Resveratrol modulates miRNA machinery proteins in different types of colon cancer cells

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Abstract

Objectives: Resveratrol (RSV) is a stilbenoid compound that shows anticancer activity in many cancer cells. Exosomes might affect carcinogenesis and the development of colorectal cancer by affecting communication between tumor cells in the tumor microenvironment via their cargo content miRNA. The aim of this study is to determine the effect of RSV on the expression of Dicer, Ago2, eIF2α, CD-9, CD-63, and exosomal miRNA levels in COLO320 and COLO741 colon cancer cell lines.

Methods: The MTT method was used for cell growth and cytotoxicity in both COLO320 and COLO741 cell lines. Dicer, Ago2, eIF2α, CD-9, CD-63 antibodies were used for the immunocytochemical evaluation. Total miRNA analysis was performed using a miRCURY Exosome Isolation Kit.

Results: As a result of immunocytochemical staining, increased CD-63 immunoreactivity was observed in RSV-treated COLO320 cells vs. RSV-treated COLO-741 cells. Dicer immunoreactivity increased after the RSV treatment in COLO320 cells. Higher eIF2α immunoreactivity was observed in RSV-treated COLO741 cells compared to both COLO741 control cells and RSV-treated COLO320 cells. Non-significant decreases were observed in miRNA concentration in RSV-treated COLO320 and COLO741 cells compared to control group cells.

Conclusions: RSV could increase miRNA biogenesis in COLO320 cancer cells and decrease it in COLO741 cancer cells.

Keywords: resveratrol; stilbenes; miRNA; exosomes; colon cancer

Introduction

Colorectal cancer is one of the cancer types with the highest incidence and mortality rates globally [1]. As result of the complex nature of the disease, treatment optimization is challenging [2].

Significantly, the tumor microenvironment (TME), composed of non-malignant cells, malignant cells, exosomes, and their secreted components, changes cancer progression and leads to drug resistance in colorectal cancer [3]. Recent experimental studies have indicated that exosomes might affect the carcinogenesis and development of colorectal cancer as important players in the communication between tumor cells in TME. Proteins, mRNA, DNA, proteins, and microRNAs (miRNAs) are the main components of exosomes in cell–cell communication [4–6]. CD-9, CD-63, CD37, and CD81 are the exosome’s markers.

In recent years, cargo content exosomal miRNAs have drawn attention as a diagnostic and therapeutic biomarker in colorectal cancer [6]. Matsumura et al. [7] showed that serum exosomal miRNAs were more plentiful in a recurrent colorectal cancer case than in a non-recurrent case. Moreover, Ogata-Kawata et al. [8] identified that 16 exosomal miRNA was more abundant in serum exosomes from colorectal cancer patients compared to healthy controls and more plentiful in conditioned medium from colorectal...
Materials and methods

Cell lines and cell culture

COLO320 (ATCC: CCL-220.1) and COLO741 (ECACC: 93052521) cell lines were used. RPMI1640 medium (Biochrom, FG1215) supplied with 10% heat-inactivated fetal bovine serum (FBS) (Capricorn Scientific, FBS-11B), 1% penicillin-streptomycin (Biochrom, A2213) and 1% glutamine (EMD Millipore, K0282) were used for the cultures of both cell lines. Cancer cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. After reaching the confluence state, the cells were subcultured using 0.25% trypsin-EDTA solution (Biochrom, L 2143).

Cell viability assay

MTT was used to determine the cytotoxicity. The MTT assay was employed in accordance with the protocol previously described by Madencioglu et al. [22]. The positive control only had the seeded cells, neither cells nor the RSV (Sigma, R5010) containing group was accepted as a negative control. RSV concentrations of 5 μg/mL, 10 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL were loaded in triplicate, and both cell lines were incubated for 24 and 48 h. After incubation at 37°C in 5% CO₂ for 4 h, 50 μL DMSO (dimethylsulfoxide) was added to dissolve the formazan salts. The absorbance was measured at 540 nm with a spectrophotometer (Versa Max, Molecular Devices, Sunnyvale, USA). All experiments were repeated three times.

