



Silibinin Upregulates E-Cadherin Expression in MKN-45 Human Gastric Cancer Cells

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Abstract

Background and objectives: Gastric cancer is currently known as one of the most important causes of cancer-driven death all over the world. In patients with gastric cancer, a significant proportion of death occurs due to metastasis. On the other hand, down modulated E-cadherin level has been reported as an important contributor to tumor cell invasion and metastasis. In this regard, the present work was aimed to evaluate the impact of silibinin, a flavonolignan with established anti-tumor efficacy, on cell viability and E-cadherin expression in the gastric cancer cell line MKN-45. **Methods:** To determine cell viability, MTT assay was performed 48 h after silibinin treatment (at concentrations of 100, 200 and 400 μ M). In addition, quantitative real-time PCR was done following total RNA extraction and cDNA synthesis, to assess E-cadherin level in cells treated with silibinin. **Results:** The MTT results showed concentration-dependent reducing effect of silibinin on viability of MKN-45 cells. The findings of quantitative real-time PCR analysis demonstrated upregulated E-cadherin expression in cells treated with silibinin (significantly ($p \leq 0.05$) at concentration of 200 μ M) compared to the control cells. **Conclusions:** The current study suggested that silibinin may exert anti-migratory/invasive effects on gastric cancer cells by enhancing E-cadherin expression, which needs to be further investigated.

Keywords: E-cadherin; gastric cancer; MKN-45; silibinin

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Introduction

The epithelial-to-mesenchymal transition (EMT) phase is increasingly known as a hallmark of epithelial cancers metastasis by which a cancer cell breaks away from cancerous tissues and can migrate to other parts of the body [1]. Downregulation or loss of function of adhesion molecules, in particular E-cadherin protein, has been reported as a distinct biological feature underlying EMT events [2]. E-cadherin is a key molecule in epithelial cell-cell adhesion, acting as a tumor suppressor protein, whose loss of expression is associated with tumor progression

and metastasis [3,4]. Growing evidence indicates that E-cadherin can be downregulated or inactivated by mechanisms such as gene mutation, epigenetic silencing and transcriptional changes [4-6]. Histone deacetylation and DNA methylation are both of the most important epigenetic mechanisms involved in downregulating E-cadherin expression in malignancies, which are mediated by Histone deacetylase (HDAC) and DNA methyltransferase (DNMTs), respectively [7-9]. HDAC enzymes remove the acetyl groups on the lysine residues

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of histone tails, increasing positive charge of histone proteins and encouraging these proteins to wrap the DNA more tightly, and therefore, preventing gene expression [10]. Furthermore, DNA methyltransferases (DNMTs) hyper methylate the gene promoter regions leading to loss of gene expression [11]. Hence, in this way, preventing the E-cadherin downregulation via HDACs suppression could be important towards lowering metastasis rate and tumor invasion in patients with cancer. Nowadays, histone deacetylase inhibitors have emerged as attractive targets for the development of novel antitumor agents [12]. Histone deacetylase inhibitors (HDACIs) have been grouped into five classes, comprising short chain fatty acids, organic hydroxamic acids and trichostatin, sulfonamide anilides, benzamides, and cyclic tetrapeptides [13].

Silibinin is a natural flavonolignan with established anti-cancer properties, which has been reported to have HDAC inhibitory activities. Silibinin ($C_{25}H_{22}O_{10}$), with a molecular weight of 482.44 g/mol, acts as the main biologically active component of a standardized milk thistle (*Silybum marianum*) extract, namely sylimarin, a combinatory mixture of flavonolignan and flavonoid ingredients including silibinin (50-60%), silichristin (20%), silidianin (10%), isosilibinin (5%), taxifolin and quercetin, and also other components such as polyphenols and aliphatic fatty acids [14]. Milk thistle, with a long history of prescription in folk medicine, has been numerously used for prevention and treatment of liver diseases such as viral hepatitis, alcohol abuse-mediated cirrhosis, drugs & industrial toxins-associated liver damages [15]. Silibinin a 1:1 mixture of two diastereoisomers (silybin A and silybin B) has been long applied in medicine, so that it has been reported to have no significant toxicity in humans and animals [16]. Previous investigations have indicated that silibinin plays several antitumor roles against some epithelium-derived malignancies (e.g. colon, prostate, and lung cancer), which may be mediated by mechanisms such as induction of tumor cell autophagy [17].

