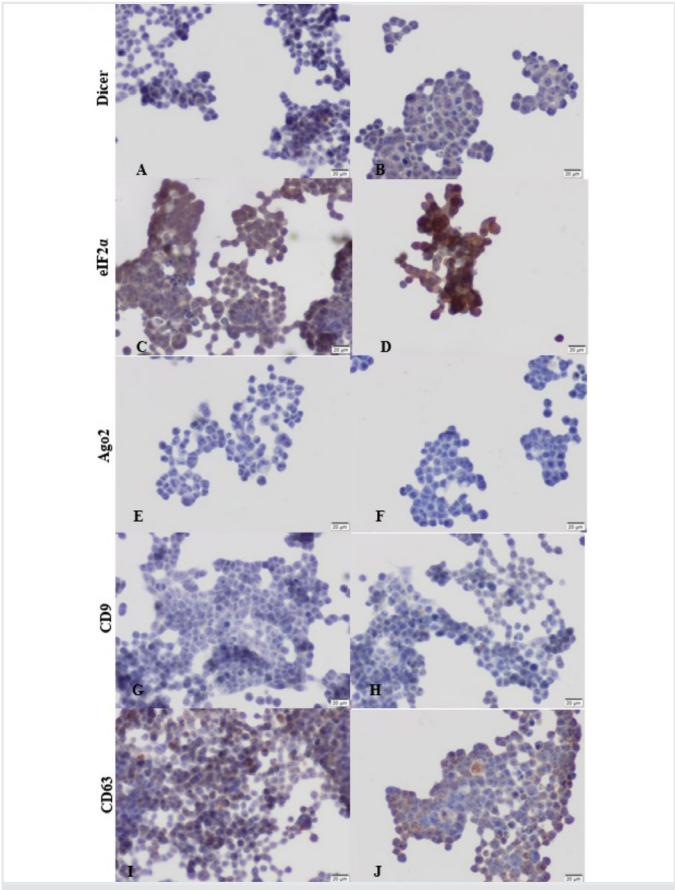

Figure 2. Dicer (A, B), eIF2α (C, D), Ago2 (E, F), CD9 (G, H), CD63 (I, J) immunoreactivity in control (A, C, E, G, I) and 25 μM quercetin-applied (B, D, F, H, J) Colo320 cells. Morphological changes were observed in the cells after quercetin application. Scale bars = 20 μm

Table 1. Evaluation of H-SCORE intensity of related antibodies on Colo320 and Colo741 cells after quercetin application

	Colo320 cell		Colo741 cell	
	Control group	Quercetin	Control group	Quercetin
Dicer	121.3±15	171.4±40.6**	277.5±33.3	325±50
eIF2α	214.3±38.4	211.2±41.6	365±19.15	275±86.6*
Ago2	107.2±6.97	102.5±5	127.5±32	110.4±12.5
CD9	107.5±9.5	108.8±4.7**	112.5±25	192±21.4*
CD63	173.8±19.6	197.7±10.4**	124.2±48	115±19.15

*Significant data after comparison with control group.
**Significant data after comparison with Colo-741 group

Discussion

CRC is associated with high mortality due to its low early detection rate owing to the lack of early-stage symptoms, and due to metastasis in the late stage. Given these diagnostic and clinical challenges, new approaches are promptly needed to diagnose effectively and improve the outcome of patients. Recent studies demonstrated that the TME played role in tumor progression and response of colorectal cancers to the therapy (27). Also, experimental data have indicated that exosomal proteins and/or miRNAs can influence this gastrointestinal malignancy at various stages (28). According to the study results of Dong et al. (29), the exosomes originating from tumoral cells affect tumor progression, formation, and metastasis. The results also show that especially specific exosomal miRNAs may play a vital role in the tumoral network. These clinical findings show the potential therapeutic effects of the regulation of exosomal miRNA secrationv (29). Besides, exosomes can improve CRC progression by elevating tumor cell proliferation via changing particular essential regulatory genes and controlling several molecular signaling pathways (30).

According to surveillance, epidemiology, and databases, the 5-year survival percentage of patients with CRC is 64%.

