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Deregulation of miR-21 and miR-155 and their putative targets after silibinin treatment in T47D breast cancer cells

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ABSTRACT

Objective(s): MicroRNAs (miRNAs) are a class of short RNAs that control the biological processes including cell proliferation, apoptosis and development. Aberrant expression of miRNAs was determined in the different stages of tumor development and metastasis. To study the effect of silibinin on miRNAs expression, we evaluated quantitative expression of miR-21 and miR-155 as two oncomiRs and several potential targets in silibinin-treated T47D cells.

Materials and Methods: The rate of proliferation and apoptosis was measured in silibinin-treated and untreated cells. The expression levels of miR-21 and miR-155 were evaluated in T47D cells treated with silibinin (100 µg/ml). Also, their putative targets were predicted in apoptotic pathways using multiple algorithms; as a confirmation, the transcription level of APAF-1, CASP-9 and BID was evaluated.

Results: In silibinin-treated cells, death was occurred in a dose and time-dependent manner. miR-21 and miR-155 was downregulated in cells treated with silibinin (100 µg/ml). It is noticeable that the expression of their potential targets including CASP-9 and APAF-1 was increased in silibinin-treated cells after 48 hr.

Conclusion: Our findings showed a correlation between the expression of miR-21 and miR-155 and apoptosis in silibinin treated T47D cells. It seems that miRNAs such as miR-21 and miR-155 were regulated by silibinin. Also, increase in the transcript level of APAF-1 and CASP-9 after downregulation of miR-21 and miR-155 might indicate that these genes were targeted by aforementioned miRNAs in T47D cells.

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Introduction

Breast cancer is one of the most common cancers in women in many countries (1). Systemic treatments for breast cancer such as hormonal and immunotherapeutic agents have severe side effects (2). Silibinin is a polyphenolic flavonoid from milk thistle (*Silybum marianum* L.) (3) with antioxidant and anticancer properties (4) that is being used as a dietary supplement and traditional medicine (3). Silibinin was reported to diminish cell growth and induce apoptosis in cancer cells (5). Consuming silibinin at doses as high as 1% (w/w) or 2 g/kg body weight does not reveal any signs of toxicity in animals or humans (2). Thus, using silibinin has been proven to be a safe and efficient therapeutic alternative in the treatment of cancers. microRNAs (miRNAs) are a group of endogenous non-coding RNA with ~22 nt length, widely existing in the eukaryotes from nematodes to humans (6). miRNAs play important roles in cell proliferation, development, differentiation, and apoptosis (7) and tumor suppression (8). miRNAs bind to the 3′-UTR of mRNAs and suppress target translation (9) or induce mRNA degradation (10). Bioinformatics analyses have estimated that up to 92% of human genes can be regulated by miRNAs. However, a small number of miRNAs targets has been identified in biological processes. Nowadays, many studies have focused on recognition of binding sites of miRNAs in mRNA targets (11-15) to find their functions in different cells. However, for some miRNAs no target has been determined, while some can repress multiple mRNAs, suggesting that gene regulation by miRNAs is complex and needs further studies.

Recent studies have reported that some miRNAs, which are called oncomiRs play important roles in cancer initiation and progression (16, 17). OncomiRs deregulation in malignancies is occurred through

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Table 1. Sequence of primers used for quantitative real-time PCR

Genes	Primers	Length of production
APAF1 forward	5'- TGCCAGCTTCAGGATCTAC-3'	277 bp
APAF1 reverse	5'- TCTCACTGACTGCACAATCCTTTT-3'	
CASP9 forward	5'- AGGGTCGCTAATGCTGTTTCG-3'	267 bp
CASP9 reverse	5'- TCGTCAATCTGGAAGCTGCTAAG-3'	
BID forward	5'- GGAGGAGGACCGGAACAGG-3'	158 bp
BID reverse	5'- GAAAGACATCACGGAGCAAGGAC-3'	
GAPDH forward	5'- GAAGGTGAAGGTCGGAGTC-3'	226 bp
GAPDH reverse	5'- GAAGATGGTGATGGGATTTC-3'	

deletion, amplification, point mutation and/or aberrant DNA methylation (16). miR-21 and miR-155 as two oncomiRs (18) that are frequently overexpressed in different cancers including breast, lung and colon cancers (19). Thus, suppression of these oncomiRs in cancerous cells could be regarded as a novel therapeutic strategy. Since silibinin is a safe herbal medicine with anti-cancer properties, we assessed its effects on the expression of miR-21 and miR-155 as two oncomiRs in breast cancer T47D cell line. Also, in these cells, the expression of some potential targets of miR-21 and miR-155 was quantitatively evaluated in the apoptotic pathway.