In this context, in the present study, the anticancer properties of the histone deacetylase inhibitor silibinin regarding the expression of E-cadherin molecule has been evaluated by its administration in gastric cancer cell line MKN-45.

Material and Methods

Cell culture

Gastric cancer cell line MKN-45 was cultured in Dulbecco's modified Eagle's medium (DMEM), containing 100 µg/ml penicillin; 100 µg/ml streptomycin; 10% heat-inactivated fetal calf serum (FCS), and then, incubated at 37 °C in a humidified 5% CO₂ incubator.

Drug treatment and proliferation assay

Performing this, silibinin (Sigma, MO, USA) was purchased, and different concentrations (100, 200 and 400 µM) were provided by dissolving silibinin in dimethyl sulfoxide (DMSO). Subsequently, for experimental treatments, a suspension of MKN-45 cells with a density of 5×10^4 cells per well was plated, and after 24 h incubation, the cells were treated by the prepared concentrations of silibinin for 48 h. Finally, 200 µL per well of MTT solution was added at concentration of 0.5 mg/mL, and the plate was further incubated for 4 h at 37 °C. The results were obtained by measuring the absorbance at 570 nm.

RNA extraction

MKN-45 cells were plated in 12 well culture plates and after gaining a confluency of 80%, and treating with the concentrations of 0, 100, and 200 µM of silibinin for 48 h. The cells treated with DMSO were considered as controls. The concentration of silibinin and the treatment time were chosen based on the MTT assay results. To obtain reliable results, these experiments were carried out in triplicate, therefore, real-time PCR analysis were done using 3 samples regarding each experiment. Afterwards, by using a TriPure total RNA isolation Kit (Roche Applied science, Indianapolis, USA), total RNA was obtained from cultured cells, which subsequently was further analyzed by spectrophotometry (Eppendorf, Germany) to assess the concentration, integrity, and purity.

cDNA synthesis

To synthesize cDNA by using M-MuLV reverse transcriptase enzyme (Fermentas, Lithuania), 1 µL of the extracted RNA (the RNA with 280/260 nm ratio between 1.8 -2), 2 µL of 10 mM dNTP, 1 µL of random hexamer, 1 µL of M-MuLV enzyme (20 u/µL), and 0.5 µL of RNase inhibitor (Vivantis Technologies, Malaysia) were mixed, followed by adjusting the total volume to 20 µL

using sterile distilled water. Thereafter, the mixture was incubated at room temperature and then at 42 °C for 10 min and 1 h, respectively. Additionally, a further incubation period (10 min at 70 °C) was applied to inactivate the reverse transcriptase enzyme; M-MuLV.

Then, real-time PCR analysis was performed using the synthesized cDNA, after diluting 10 times with distilled water.

Quantitative real-time PCR

A SYBR green master mix (Premix Ex Taq II kit provided by TAKARA Bio INC., Otsu, Japan) was applied to quantify E-cadherin mRNA levels via quantitative real-time PCR assay. Performing the reactions, E-cadherin gene (Forward primer: 5'-AGGGGTAAAGCACAACAGCA-3', Reverse primer: 5'-CTTAGCCTCTCCATCGGACT-3'), and GAPDH gene primers (Forward primer: 5'-ATGTTTCGTCATGGGTGTGAA-3', Reverse primer: 5'-GGTGCTAAGCAGTTGGTGGT-3') were used to prepare the reaction mixture containing the following materials in a total volume of 20 μ L; SYBR premix Ex Tag II (10 μ L), ROX reference dye (0.4 μ L), primer pairs (0.8 μ L), and the diluted cDNA (2 μ L). Amplification was done at 95 °C (1 min), and then, 40 cycles at 95 °C (15 s), 55 °C (30 s), and 72 °C (30 s). Quantity of expressed E-cadherin mRNA in each sample was normalized to that of GAPDH, as an endogenous housekeeping gene, using the $2^{-\Delta\Delta Ct}$ method.

Statistical analyses

Statistical analysis was carried out using Graph-Pad Prism software; data was analyzed using the unpaired, two-tailed student's t and ANOVA tests. Moreover, statistically significant differences were considered at $p \leq 0.05$.