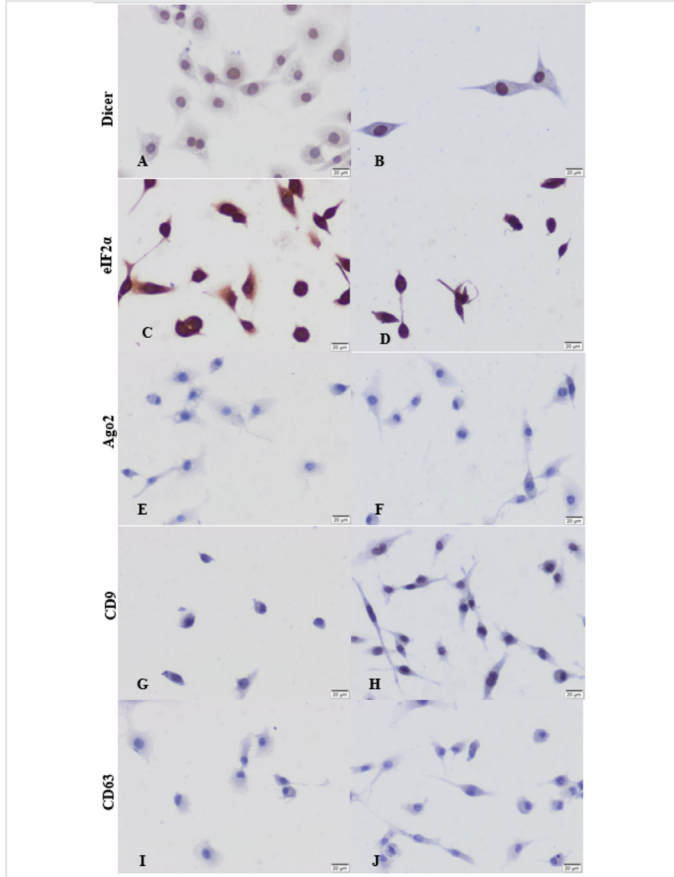

Figure 3. Dicer (A, B), eIF2α (C, D), Ago2 (E, F), CD9 (G, H), CD63 (I, J) immunoreactivity in control (A, C, E, G, I) and 25 μM quercetin-applied (B, D, F, H, J) Colo741 cells. Morphological changes were observed in the cells after quercetin application. Scale bars = 20 μm

Surgical intervention, chemotherapy, and radiotherapy are the most common treatment methods for CRC (31). The effects of natural polyphenolic compounds on CRC cells were searched and it was suggested that they could inhibit the carcinogenesis process by triggering of cell death signaling pathways or diverse molecular mechanisms. Quercetin, which is primarily found in apples, onions, strawberries, and red wine, is a natural polyphenolic compound and is the most commonly studied polyphenolic compound on cancer cells (32).

Previously, it was reported that quercetin inhibited the viability and proliferation of cancer cells. On the B16F10 (melanoma cell line), 5μM quercetin reduced cellluar viability (33). In addition, 20 μM quercetin also affected viability of SW- 620 and Caco- 2 cells (34). Another experimental study reported that the viability of Caco- 2 cells was inhibited after quercetin (20 μM for 24h) application (35). According to our study, the effective quercetin dosage was 25 μg/mL for 48h in Colo320 and Colo741 cells (24).

Exosomes are nano-sized and membrane-bound vesicles that are crucial components of the TME. Also, exosomes, proteins, and exosomal miRNAs originating from cancer cells may control the progression or survival of CRC cells. Accordingly, there has been increased interest in micro-stuffed molecules found in exosomes as possible cancer cell biomarkers, and targets for CRC (5). Also, these microvesicles transfer mRNAs, miRNAs, fragments of DNA, and proteins from active cancer cells to distant cells (36). In the first decade, accumulating evidence showed that different protein and miRNA contents in the plasma exosomes of patients might be beneficial prognostic biomarkers and would encourage the specification of new therapeutic strategies in CRC (37,38). Although polyphenolic compounds can potentially affect CRC prognosis by affecting miRNA concentrations, this effect may differ in Colo320 primary and Colo741 metastatic cell lines. Accordingly, our experimental showed increased miRNA concentrations in Colo741 cells after quercetin administration. This increase was not statistically significant.

In the present study, we showed that the Dicer and eIF2α immunoreactivities were higher in Colo741 cells than in Colo320 cells. After quercetin application, Dicer immunoreactivity was highest; in contrast, eIF2α immunoreactivity decreased after quercetin application, especially in Colo741 cells. Metastatic colon adenocarcinoma cells are more aggressive than primary cells, and survival and response to the therapy are worse than primary cancer cells. Therefore, therapeutic strategy for primary and metastatic CRC should be different. While CD9 immunoreactivity was statistically significantly higher in quercetin-applied Colo741 cells, CD9 immunoreactivity was similar in both Colo320 and control Colo741 cells. However, the CD63 immunoreactivity was elevated in Colo320 cells and reduced in the Colo741 cells after the quercetin application. Therefore, quercetin may trigger exosome secretion from Colo320 cells.

miRNAs are small non-conding RNAs that are differentially expressed and arrange various cellular pathways. Molecular studies suggested that miRNAs had crucial roles in CRC progression and metastasis, therefore, miRNAs could be a strong biomarker for CRC diagnosis. Recent evidence indicated that the biogenesis of miRNAs and related proteins, including Ago2 and Dicer, affected several cancers' development. While deletion of the Dicer gene locus d was associated with pre-cancerous lesions and cancer cell invasion in lung adenocarcinomas (39), and elevated expression of Dicer protein was related to poor prognosis in CRC (18,40), and colon cancer stem cells (41), the Dicer-related molecular mechanism involved in CRC was still unclear.