Materials and Methods

Cell culture

T47D human carcinoma breast cancer cell line was purchased from National Cell bank of Iran (NCBI, Pasteur Institute of Iran). Then, T47D cells were seeded in 0.2 ml 96-well tissue culture plates and cultured in RPMI1640 medium (with glutamine) supplemented with 10% FBS at 37ºC and 5% CO2.

Cell proliferation assay

We used MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) assay to evaluate cell proliferation. Briefly, 7×10^3 cells per well were cultured in 96-well plates and treated with silibinin (Sigma Aldrich) at different doses (0, 50, 75, 100, 150, 200, 250, and 300 µM) for 24, 48 and 72 hr. Then, MTT dye (0.5 mg/ml; Sigma Aldrich) was added and incubated at 37 ˚C for 4 hr. Then, to dissolve the formazan crystals, 100 µl of DMSO was added. Absorbance was read at 570 nm using an ELISA plate reader.

Cell cycle and apoptosis analysis

To evaluate cell cycle and death, 0.5-1 × 10^6 cells treated with silibinin were harvested, washed with PBS, suspended in 5 ml PBS, fixed in 70 % ethanol and stored at -20 ºC for ≥2hr. The fixed cells were washed with PBS and stained with 0.02 mg/ml propidium iodide (PI) (Sigma Aldrich) in a 0.1 % Triton X-100 solution with 0.2 mg DNase-free RNase A. The stained cells were incubated at 37 ºC for 15-30 min. Then, flow cytometric analysis was carried out using CyFlow®-SL system (Partec, Germany) and FlowMax software.

miRNAs expression analysis by Q-RT-PCR

RNA isolation was carried out using miRCURY™ RNA isolation kit (Exiqon,Vedbaek, Denmark) according to the manufacturer's instructions. miR-Amp kit (Parsgenome, Tehran, Iran) was used for cDNA synthesis. First, poly-(A) tail was added to miRNAs with polyA polymerase at 37 ˚C. RNA polyA tail was mixed with RT-enzyme, reaction buffer, and miR specific primers for cDNA synthesis, then, incubated at 45 ˚C for 60 min and inactivated at 85 ºC for 1 min. Quantitative real-time PCR was performed with SYBR® Premix Ex Taq™ II (Takara bio, Japan) and monitored by Applied Biosystems StepOne™ instrument according to this program: 95 ˚C for 10 sec, 40 cycles at 95 ˚C for 5 sec, 62 °C for 20 sec, and 72 ˚C for 30 sec. As an internal control, U6 snRNA was used for miR-21 and miR-155 template normalization. The primer pairs were purchased from Parsgenome (Tehran, Iran). All reactions were run in triplicate. The expression of miRNAs was analyzed using the equation $2^{-\Delta\Delta CT}$.

Prediction of miRNA target genes

The bioinformatics methods were used to identify the potential targets of miR-21 and miR-155. Their targets were predicted in apoptotic pathways by some algorithms such as TargetScan, miRWalk and Diana microT.

Quantitative analysis of target genes

The expression levels of three potential target genes of miR-21 and miR-155 including APAF-1, CASP-9 and BID were measured by Q-RT-PCR.

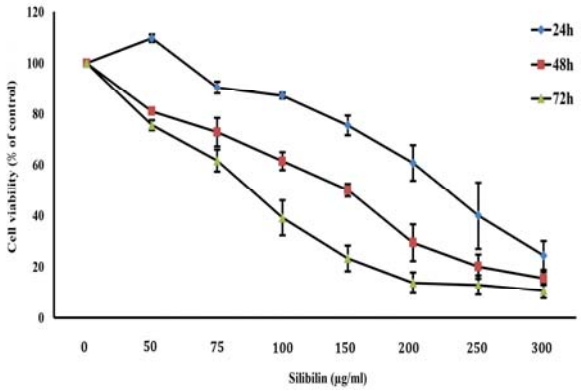

Figure 1. Cell viability after silibinin treatment. T47D cells treated with different doses of silibinin (0-300 µg/ml) were cultured for 24–72 hr. Viability of cells was measured by MTT assay. Results were representative of three experiments and each concentration was repeated at least four times in each experiment. The results are presented as mean±SD

Isolated RNA from miRCURY™ RNA Isolation kit was used for cDNA synthesis. cDNA synthesis was carried out with PrimeScript™ RT reagent Kit (Takara bio inc, Japan). Quantitative expression of target genes was analyzed using SYBR® Premix Ex Taq™ II (Takara bio inc, Japan) and Applied Biosystems StepOne™ instrument. The PCR was programmed with: 95 ˚C for 15 sec, 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. The used primers were listed in Table 1. The gene expression level was normalized by GAPDH gene as endogenous control. The fold change of genes were calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

The results are presented as mean ± SD. *t*-test and one-way ANOVA were used to compare the differences between two cell groups.

