Silibinin reduces cell proliferation and migration via EMT pathway in TFK-1 cell line

Abstract

Objectives: Cholangiocarcinoma (CCA) is usually diagnosed at a late stage due to resistance to chemotherapeutic drugs. Epithelial mesenchymal transition (EMT) is a biological process in cancer that allows multiple biochemical changes that enable epithelial cells to acquire a mesenchymal phenotype. In the present study, we focused on the EMT process which is an important in carcinogenesis and metastatic progression, and also investigate the effect of silibinin on cell proliferation, colony formation, migration, apoptosis, cell cycle and EMT.

Methods: Cell viability, apoptosis and cell cycle were measured by Muse Cell Analyzer. All the protein levels were determined by ELISA method.

Results: We found that silibinin significantly reduced cell proliferation in a dose-dependent manner and the IC50 value was 200 μM. Silibinin, significantly inhibited colony formation, inhibited cell migration of cancer cells induced total apoptosis due to the induction of early and late apoptosis, arrest cancer cells in the G0/G1 phase of the cell cycle compared to the control group. We found that E-cadherin, N-cadherin, Vimentin and α-SMA protein levels were significantly decreased in the silibinin group compared to the control group.

Conclusions: Our results showed that silibinin could significantly prevent tumor proliferation, reduce colony formation, prevent migration, increase the arrest of the G0/G1 phase and induce apoptosis progress in human extracellular cholangiocarcinoma cell line. Another important data is that silibinin inhibits EMT in the cholangiocarcinoma cell line (TFK-1). Our study shows significant effects of silibinin in the TFK-1 cell line, which may be exciting to explore its implications in future animal studies.

Keywords: apoptosis; E-cad; EMT; N-cad; silibinin; TFK-1

Introduction

Cholangiocarcinoma (CCA) is a hepatic malignant tumor and is known as the second most common after liver cancer [1]. Most of the patients are not eligible for curative surgery due to its late on set clinical symptoms and its high metastatic potential. The incidence of extrahepatic cholangiocarcinoma (eCCA) is more common according to intrahepatic cholangiocarcinoma and early diagnosis is lifesaving in eCCA patients [2]. In this study we focused on eCCA and we used TFK-1 cells, to find out possible effect of silibinin in eCCA cells.

Chemotherapy has always side effects. Therefore, curative drugs with low side effects are always preferred [3]. Silibinin draws attention with its anti-cancer effects along with its healing effects in most cancer studies and has been seriously investigated for the last two decades [4]. Silibinin has been shown to have an anti-cancer activity such as cell cycle arrest, apoptosis induction, and metastasis inhibition in breast, liver, colon, pancreas, endometrium, ovarian, bladder, lung and prostate cancers [5].

Epithelial-mesenchymal transition (EMT) is known as epithelial cells losing their apical-basal polarity and cell-cell adhesions, changing their migration capacity and gaining a more mobile and spindle-shaped mesenchymal-like phenotype [6]. EMT contributes to cancer progression by enhancing metastasis, chemoresistance, and tumor stemness. Because of this crucial role of EMT in tumorigenesis, targeting EMT with therapeutic agents is of great interest in cancer studies [7]. EMT process is characterized by the upregulation of mesenchymal marker N-cadherin (N-cad), Vimentin (Vim), α-SMA followed by downregulation of epithelial marker E-cadherin (E-cad), and this process is
regulated by many signaling pathways and transcription factors [6].

Apoptosis is one of the most studied topics because it is an orchestration of the cellular process necessary for homeostasis that occurs in physiological and pathological conditions. Apoptosis plays a crucial role in many diseases and is important to understand underlying mechanisms [8].

The normal process of cell division occurs via the cell cycle. In the cell cycle, there are regulatory signals that control cell division (G0), Gap 1 (G1), DNA replication/synthesis (S), Gap 2 (G2), and mitosis (M) [9]. However, the deregulation of the cell cycle progression and apoptosis in CCA still remains incompletely understood.

Silycmarin is obtained from the seeds of the Silybum marinus plant, also called milk thistle, which belongs to the Asteraceae family. In the last decade, in vitro and in vivo studies have reported that Silibinin has anti-cancer activity [10, 11].

To our knowledge, this is the first study to evaluate anticancer effects of silibinin in TFK-1 cell line. We focused on the EMT process which is important in carcinogenesis and metastatic progression, and also investigate effect of silibinin on cell proliferation, colony formation, migration, apoptosis, cell cycle and EMT.