Immunocytochemistry

Cultured COLO320 and COLO741 cells were assessed immunocytochemically for antibodies binding against Ago2, CD-9, CD-63, Dicer, and eIF2α. The indirect immunoperoxidase method was conducted as described previously [24]. Primary antibodies CD-9, CD-63, Dicer, EIf2a, and EIf2c were used in the assay. Also, biotinylated secondary antibody (Histostain-Plus, IHC Kit, HRP, 859043, Thermo Fischer) was used for immunocytochemically staining. In the last step, diaminobenzidine (DAB) was added and incubated for 5 min to enhance immuno-labeling. DAB was washed with distilled water. Cells were counterstained with Mayer’s hematoxylin for 5 min and mounted using the mounting medium (Merck Millipore, 107961, Germany). All specimens were examined under a light microscope (Olympus BX40, Tokyo, Japan).

Staining of CD-9, CD-63, Dicer, eIF2a, and Ago2 was also graded semi-quantitatively using the H-SCORE, which was calculated with the following equation: H-SCORE=Σxi (i=1), where i is the intensity of staining with a value of 1, 2 or 3 (mild, moderate, or vigorous, respectively) and x is the percentage of cells stained at each intensity, ranging between 0 and 100%.

Total miRNA analysis

After the cells were thawed at 4°C, they were centrifuged to remove dead cells and other residues. Then, a settling buffer was applied and incubated at 4°C for 60 min. After the incubation, the supernatant obtained from the centrifuged cells was discarded, and the pelleted part was resuspended. Subsequently, miRCURY™ Exosome Isolation Kit (Exiqon 300102) was used, and total miRNA was obtained.
**Statistical analysis**

Results are expressed as mean±standard deviation (SD). GraphPad Prism 7 statistical software program was used for data analysis. The Mann Whitney U test was used for the determination of the differences among groups. For statistical significance, a p-value below 0.05 was required.

**Results**

**Cell viability and cytotoxicity**

MTT assay was used to evaluate the cytotoxic effect of RSV in COLO320 and COLO741 cells. According to the MTT results, 48 h of RSV treatment led to more significant inhibition of cell proliferation at 25 μg/mL concentration for COLO320 cells (Figure 1A) and 10 μg/mL concentration for COLO741 cells (Figure 1B) in comparison to other engagements.

**Immunocytochemical evaluation**

CD-9 immunoreactivity was weak or negative in RSV-treated COLO320 and COLO741 cells (Figures 2B and 3B) (Table 1). H-SCORE values of CD-9 in COLO320 control and RSV-treated groups were similar and nonsignificant (p>0.05, Table 2). RSV-treated COLO741 cells showed the highest H-SCORE value vs. the control group, but the difference was not significant (p>0.05, Table 2).

Immunoreactivity of CD-63 was weak in control COLO320 cells (Figure 2C) and increased to a strong level after RSV administration (Figure 2D). However, no significant differences between the groups were observed (p>0.05, Table 1). In COLO741 cells, CD-63 intensity was very weak or negative (Figure 3C and D). The H-SCORE value of CD-63 was significantly higher in RSV-treated COLO320 cells in comparison to RSV-treated COLO741 cells (p<0.05, Table 2).

The immunostaining intensity of Dicer was moderate in RSV-treated COLO320 cells vs. the control group (Figure 2E and F). The H-SCORE value of Dicer was significantly higher in RSV-treated COLO320 cells compared to the control group (p<0.05, Table 2). In addition, a similar intensity of Dicer was detected in RSV-treated COLO741 cells and the control group (Figure 3E and F), but the H-SCORE value for this intensity was not significant (p>0.05, Table 2).

**microRNA analysis**

Decreased miRNA concentration was detected in RSV-treated COLO320 and COLO741 cells compared to the control groups. Nevertheless, this decrease was not significant (p>0.05, Table 3). The highest miRNA concentration was

![Figure 1: Dose–response curves and IC50 values of RSV COLO320 (A) and COLO741 (B) cells treated with different concentrations of RSV for 24 and 48 h, respectively.](image-url)
Table 1: Immunostaining analysis of CD-9, CD-63, Dicer, eIF2α, and Ago2 for control and RSV groups of COLO320 and COLO741 cells.

<table>
<thead>
<tr>
<th></th>
<th>COLO320 cell</th>
<th>COLO741 cell</th>
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<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>RSV group</td>
</tr>
<tr>
<td>CD-9</td>
<td>–</td>
<td>+/−</td>
</tr>
<tr>
<td>CD-63</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Dicer</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>eIF2α</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Ago2</td>
<td>–</td>
<td>+</td>
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Two Way ANOVA. aThe data was significant compared with the control group (p<0.05). bThe data was significant when compared with the Colo-741 group (p<0.05).