Results

Effect of silibinin on T47D cells proliferation

T47D cells were cultured by different doses of silibinin (0, 50, 75, 100, 150, 200, 250, and 300 µM) during 72 hr. The results indicated that silibinin inhibited the proliferation of T47D cells in concentration- and time-dependent manners. Treatment of cells with silibinin (100 and 150 µg/ml) decreased cell proliferation by ~61% and ~49% after 48 hr, respectively (Figure 1).

Cell cycle arrest and apoptosis induction by silibinin

To assess the inhibitory effect of silibinin on T47D cells growth, flowcytometric analysis was performed after 24 and 48 hr for cells treated with silibinin (100 and 150 µg/ml) and compared to untreated cells. Silibinin 100µg/ml led to significant cell cycle arrest at the G2/M phase after 24 hr. Also,

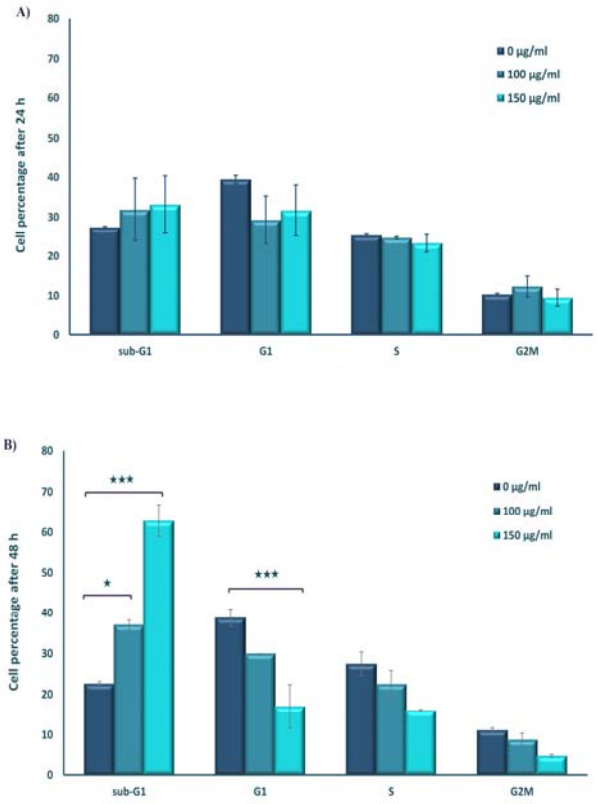

Figure 2. Cell percentage in the cell cycle phases after silibinin (0, 100 and 150 µg/ml) treatment. The percentage of cells in each phase was measured using flowcytometry after staining with propidium iodide (PI) following 24 (A) and 48hr (B). Asterisks indicate significant difference among different cell groups (**P*<0.05, ***P*<0.01, ****P*<0.001). The results are presented as mean ± SD

percentage of G1, S and G2/M phase cells was decreased after 48 hr treatment with silibinin (100 and 150 µg/ml) and a greater number of treated cells entered sub-G1 phase after 48 hr treatment as compared to 24 hr treatment. Hence, our results revealed that silibinin significantly increased the rate of cell death after 48 hr from ~23% in the control to ~37% and ~63% at 100 and 150 µg/ml, respectively (Figure 2).

Quantitative evaluation of miR-21 and miR-155

Real-time PCR was used to identify the expression levels of miR-21 and miR-155 in T47D cells treated with silibinin (100 µg/ml). Quantitative analysis showed that aforementioned miRNAs were significantly (*P*<0.05) downregulated in silibinin-treated cells compared to untreated ones after 48 hr (Figure 3).