Results and Discussion

The present study was performed to determine the effect of silibinin on the viability of MKN-45 cells, by measuring the amount of living cells at concentrations of 0, 100, 200 and 400 μ M of silibinin 48 h after treatment, in a well-established viability assay; MTT assay. The results have been shown in figure 1. Cell viability in 200 μ M Silibinin was approximately high (more than 50%). As illustrated, silibinin treatment decreased the viability of MKN-45 cells in a concentration-dependent manner. Real-time PCR analysis of control and treated MKN-

45 cells showed that silibinin treatment resulted in a concentration -dependent increase in E-cadherin expression levels in silibinin-treated cells 48 h after treatment; indeed, the expression level of E-cadherin mRNA at concentration of 200 μ M (in contrast to that of 100 μ M) was significantly higher than control MKN-45 cells, which did not receive silibinin ($p \leq 0.05$) (figure 2).

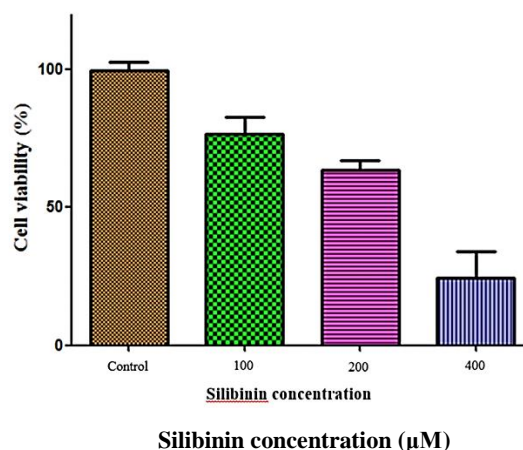


Figure 1. Silibinin treatment decreased the viability of MKN-45 cells in a concentration-dependent manner. The cells were treated with DMSO (control) or by 100 or 200 and 400 μ M silibinin.

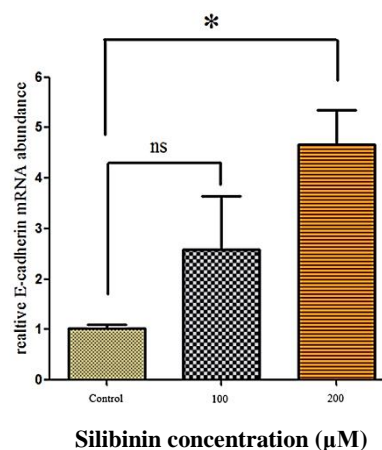


Figure 2. Silibinin treatment increased E-cadherin mRNA expression in MKN-45 cells, 48 hours after treatment. The cells were treated with DMSO (control) or by 100 or 200 μ M silibinin and were collected 48 h after exposure. Real-time PCR analysis represented the highest level of the E-cadherin expression at concentration of 200 μ M of silibinin. The experiments were performed in triplicate. * $p = 0.0346$, ns; no significant.

Cadherins are a prominent class of cell-adhesion molecules which are well-known for regulating cell-to-cell and cell-to matrix adhesion [18]. E-

cadherin is a prototypical member of cadherin family that is localized at the basolateral membrane of epithelial cells [19]. Physiologically, this calcium-dependent adhesion molecule plays a critical role in various processes such as embryonic development, epithelial cell behavior and tumor suppression [20]. Nonetheless, it has been shown that loss of function of E-cadherin is mostly associated with tumor invasiveness and metastasis [21]. Therefore, enhancing E-cadherin expression by utilizing agents such as non-toxic phytochemicals could be regarded as an ideal strategy towards reducing tumor invasiveness and cancer metastasis-caused mortality. In the present study, the effects of a natural compound, silibinin, on a gastric cancer (GC) cell line, known as MKN-45, have been investigated by assessing cell viability and E-cadherin expression. Silibinin is known as a useful antioxidant that mediates its function by scavenging hydroxyl radicals and suppressing lipid peroxidation via playing a chain breaking antioxidant role. In addition to this function and hepatoprotective effects, it has also been reported to have considerable anti-tumor as well as cancer chemopreventive potentials in pre-clinical of some malignancies (e.g. skin, bladder, colon, prostate and lung tumors) [15].