Materials and methods

Chemical

Silibinin was obtained from Sigma Aldrich (22888-70-6). RPMI, penicillin-streptomycin, trypsin-EDTA and L-glutamine in the medium used to culture cells were obtained from Biological Industries. DMSO used for negative control was purchased from Merck (Merck KGaA, Darmstadt, Germany). Count&Viability, Annexin V&Dead, Cell and Cell Cycle kits were purchased from Luminox.

Cell lines and culture conditions

The TFK-1 cell line was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures cell bank. TFK-1 was used as the extrahepatic human cholangiocarcinoma cell line. TFK-1 was used as the human cholangiocarcinoma cell line. The doubling time of cell proliferation is 40 h. TFK-1 cells were cultured with RPMI which medium was supplemented with 10 % FBS, 1 % glutamine and 1 % penicillin-streptomycin and passaged according to the appropriate protocol. Cell culture conditions were determined as 5 % CO2 and 37°C. We formed two groups of non-treated (control group) and treated groups (treated with silibinin). Ethics committee approval is not needed since that is a cell culture study.

Cell viability assay

For cell viability, TFK-1 cells were seeded into 6-well plates at 4.0 × 10^5 cells per well. After the density of the cells reached 80 %, different concentrations (5, 10, 25, 50, 75, 100, 200 μM) of silibinin were applied for 48 h to determine the drug concentration. Cell viability was measured by Muse Cell Analyzer, a device based on the flow cytometry principle. The IC50 value was calculated.

Colony forming assay

TFK-1 cells were seeded on 6-well plates with 100 cells per well. After 24 h of incubation, 200 μM silibinin, determined by cell viability test, was applied to the treated group for 48 h. Colony formation was examined under a microscope for about 7 days and the experiment was terminated before the colonies coalesced. At this stage, the medium in each well was aspirated. Colonies were stained with 0.05 % crystal violet and incubated for 20 min for counting. Each well was washed slowly with distilled water and photographed then counted colonies. Each experiment was repeated in three times.

Migration assay

TFK-1 cells were seeded into 6-well plates at 4.0 × 10^5 cells per well. After the density of the cells reached 80 %, 200 μM silibinin was applied to the treated group for 48 h. Then, a pipette tip was used to scratch surface of cells with smooth and equal angles. The time at which the first wound was created was accepted as 0th hour, and the wound width was photographed. Then, wound width was calculated by measuring with the ImageJ program to compare with the control group. Each experiment was repeated in three times.

Apoptosis assay

TFK-1 cells were seeded into 6-well plates at 4.0 × 10^5 cells per well. After the density of the cells reached 80 %, 200 μM silibinin was applied to the treated group for 48 h. Cells were washed 2 times with PBS and trypsin/EDTA was added to the cells and incubated for 7 min. In order to inhibit trypsin/EDTA activation, medium was added on the rapidly released cells and pipetted, and the cells were taken into ependorf. Apoptosis was analyzed by Muse Cell Analyzer device based on the flow cytometry principle. Each experiment was repeated in three times.

Cell cycle analysis

TFK-1 cells were seeded into 6-well plates at 4.0 × 10^5 cells per well. After the density of the cells reached 80 %, 200 μM silibinin was applied to the treated group for 48 h. Cells were washed 2 times with PBS and trypsin/EDTA was added to the cells and incubated for 7 min. In order to inhibit trypsin/EDTA activation, medium was added on the rapidly released cells and pipetted, and the cells were taken into ependorf. After centrifugation, supernatant was aspirated and 70 % ice-cold ET-OH was added to fix the cells on the pellet. After incubation, cell cycle was measured by Muse Cell Analyzer device based on the flow cytometry principle. Each experiment was repeated in three times.

Assessment of EMT proteins concentration

TFK-1 cells were seeded into 6-well plates at 4.0 × 10^5 cells per well. After the density of the cells reached 80 %, 200 μM silibinin was applied to the
treated group for 48 h. Then supernatant was collected and E-cad, N-cad, Vim and α-SMA concentrations were analyzed by ELISA (Bioassay Technology Laboratory E0209Hu, E0305Hu, E1673Hu, E1208Hu) respectively. Non-linear curve was obtained according to standard concentrations and protein concentrations were calculated. Each experiment was repeated in three times.

**Statistical analysis**

SPSS 22.0 statistical software (SPSS Inc, Chicago, IL, USA) was used. Groups were compared by Student’s t-test.

