Research Article

Gülden Başkol, Merve Özel*, Çiğdem Uçar, Büşra Nur Doğru, Esra Hilal Yüksek, Fatma Güneş and Mevlüt Başkol

SAHA modulates cell proliferation, colony forming and epithelial-mesenchymal transition in CCA cells

SAHA, CCA hücrelerinde hücre proliferasyonunu, koloni oluşumunu ve epitelyal mezenkimal geçişi modüle eder

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Abstract

Background: Therapeutic options for advanced cholangiocarcinoma (CCA) are limited and ineffective due to the largely incomplete understanding of the molecular pathogenesis of this deadly tumor. So that, we planned to investigate epigenetic regulation of epithelial-mesenchymal transition (EMT) in cholangiocarcinoma cell line by applying Suberoylanilide hydroxamic acid (SAHA). We studied the effect of SAHA on cell proliferation, colony forming, migration and protein level of E-cadherin (E-cad) as an epithelial EMT marker, N-cadherin (N-cad) and Vimentin (Vim), as a mesenchymal markers of EMT, in human CCA cell line.
Materials and methods: Cell proliferation and migration measurements were performed by flow cytometry and wound healing assay, respectively. E-cad, N-cad and Vim protein levels were determined by Western blot analysis.
Results: It was found that SAHA significantly inhibits cell viability, proliferation and migration of TFK-1 cells, accompanied by reversing of EMT markers. SAHA, upregulated protein level of E-cad, while downregulated the protein levels of N-cad and Vimentin.
Conclusions: SAHA treatment may bebeneficial for CCA patients and SAHA might be a potential therapeutic agent for the treatment of CCA. However, future studies are needed to evaluate the clinical applicability of SAHA as a part of the chemotherapeutic regimen for CCA.

Keywords: Cholangiocarcinoma; Suberoylanilide hydroxamic acid; Epithelial-mesenchymal transition; Epigenetic; E-cadherin.

Öz

Giriş: İlerlemiş kolanjiyokarsinoma (CCA) için tedavi seçenekleri sınırlıdır ve bu ölümcül tümörün moleküler patogenezinin büyük ölçüde anlaşılamaması nedeniyle etkisizdir. Böylece, suberoilanilid hidroksamik asit (SAHA) uygulayarak kolanjiokarsinoma hücre hattındaki epitelyal mezenkimal geçişin (EMT) epigenetik düzenlenmesini araştırmayı planladık. İnsan CCA hücrelerinde; SAHA'nın hücre proliferasyonuna, koloni oluşumuna, migrasyona ve epitelyal belirteç olan E-kaderin (E-kad) protein seviyesi, mezenkimal belirteç olan N-kaderin (N-kad) ve Vimentin (Vim) protein seviyesine olan etkisini araştırdık.
Materyal ve metod: Hücre canlılığı ve hücre göçü ölçümleri sırasıyla, flow sitometri ve yara kapanması testi ile gerçekleştirildi. E-kad, N-kad ve Vim protein seviyeleri Western blot analizi ile belirlendi.
Bulgular: SAHA'nın, EMT belirteçlerini tersine çevrilmesi ile birlikte, hücre canlılığını, hücre proliferasyonunu ve TFK-1 hücrelerinin migrasyonunu önemli ölçüde inhibe

***Corresponding author: Merve Özel,** PhD Student, Department of Biochemistry, Erciyes University School of Medicine, Kayseri, Turkey, Phone: 05428924774, e-mail: ozelm381@gmail.com.
https://orcid.org/0000-0002-0434-9731
Gülden Başkol: Department of Biochemistry, Erciyes University School of Medicine, Kayseri, Turkey; and Betül-Ziya Eren Genome and Stem Cell Center, Erciyes University, Kayseri, Turkey
Çiğdem Uçar, Büşra Nur Doğru, Esra Hilal Yüksek and Fatma Güneş: Department of Biochemistry, Erciyes University School of Medicine, Kayseri, Turkey
Mevlüt Başkol: Department of Gastroenterology, Erciyes University School of Medicine, Kayseri, Turkey

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ettiği bulundu. SAHA E-kad protein seviyesini artırırken, N-kad ve Vim protein seviyesini azaltmıştır.
Sonuç: SAHA tedavisi CCA hastaları için yararlı olabilir ve SAHA CCA tedavisinde potansiyel bir terapötik ajan olabilir. Bununla birlikte, CCA için kemoterapötik tedavinin bir parçası olarak SAHA'nın klinik uygulanabilirliğini değerlendirmek için gelecekteki çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Kolanjiokarsinoma; Suberoilanilid hidroksamik asit; Epitelyal mezenkimal geçiş; Epigenetik; E-kaderin.

Introduction

Cholangiocarcinoma (CCA) is the second most common primary hepatic cancer, after hepatocellular carcinoma. It is common in elderly patients. The peak incidence is in the seventh decade of life [1]. The majority of patients with cholangiocarcinoma are diagnosed with advanced stage of diseases. Curative surgery is the only treatment modality in early stage cases unfortunately tumor progresses insidiously in most cases and the majority of the patients lose the chance of curative surgery because of metastasis. In advance stage of the CCA chemotherapy is another therapeutic option nevertheless, chemoresistance is an important problem in CCA [2]. Mechanisms of metastasis has not been clearly defined yet but it has been revealed that cancer cells must have acquire migratory and invasive properties by a cell plasticity-promoting phenomenon known as epithelial-mesenchymal transition (EMT) to metastasize from the primary origin [3].