Prediction of potential targets

After silibinin treatment, *in silico* analysis predicted putative targets of miR-21 and miR-155 in

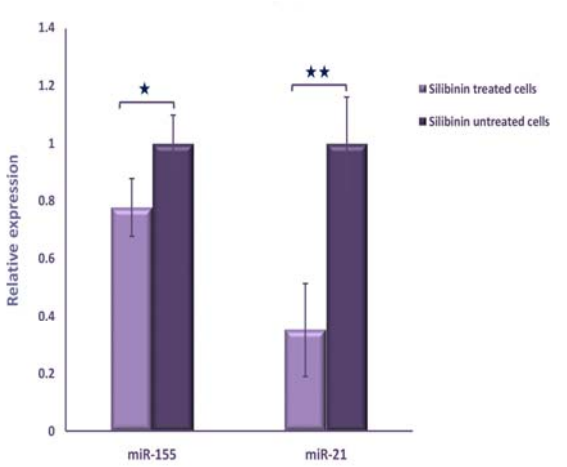

Figure 3. Relative expression of miR-21 and miR-155 in silibinin (100 µg/ml)-treated and untreated cells. Relative expression of miR-21 and miR-155 was normalized to U6 snRNA (an endogenous control). Asterisks indicate significant differences among different groups (**P*<0.05, ***P*<0.01). The results are represented as mean ±SD

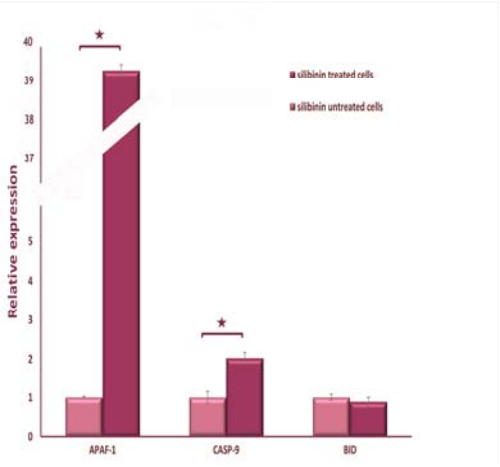

Figure 4. Quantitative expression of APAF-1, CASP-9 and BID in silibinin-treated cells compared to untreated ones. Relative expression of these genes was normalized to GAPDH. Asterisks indicate significant differences among differen groups (*P<0.05). Results are represented as mean ±SD

mitochondrial apoptosis pathway. Among their putative targets, APAF-1, CASP-9 and BID were predicted by at least four algorithms and selected for further analysis.

APAF-1 and CASP-9 upregulation after silibinin treatment

The expression level of APAF-1, CASP-9 and BID, as putative targets of miR-21 and miR-155, was quantitatively assessed in both cell groups. Silibinin-treated cells had an increase in APAF-1 and CASP-9 expression compared to untreated ones (Figure 4). The expression level of APAF-1 in silibinin-treated cells showed ~ 40-fold elevation compared to untreated cells, as well as for CASP-9, which displayed a 2-fold increase. Note that there was no significant difference between silibinin-treated cells and untreated cells in the expression of BID.

Discussion

Silibinin is a major constituent of the traditional herb, milk thistle (*Silybum marianum* L.) which is widely used in treatment of hepatic diseases. Recently, silibinin has been reported to be a potential adjunctive agent for cancer therapies (20). Inhibitory effects of silibinin on DNA synthesis, cell cycle and cell differentiation have been determined in several types of cancer cells (21). Also, silibinin initiates apoptosis through activation of caspases and decrease of anti-apoptotic protein Bcl-2 and survivin (22). In the present study, we evaluated the effects of silibinin on T47D breast cancer cell line. Our results revealed that silibinin arrests the growth of T47D cells at the G2/M phase and additionally induces apoptosis as identified by flow cytometry. The increase in the percentage of apoptotic cells was dose and time-dependent. Following 48 hr or treatment, the highest percentage of apoptotic cells was found when the cells were treated with 150 µg/ml silibinin rather than 100 µg/ml. Since, the higher dose of silibinin (150 µg/ml) killed most of treated cells, the expression of apoptosis related genes could not be precisely evaluated and we selected silibinin (100 µg/ml) for next experiments. Further studies revealed that silibinin led to downregulation of oncomiRs including miR-21 and miR-155 and upregulation of several apoptotic related genes.