**Results**

**Determination of silibinin concentration by cell viability assay**

The concentration of silibinin was determined by cell viability assay in TFK-1 cells. Silibinin was applied to TFK-1-cells at different concentrations (5, 10, 25, 50, 75, 100, 200 µM) for 48 h. Silibinin decreased the proliferation of TFK-1. The IC$_{50}$ was calculated according to the formula below. IC$_{50}$ value was found to be 200 µM (Figure 1).

$$\text{IC}_{50} = 100 - \left( \frac{\text{non-treated group} - \text{treated group}}{\text{non-treated group}} \right) \times 100.$$

**Effect of silibinin on colony formation in TFK-1 cells**

The effect of silibinin on colony formation was observed under the microscope for 7 days in TFK-1 cells. The experiment was completed before the colonies on the plate merged with each other and the visibility of the colony-forming cells was ensured with crystal violet (Figure 2A). Silibinin significantly reduced the colony-forming compared to control group in TFK-1 cell line (Figure 2B).

**Effect of silibinin on migration in TFK-1 cells**

We observed the effect on migration following applied of 200 µM silibinin to TFK-1 cells. Cells were photographed every 24 h (Figure 3A). Silibinin was not applied to the control group and the wound was observed to be closed at the end of the 48th hour. Wound widths were measured with Image J and the significant change in wound width was shown as graphically. Silibinin significantly reduced migration compared to the control group (Figure 3B).

**Effect of silibinin on apoptosis in TFK-1 cells**

For investigating the effect of silibinin on apoptosis, TFK-1-cells were treated with silibinin 200 µM for 48 h. The early apoptosis rate was determined by the Muse Cell Analyzer by binding the Annexin V protein included in the kit to PS. TFK-1 cells were not stained with 7-AAD because of preserved membrane integrity. As seen in Figure 4a, early apoptosis rate was 11.30 % when the TFK-1 cells were treated with silibinin concentration of 200 µM for 48 h, while in the control group, the apoptosis rate was 1.30 %. Cells were stained with Annexin V and 7 amino actinomycin D (7AAD), and late apoptosis was determined accordingly. The late apoptosis rate was 4.95 % when the TFK-1 cells were treated with silibinin concentration of 200 µM for 48 h, while in the control group, the apoptosis rate was 3.55 %. TFK-1 cells were treated with silibinin concentration of 200 µM for 48 h, silibinin significantly induced total apoptosis consisting of early and late apoptosis (16.25 % of the total apoptotic cell) (Figure 4A). Silibinin increased total apoptosis compared to controls. A comparison graph of apoptosis (early, late and total) percentages in the control group and silibinin-treated group is shown (Figure 4B).

**Effect of silibinin on cell cycle in TFK-1 cells**

The effect of 200 µM silibinin on the cell cycle was measured by Muse Cell Analyzer device based on the flow cytometry principle. The population of cells was arrested in G1 phase (54.2 % compared with 27.7 % in control group). Silibinin significantly increased the G0/G1 phase compared to the control group in TFK-1 cell line. There was no statistically
significant difference in the G2/M phase (Figure 5A). A comparison graph of cell cycle phase percentages in the control group and silibinin-treated group is shown (Figure 5B).

**Effect of silibinin on EMT markers**

In order to evaluate the effect of silibinin on EMT, E-cad (EMT marker in cell adhesion), N-cad (EMT marker in cell adhesion), Vim (EMT marker involved in migration) and α-SMA (mesenchymal marker) levels were investigated in TFK-1 cells. TFK-1 cells were treated with 200 µM silibinin for 48 h. Protein concentrations of EMT markers were determined by ELISA method. Silibinin significantly reduced EMT markers (Figure 6A–D).

**Discussion**

Various studies have been conducted to investigate the cytotoxic effects of silibinin on cancer cells. Silibinin’s anticancer activity has been proven in many studies; It has demonstrated potent antitumor activities against various tumors such as liver, over, colon, lung, breast, prostate and colon cancer by influencing cancer cell growth, proliferation, apoptosis and angiogenesis through multiple signaling pathways [5].

Firstly, we investigated effects of silibinin on cell viability, colony forming, migration in TFK-1 cell line. In the literature, there is no study evaluating the cytotoxic effects of silibinin in the TFK-1 cell line. In our study, we found that silibinin reduced cell proliferation, migration and colony formation in TFK-1 cells in dose of 200 µM. Similar results have been demonstrated in various cancer studies [12–15].

Secondly, we considered the effects of silibinin on cell cycle and apoptosis in TFK-1 cell line. In cancer, one of the main goals to prevent uncontrolled proliferation is to arrest in the G0/G1 phase of the cell cycle and prevent them from going to mitosis. Besides, various cancer studies have emphasized that induction of apoptosis with anticancer agents is one of the anticancer effects [16, 17]. In our study, we observed that silibinin significantly increased G0/G1 phase and induced apoptosis in TFK-1 cells.