Our results suggested that the Dicer immunoreactivity was elevated in both quercetin applicated Colo320 and Colo741 cells compared to control groups. Furthermore, increased and significant Dicer immunoreactivity was detected in quercetin applied Colo741 cells compared to quercetin applied Colo320 cells. Thus, quercetin could upregulate tumor suppressive miRNA and have protective effects in CRC by increasing the expression of Dicer.

Ago2 protein is a key and essential regulator of miRNAs secretion. Overexpression of Ago2 has been found in CRC, ovarian, and gastric carcinoma cells (42-44,14). Feng et al. (45) showed that elevated Ago2 was associated with cancer aspects such as its cell growth, proliferation, and also survival of patients. The upregulation of Dicer mRNA expression was not positively correlated with mRNA expression levels of Ago2 (46). Our results demonstrated that the immunoreactivity of Ago2 decreased but this decrease was not significant in both quercetin applied Colo320 and Colo741 cells compared with control groups. The Ago2 changes should be examined at mRNA levels before and after quercetin administration in both type of cells.

Recent experimental studies prescribed that quercetin could reveal conspicuous endoplasmic reticulum stress in different cancer cell types (47,48). The other cellular stress type leads to inhibition of cell translation, which is thought to encourage survival and save energy. In eukaryotic cells, the best-characterized inhibition mechanism of translation regulation is the phosphorylation of eukaryotic initiation factor eIF2α. Additionally, translational attenuation can induce autophagy and apoptosis in cells (49). Accordingly to our data, the eIF2α staining intensities were decreased in both Colo320 and Colo741 cells after quercetin administration, but significant decrease was only detected in the Colo741 cells. In another publication, we showed quercetin initiated cell death in Colo320 and Colo741 cells, which was in line with the results of this study (24). Our results demonstrated that quercetin might have preventive effects by inhibiting the eIF2α protein expression and stimulating cell death in Colo741cells.

miRNAs are single small stand noncoding RNA molecules that inhibit the translation of mRNA and induce degradation

of mRNA. Moreover, experimental miRNA studies reported that varying miRNA levels might regulate tumor formation in CRC (9). According to our exosomal total miRNA levels, they were elevated in Colo320 and Colo741 cells after quercetin administration, but the increase was not statistically significant.

Study Limitations

The present study used a total miRNAs analysis kit to define the exosomal miRNA concentrations in CRC cell lines after quercetin application. This miRNA analysis can be done with commercial kits that determine the specific miRNAs that affect the pathogenesis and may change concerning the cancer type. Finally, to define the exact anti-cancer properties and activities of quercetin on CRC cells. Furthermore, assessment with various mechanisms and various signaling pathway molecules that include probable carcinogenesis mechanisms is necessary.

Conclusion

In conclusion, using five different doses, we showed the anti-cancer effects of quercetin in Colo320 and Colo741 cells. Accordingly, quercetin increased CD9 exosomal biomarker expressions in Colo741 cells. Moreover, high Dicer and exosomal miRNA levels in both two Colo320 and Colo741 cell lines showed the effectivity of quercetin. These results suggest that reduced eIF2α immunoreactivty may be associated with the quercetin-stimulated apoptosis in Colo741 cells. However, proteins that were related with exosomal proteins or miRNAs were similar in Colo320 and Colo741cancer cells before and after quercetion application.

In the current study, we showed that CD9 immunoreactivity elevated significantly in quercetin-administrated Colo741 cells compared to the control group. Moreover, CD9 immunoreactivity was lower and this decrease was significant in Colo320 cells than in Colo741 cells after the application of quercetin. Otherwise, after quercetin application, CD63 immunoreactivity was elevated in Colo320 cells and decreased in Colo741 cells. The increase in CD63 immunoreactivity was statistically significant in quercetin-administrated Colo320 cells compared with quercetin-administrated Colo741 cells. We concluded that secretion of exosomes occurred in response to the quercetin administration in both primer and metastatic colon cancer cell lines, therefore, exosomes and their surface proteins, CD63 and CD9, could be used for colon carcinoma identification.

Ethics

Ethics Committee Approval: Colo-320 (ATCC: 220.1) primary and COLO-741 (ECACC: 93052621) metastatic colon cancer cell lines were used in our study. Therefore, the study does not require ethics committee approval.

Peer-review: Externally and internally peer reviewed.

Authorship Contributions

Concept: E.B., H.S.V., Design: E.B., S.Ö., H.K., Data Collection or Processing: H.K., H.S.V., Analysis or Interpretation: E.B., H.K.E, H.S.V., Literature Search: E.B., S.Ö., Writing: E.B., S.Ö., H.S.V.

Conflict of Interest: No conflict of interest was declared by the authors.

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