Table 2: H-SCORE analysis of CD-9, CD-63, Dicer, eIF2α, and Ago2 for control and RSV groups of COLO320 and COLO741 cells.

<table>
<thead>
<tr>
<th></th>
<th>COLO320 cell</th>
<th>COLO741 cell</th>
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<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>RSV group</td>
</tr>
<tr>
<td>CD-9</td>
<td>107.5±9.6</td>
<td>102.9±5.7</td>
</tr>
<tr>
<td>CD-63</td>
<td>173.8±19.7</td>
<td>204±36.2a</td>
</tr>
<tr>
<td>Dicer</td>
<td>121.3±15</td>
<td>156.3±11.9ab</td>
</tr>
<tr>
<td>eIF2α</td>
<td>214.3±38.4</td>
<td>215.9±9.1ab</td>
</tr>
<tr>
<td>Ago2</td>
<td>102.2±7.0</td>
<td>134.4±9.1ab</td>
</tr>
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Two Way ANOVA. aThe data was significant compared with the control group (p<0.05). bThe data was significant when compared with the Colo-741 group (p<0.05).
found in RSV-treated COLO741 cells in comparison to RSV-treated COLO320 cells, albeit non-significant (p>0.05).

Discussion

Recent studies have shown that exosomes, through the RNA and proteins they contain, are important biomarkers of cancers in humans, such as colorectal cancer [6, 25, 26]. miRNAs are the content of the exosomes and affect colorectal cancers at different stages, so they are considered one of the markers of the diagnosis or progression of colorectal cancer [25, 27]. miRNAs act as onco-miRNA or tumor-suppressing miRNAs [28]. However, tumor suppressor miRNAs content is decreased in the exosomes by the colorectal cancer cell to promote metastasis [25]. On the other hand, exosomal miRNAs can influence the chemoresistance of the cancer cell to chemotherapeutic agents [5, 29, 30]. It was reported that RSV modulates some miRNAs and inhibits proliferation, migration, invasion, and induced apoptosis and cell cycle arrest [28, 31]. The current study observed a non-significant decrease in both cell lines after the RSV treatment. Oncogenic miRNA expression may be increased despite decreased miRNA levels. From this point of view, it will be helpful to determine the changes in specific miRNA levels by performing detailed miRNA analysis in future studies.

Tetraspanins (CD-9 and CD-63) are exosome-enriched proteins that are markers of exosome recognition [6]. Elevated CD-9 expression suppresses metastasis and tumorigenesis in cancer cells [32, 33]. Ovalle et al. [32] showed that CD-9 inhibits the proliferation and tumorigenesis in COLO320 cells. On the other hand, this study is the first to show the effect of RSV on CD-9 expression in different types of colon cancer cells. In the current study, RSV caused a non-significant decrease in COLO320 cells and an increase in COLO741 cells. These results suggest that RSV causes more stress in COLO320 cells, and cells may activate the proliferation to escape the stress. On the other hand, RSV suppresses the proliferation and metastatic potential of COLO741 cells by increasing CD-9 levels.

Table 3: miRNA concentrations after exosome analysis in COLO320 and COLO741 cells.

<table>
<thead>
<tr>
<th></th>
<th>COLO320 Cell</th>
<th>COLO741 Cell</th>
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<tbody>
<tr>
<td>Control group</td>
<td>11.0 ng/µL</td>
<td>15.2 ng/µL</td>
</tr>
<tr>
<td>RSV group</td>
<td>10.8 ng/µL</td>
<td>13.0 ng/µL</td>
</tr>
</tbody>
</table>

p<0.05

Data compared with Mann-Whitney U.

Tetraspanin CD-63 is considered one of the colorectal cancer biomarkers. Elevated CD-63 expression was detected in colon cancer cells compared to healthy colon cells [34]. Increased CD-63 levels activate the CD-63/integrin complex, increases cell survival pathways, and inhibits apoptotic pathways [35]. No data was found on RSV’s effects on CD-63 expression in colon cancer cells. The current study showed that RSV treatment caused elevated CD-63 expression in COLO320 cells compared with COLO741 cells. This might be explained by the fact that RSV causes more stress in COLO320 cells than in COLO741 cells; this is consistent with our previous study results [22].