Gastric cancer (GC) is one of the most leading causes of cancer-mediated death, globally [22, 23]. In majority of patients with GC, death occurs owing to tumor metastasis to various anatomical sites, particularly the peritoneal membrane [24, 25], which demands more investigations to develop novel antitumor agents towards preventing this process. The findings of the current study showed that silibinin treatment could enhance E-cadherin expression, and on the other hand, decrease cell viability in this gastric cancer cell line. Indeed, the results of MTT assay indicated that silibinin functions in a concentration-dependent manner; it exerts an inhibitory effect on the proliferation of MKN-45 cells, more efficiently at higher concentrations (400 >200 >100). Furthermore, the E-cadherin expression data from the present study clearly showed that silibinin treatment (at low non-cytotoxic doses) resulted in a concentration-dependent increase in E-cadherin mRNA levels in gastric cancer cell line. The E-cadherin findings are in accordance with the data reported by previous investigations suggesting that silibinin treatment leads to enhanced E-cadherin expression in other tumor cell lines, in vitro

assays [26,27]. In a study by Deep et al., it was shown that silibinin administration could prevent the migratory/invasive potentials of prostate cancer (PCA) cells, in-part via upregulating E-cadherin expression [26]. Additionally, silibinin also down modulated the levels of factors involved in E-cadherin expression and function, such as Slug, phospho-Akt, Snail, phospho-Src, Hakai and nuclear β -catenin. Similarly, Mateen et al. evaluated the effect of silibinin and epigenetic drugs (HDAC or DNMT inhibitor) on different sub-types of non-small cell lung cancer (NSCLC) cells (H1299, H460 and H322) [27]; silibinin did not increase E-cadherin levels in NSCLC H1299 cells re-expressed E-cadherin in NSCLC H322 and H358 Cells, but also concomitantly decreased Zeb1 levels, a transcriptional repressor of E-cadherin molecule. Their findings also demonstrated that silibinin treatment alone prevented the migratory/invasion potentials of H322 cells, and that it synergized with HDACIs and DNMTIs towards increasing E-cadherin levels. In contrast to these observations, the exact molecular targets underlying silibinin's mechanism of action remain to be fully defined. However, various factors have been shown to be modulated by silibinin treatment which may be responsible for the anti-cancer efficacy of silibinin; for instance, as mentioned earlier, silibinin has been demonstrated to decrease multiple transcriptional factors (e.g. Zeb1, Snail, Slug, Twist etc.), which down modulate E-cadherin mRNA expression levels [26,27]. Silibinin also reduces phosphorylated-Src and Hakai, which predispose E-cadherin molecules to lysosomal-induced proteolysis [26]. Further, as was shown in Mateen et al. study, silibinin re-expressed E-cadherin in NSCLC cells, which varied in their basal level of E-cadherin [27]. The importance of this observation is on the basis of the fact that E-cadherin is predominantly down modulated in NSCLC cell types owing to epigenetic modulations (through HDACs and DNMTs) [27], and that silibinin has been shown to play HDAC/DNMT inhibitory roles [14,28]. However, more investigation should be done to clearly elucidate the molecular mechanisms underlying silibinin function. Taken together, these findings show that silibinin has exerted diverse effects on different cell lines, but it could be concluded that silibinin has anti-cancer potency at doses in which it exerts minimal cytotoxicity against MKN-45 cells.

In fact, in the present study, we showed that silibinin increased E-cadherin in gastric cancer cell line, and given the anti-migratory/invasive roles of E-cadherin, it seems possible that silibinin could limit the migratory and invasive potentials of MKN-45 cell line which should be more investigated in future studies.

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Author contributions

Ebrahim Faghihloo performed the experiments and wrote the paper. Habibollah Mirzaei performed the experiments. Abolfazl Akbari analyzed the data, Hossein Goudarzi revised this article.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

EMT; Epithelial-to-mesenchymal transition; HDAC: Histone deacetylase; DNMT: DNA methyltransferases; HDACI: Histone deacetylase inhibitor; DMEM: Dulbecco's modified Eagle's medium; MTT: Methyl-thiazol-tetrazolium; FBS: Fetal bovine serum; DMSO: Dimethyl sulfoxide; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PCR: Polymerase chain reaction; cDNA: Complementary deoxyribonucleic acid; GC: Gastric cancer; PCA: Prostate cancer; NSCLC: non-small cell lung cancer