Aberrant expression of miRNAs was determined in the different stages of tumor development and metastasis (19, 24-26). On the other hand, miRNAs seems to be efficient candidates for cancer therapy. A miR-21 inhibitor was recently reported to reduce MCF-7 cell growth and increase cell death (up to 40%) in cells treated with the anticancer drug topotecan (TPT) (23). Nevertheless, there are a few documents about effects of silibinin on miRNAs expression in cancerous cells. Thus, herein we evaluated effects of silibinin on the expression of two oncomiRs (miR-21 and miR-155) that were upregulated in different breast cancer cells (19, 25-27). Previous studies revealed that some of miRNAs were upregulated in breast cancers including miR-21, miR-155 (26), miR-221 and miR-222 (28, 29) while miRNAs such as miR-10b, miR-125b and miR-145 (26, 30) were downregulated in the breast cancer cells. It seems that upregulated miRNAs that play roles in tumorogenesis and downregulated miRNAs are implicated in tumor suppression. Our results showed anticancer activities of silibinin were

mediated through downregulation of two oncomiRs, miR-21 and miR-155, in the silibinin-treated cells compared to untreated cells. miR-21 is upregulated in various types of cancerous cells or tissues such as glioblastoma, lung, liver, ovarian, and breast cancers (31). Overexpression of miR-21 is also associated with advanced stage of breast cancer, metastasis and poor prognosis (25). In a study, knock-down of miR-21 was associated with increased cell death and decreased cell proliferation in MCF-7 breast cancer cells. Such studies suggest that miR-21 has some oncogenic properties (32). Regarding downregulation of miR-21 in silibinin-treated cells, it seems that silibinin induced apoptosis in T47D breast cancer cells partly through miR-21 suppression.

On the other hand, overexpression of miR-155 was determined in a variety of human solid tumors including breast, lung, thyroid (31), and colon cancer (33). Recent studies have indicated that deregulation of miR-155 led to tumors development, invasion and metastasis (34). Thus, oncogenic miR-155 may be used as a biomarker in tumor diagnosis or prognosis in future. In this study, downregulation of miR-155 induced by silibinin which was associated with decreased proliferation and increased apoptosis could be a result of its oncogenic activity in T47D breast cancer cells.

Nowadays, lack of knowledge about bona fide miRNA targets does not allow us a better understanding of the biological functions deregulated by aberrant expression of miRNAs in the different cancers. To overcome problems, computational approaches have been designed and were able to potentially predict gene targets of various miRNAs (35). It has been demonstrated that upregulation of miR-21 was associated with downregulation of apoptosis related genes. Previously, miR-21 target genes were identified in several cancers such as PTEN in hepatocellular carcinomas, PDCD4 and tropomyosin 1 in breast cancer cells (36). Also, miR155 targets have been defined in the several cancer cells. miR-155 targets TP53INP1 in pancreatic cancer, MSH2 and MSH6 in colon cancer, SOCS1 in Hodgkin and B cell lymphoma and BACH1 in renal cancer (37). In this study, not-validated targets of miR-21 and miR-155 were quantitatively analyzed in apoptotic pathways. We found that the decrease in miR-21 expression was more marked in MCF-7 cells (data not shown) compared to T47D cells. As no significant difference has been shown in relative expression of BID between treated and untreated cells, it seems that cell death induced in two groups was mediated by similar level of BID. Therefore, similar levels of BID transcript in both silibinin-treated and untreated T47D cells may play an important role in apoptosis induction. Caspase-9 is a key component of intrinsic apoptosis pathway that is recruited and activated by the Apaf-1 (apoptotic protease activating factor-1) apoptosome. Activated caspase-9, in turn, activates executioner caspases, caspase-3 and caspase -7 leading to apoptosis induction (38). In this study, overexpression of caspase-9 revealed silibinin-induced activation of intrinsic pathway of apoptosis in T47D cells. Conversely, the expression of APAF-1, another key component in intrinsic pathway of apoptosis (38) and a potential target of miR-21 was considerably increased in silbinin-treated T47D cells. Apaf-1 is associated with cytochrome c and caspase-9 and thus activates caspase-3. Then, caspase-3 cleaves poly-(ADP-ribose) polymerase, protein kinase C-δ, and other; proteins (39). These results suggested that silibinin, through overexpression of crucial components in mitochondrial pathway induces greater degree of apoptosis in T47D cells.

Conclusion

In this study, the expression pattern of miR-21 and miR-155 was evaluated in silibinin-treated T47D cells. Our findings showed that silibinin decreased expression of miR-21 and miR-155 in treated cells. Also, overexpression of APAF1 and CASP-9 may be the result of downregualtion of miR-21 and miR-155 in silibinin-treated cells.

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