Dicer is one of the miRNA machinery proteins related to tumor relapses and death [36]. Moreover, Dicer expression is associated with liver metastasis in colorectal cancer [27]. Aggressive tumors might decrease miRNA levels to promote their poor differentiation. Therefore, it might suggest a lower Dicer expression instead of elevated expression in aggressive tumors [36]. On the contrary, Kim et al. showed that Dicer expression was not associated with CRC [13]. Dicer expression and the effect on the cancer cell differ according to the tumor cell type [37]. RSV decreased on-miRNA levels, targeting genes encoding Dicer in SW480 human colon cancer cells and increased the tumor suppressor miRNA levels [22]. However, this effect has not been tested in other colorectal cancer cell lines. Dicer levels significantly increased after RSV treatment in COLO320 cells in this study. miRNAs expression which encode the Dicer in COLO320 cells, might increase to promote the survival pathways.

On the other hand, reduced Dicer levels were observed in COLO741 cells after RSV treatment. Decreasing Dicer levels in metastatic tumors causes decreased mRNA concentration. This is one of the factors initiating senescence in the cells, which could help cancer cells hide in a dormant phase [38]. This result is consistent with our previous study results [22]. Although the Dicer level decreased after RSV treatment in COLO741 cells, it was higher in this cell line compared to RSV-treated COLO320 cells.

eIF2α (Eukaryotic initiation factor 2) phosphorylation increased in response to stress and caused a decrease in protein synthesis in the cell [16]. Decreasing protein synthesis in the cell initiates signal cascades such as cell cycle arrest, gene expression regulation, and apoptosis induction in response to cell stress [39]. Yu et al. [40] reported that pterostilbene, the RSV analog, inhibited the eIF2 α dephosphorylation and induced endoplasmic reticulum (ER) stress-related autophagy in hepatocellular carcinoma cells without inducing apoptosis. Moreover, pterostilbene caused inhibition of cell growth time and was dose-dependent [40]. RSV
activated PERK/eIF2α/starting transcription factor 4 (ATF4)/CHOP signaling pathway and induced ER stress-mediated apoptosis and cell cycle arrest in gastric cancer cells [40, 41]. In vivo studies showed that high-dose RSV (40 mg/kg day) decreased the eIF2α expression in renal and brain cells [42, 43]. Despite the findings in the literature, the effect of RSV on eIF2α expression in colon cancer cells remains unclear. In the current study, eIF2α levels decreased in RSV-treated COLO741 cells vs. the control group, whereas they were similar in COLO320 cells. The reduced eIF2α levels in RSV-treated COLO741 cells might be related to the escape to apoptosis in response to RSV. However, eIF2α levels in RSV-treated COLO741 cells were higher than in RSV-treated COLO320 cells. This might suggest that RSV causes more inhibition of tumorigenesis vs. COLO320 cells.

Elevated Ago2 level was observed in RSV-treated COLO320 cells, which might be related to increased cell proliferation to survive. The increased levels of both Dicer and Ago2 levels might be explained by the increase in protein translation related to miRNA proteins in the cancer cells.

On the other hand, non-significant decreases in Ago2 levels in RSV-treated COLO741 cells were observed. The reduced Dicer and Ago2 levels in this cell line might be explain by decreased RISC complex and gene silencing as result of inhibited protein translation. RSV may suppress cancer cells by affecting miRNA machinery proteins. In addition, a significant Ago2 level increase was observed in RSV-treated COLO320 cells compared to RSV-treated COLO741 cells. RSV might cause more stress in the primary cancer cells and promote protein translation while decreasing the RSV-treated COLO741 cells.

In conclusion, the effects of RSV on exosomes and miRNA biogenesis were evaluated in primary (COLO320) and metastatic (COLO741) colon cancer cells. The results showed that RSV could increase miRNA biogenesis in primary cancer cells and decrease it in metastatic cancer cells. The results of the current study can form the basis for further studies to better understand the effects of RSV on exosomes and miRNA biogenesis.

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Informed consent: Not applicable.

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Competing interests: Authors state no conflict of interest.

Research funding: None declared.

Data availability: Not applicable.

References