Similarly, Sameri et al., have investigated effect of silibinin on CT26 mouse colon cell line and observed that silibinin decreased cell proliferation. The effect of silibinin on apoptosis was also investigated, and it was shown that the proapoptotic markers Bax and caspase 3, which play a role in
Figure 4: (A) Demonstration of the effect of silibinin on apoptosis in TFK-1 cells with the muse device based on the flow cytometry analysis principle. Left: representation of cell population with dot plot and right image of representative event of the four quadrants of the apoptosis; lower left quadrant: viable cells, lower right quadrant: cells in the early stage of apoptosis, right upper quadrant: cell, late stage of apoptosis, and left upper quadrant: cells that die by necrosis (not by apoptotic die). Early apoptosis rate was 11.30 % when the TFK-1 cells were treated with silibinin concentration of 200 μM for 48 h, while in the control group, the apoptosis rate was 1.30 %. Cells were stained with Annexin V and 7 amino actinomycin D (7AAD), and late apoptosis was determined accordingly. The late apoptosis rate was 4.95 % when the TFK-1 cells were treated with silibinin concentration of 200 μM for 48 h, while in the control group, the apoptosis rate was 3.55 %. TFK-1 cells were treated with silibinin concentration of 200 μM for 48 h, silibinin significantly induced total apoptosis consisting of early and late apoptosis (16.25 % of the total apoptotic cell). (B) Silibinin significantly increased the early, late and total apoptosis compared to the control group in TFK-1 cell line (*, p<0.001). Error bars indicate mean ± SD, each experiment was repeated three times.
apoptosis, increase gene expression levels and decrease the antiapoptotic marker Bcl-2 gene expression levels [12].

In another study, Sameri et al., have shown that silibinin reduces colony formation, inhibits cell migration both time and concentration-dependent, and reduce levels of MMP2 and MMP9 which plays a crucial role in migration, invasion and metastasis in a colorectal cancer cell line (HCT-116). Besides, it has been also shown that silibinin increased levels of proapoptotic markers Bax and caspase 3 and decreased level of anti-apoptotic marker Bcl-2 [13].

Shi et al., have investigated effect of silibinin on endometrial cancer cell line and observed that Silibinin inhibits cell proliferation depending on dose and time, decreases the growth of cells and colony formation, affects the cell cycle (keeps cells in G2/M arrest), induces apoptosis, and as a mechanism. It has also thought that silibinin exerts these effects by inhibiting STAT3 and sterol regulatory element-binding protein 1 (SERBP1) signaling pathways [14].

In cancer, one of the most important issues is metastasis [18]. In metastasis phase, cancer cells gain motility, invade locally, and enter the systemic bloodstream, called invasation, to survive in the circulation and thus grow in distant organs [19]. In this complex process, cancer cells lose their epithelial polarity and undergo severe phenotype changes, resulting in a highly mobile and invasive mesenchymal phenotype, a process defined as EMT [20].

Figure 5: (A) Demonstration of the effect of silibinin on cell cycle in TFK-1 cells with the muse cell analyzer device based on the flow cytometry analysis principle. Left: representation of cell population with dot plot and right image of representative event of the three phases of the cell cycle, G0/G1, S, and G2/M quadrants. (B) The population of cells was arrested in G1 phase (54.2 % compared with 27.7 % in control group). Silibinin significantly increased the G0/G1 phase compared to the control group in TFK-1 cell line (*, p<0.001). There was no statistically significant difference in the G2/M phase (p>0.05). Error bars indicate mean ± SD, each experiment was repeated three times.
Thirdly, after demonstrating the antiproliferative effects of silibinin, we evaluated its relationship with the EMT mechanism, which plays an important role in carcinogenesis and metastatic progression. The EMT process is characterized by the loss of membranous localization of the epithelial marker E-cad, as well as a decrease in polarization, and an increased level of N-cad, Vim, fibronectin and α-SMA [21]. We evaluated the effects of silibinin on the level of E-cad, an epithelial marker that plays a role in the EMT pathway, and N-cad, Vim and α-SMA, which are mesenchymal markers, in TFK-1 cells. Silibinin significantly decreased N-cad, Vim and α-SMA protein concentrations compared to the control group. Surprisingly, in our study, silibinin significantly reduced E-cad protein concentration because, in most cancers, decreased E-cad level is associated with oncogenesis [21–23]. On the contrary, a few studies reported that high E-cad levels are associated with tumor aggressiveness and metastasis such as prostate, ovarian and glioblastoma cancers [24–26].

In another study conducted in a renal cancer cell line, it was shown that silibinin increased the expression level of E-cad and decreased the expression of mesenchymal markers N-cad and Vim both in a concentration and time-dependent manner [23]. Similarly, it was shown that the expression level of E-cad increased, and the expression levels of N-cad and Vim decreased as expected, with the administration of low concentrations of silibinin to MDA-MB-231 cells. In addition, it has been also shown that Silibinin decreased MMP2 and MMP9 levels, relation to cell migration inhibition [22].

In most studies, the aim is to reverse the EMT process and inhibit the invasive and metastatic process with anti-cancer agents [21]. Although low levels of E-cad are thought to contribute to EMT, a few studies argue that elevated levels of E-cad, an epithelial marker of EMT, are specifically associated with various invasive and metastatic cancers [24–26]. Depending on the EMT process, it has been reported that the epithelial marker E-cad levels decrease in metastatic cancers [21–23].

Bone metastasis is the last stage of prostate cancer and the expected finding is low E-cad level in metastatic sites of patients with metastatic prostate cancer, but in the prostate cancer study, it has been reported that E-cad expression was mostly increased in the DU-145 cell line (prostate cell line). In addition, in this study, high E-cad expression in metastatic prostate cancer in xenograft modeling was thought to be associated with aggressive tumor growth. A cell line with tighter epithelial cell morphology (high E-cad

Figure 6: (A) Silibinin decreased protein concentration of E-cad. (B) Silibinin decreased protein concentration of N-cad. (C) Silibinin decreased protein concentration of Vim. (D) Silibinin decreased protein concentration of α-SMA (*, p<0.001). Error bars indicate mean ± SD, each experiment was repeated three times.
level) responsible for intercellular association has proven to be associated with poor prognosis in both tumor tissues and xenograft study due to E-cad expression [24].

In another study, Reddy et al., have investigated E-cad which is a well-characterized adhesion molecule in both human normal ovarian tissues and ovarian cancer tissues [25]. It has been observed that E-cad expression is very little or no expression in normal human tissues and E-cad expression has been high in most ovarian epithelial cancers regardless of tumor type, stage of malignancy, or stage of differentiation. Reddy et al., have also investigated whether mitogen-activated protein kinase (MAPK) and phospho-inositol 3-kinase (PI3K)/AKT signaling pathways were associated with the growth and survival of cancer cells [25]. E-cad neutralizing antibodies were applied to the OVCAR-3 cells to reduce E-cad levels by authors [26] and it has been shown that the PI3K and MAPK pathways were blocked and cell proliferation was decreased significantly in OVCAR-3 cells [26]. Reddy et al., have suggested that E-cad may function as a tumor enhancer in the development of ovarian epithelial cancers [25].

In another study conducted in glioblastoma multiforme or glioblastoma (GBM) tissues, Lewis-Tuffin et al., have investigated E-cad expression by immunohistochemistry staining in Glioblastoma multiforme or glioblastoma (GBM) tissues with epithelial/pseudoepithelial differentiation. Tumors that have an E-cad expression have shown to be related to poorer survival overall. E-cad expression is rare in normal central nervous system tissue, and it has been suggested that E-cad levels are associated with worse outcomes in patients with GBM. Lewis-Tuffin et al., have also investigated E-cad levels by constructing a GBM xenograft model. It has been shown that E-cad protein levels were significantly expressed in five cell lines from the group formed as highly/moderately invasive cell lines, and E-cad expression was absent in 10 minimally/non-invasive cell lines group by Western blotting method. It has been thought that E-cad maybe contributes to GBM tumor aggressiveness due to E-cad expression being higher in more invasive xenograft lines [26].

In our study, the reduction of E-cad levels by silibinin in the TFK-1 cell line may have decreased independently of the EMT process. As mentioned in the above studies [24–26] the reduction of E-cad levels by silibinin may be an antimitastatic feature of silibinin rather than a negative indicator of EMT. In addition, as we have shown in our results may indicate that silibinin has a positive effect on EMT through the decreased levels of N-cad, Vim and α-SMA in TFK-1 cell line.

There are some limitations to our study: (1) We could not evaluate the cyclin dependent kinase proteins, cell cycle related phase (2) We could not evaluate the apoptotic markers of BCL-2 proteins or PS.

In conclusion, our results showed that silibinin could significantly prevent tumor proliferation, reduce colony formation, prevent migration, increase the arrest of the G0/G1 phase and induce apoptosis progress in human extracellular cholangiocarcinoma cell line. Another important data is that silibinin inhibits EMT in the TFK-1 cell line. Our study shows significant effects of silibinin in the TFK-1 cell line, which may be exciting to explore its implications in future animal studies.

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References