EMT is a biological process that allow a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of extracellular matrix components (ECM) [4–6].

Pathogenesis of CCA is not well defined. Mesenchymal transition of epithelial tumor cells may contribute to the invasion ability and chemoresistancy of CCA [7, 8]. Changes in gene expression levels occur during EMT including down-regulation of epithelial markers such as E-cadherin (E-cad) and up-regulation of mesencyhmal markers such as vimentin (Vim) and N-cadherin (N-cad). EMT is fundamental in many physiological processes, such as embryogenesis and wound healing. Many clinical studies have been reported that EMT expressions correlated with an increased metastatic potential and poor clinical outcome [4, 5].

The neoplastic transformation of biliary epithelial cells and malignant progression of CCA has genetic and epigenetic alternations. The concept of epigenetic therapy is now well-established in the field of oncology, with the successful clinical application of histone deacetylases (HDACs) described for many types of cancers [9–11]. Suberoylanilide hydroxamic acid (SAHA) is a known HDAC inhibitor that shows anticancer activity by relieving gene silencing against hematologic and solid tumors [12]. SAHA is currently being after investigated a number of clinical trials. SAHA is approved as HDAC inhibitor by FDA to treat cutaneous T-cell lymphoma Cell culture studies have shown that SAHA inhibits cell proliferation and induces apoptosis and cell cycle arrest in CCA cell lines [13]. In other study has been shown to HDAC inhibitor of SAHA reduce cell proliferation in CCA cells (M213, M214 and KKU-100) [14]. Although SAHA is widely accepted as an HDAC inhibitor, it has been reported to suppress EZH2 expression in cancer cells and to exert an anticancer effect, indicating that SAHA also functions as an EZH2 inhibitor [15]. Overexpression of EZH2 positively correlates with the progression of multiple malignancies including breast cancer, lymphoma, myeloma, colorectal cancer, endometrial cancer, bladder cancer and prostate cancer [16]. However, the potential effect for in vitro SAHA on EMT has not been investigated in CCA yet.

Therapeutic options for advanced CCA are limited and ineffective due to the largely incomplete understanding of the molecular pathogenesis of this deadly tumor. So that, we planned to investigate epigenetic regulation of EMT in cholangiocarcinoma cell line by applying SAHA. We studied the effect of SAHA on cell proliferation, colony forming, migration and protein level of E-cad as an epithelial EMT marker, N-cad and Vim, as a mesenchymal markers of EMT, in human CCA cell line.

Materials and methods

Chemicals

SAHA was obtained from Cayman Chemical (MI, USA). Count and Viability kit (Merck Millipore, Billerica, MA, USA, cat. no. MCH 100102) was obtained from Merck Millipore. E-cad, N-cad, Vim, enhancer of zeste homolog 2 (EZH2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Tubulin were purchased from Proteintecht (Chicago, IL, USA). Radioimmunoprecipitation assay (RIPA) buffer, Phosphatase Inhibitor Cocktail3, Phosphatase Inhibitor Cocktail2 were obtained from SIGMA Chemical Co. (Darmstadt, Germany).

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Cell lines and culture conditions

We used cholangiocarcinoma cell line TFK-1. TFK-1 cell line was purchased from DSMZ. Those cells were seeded in RPMI-1640 medium. Medium was supplemented with 10% FBS and 1% penicilin-streptomycin. The stock concentration of SAHA was determined as 1000 μM and dimethyl sulfoxide (DMSO; BioChemical) was used as the negative control. Cell line was treated with 2 μg/mL of the HDAC inhibitor SAHA for 48 h. Ethics committee approval is not needed.

Cell viability analysis

Cells were cultured in a six-well plate and treated with different doses of SAHA (1–10 μM). After 48 h of incubation, cells were harvested by trypsin. Then cells were washed with sufficient PBS. The samples were then stained with 450 μL Count and Viability Assay kit at room temperature for 5 min in darkness. Using a flowcytometry. IC_{50} value was calculated by Graph Pad Prism Software. The IC_{50} value was determined as to be 2 μM according to this formula;

$$IC_{50} = 100 - [(\text{control} - \text{treated group})/\text{control} \times 100]$$

(control is cell number in nontreated group, treated group is the number of cells in the SAHA-treated group at different concentrations).

Colony forming assay

Cells were cultured in a six-well plate and treated with 2 μM SAHA (500 cells per well) plates. The culture medium was replaced every 2 days. After 10 days colonies were fixed in 10% formaldehyde, stained with crystal violet solution and then counted.

Wound healing assay

TFK-1 cells were seeded in six wells and grown in duplicate as 2.5×10^5 cells in each well. When the cells reached 80–90% density, normal medium was added to the control group and SAHA at the dose of 2 μM was added to the SAHA group. After 48 h in this way, a 200 μL pipette tip was wound on the cells. When the wound was created, it was taken as 0 h and the image was taken with a camera microscope. Then, the cells were left in normal medium until the wound in the control group was closed, and images were taken every 24 h with a camera microscope. The wound widths pixel were then measured using the Image J (National Institute of Mental Health, MA, USA).

Western blot analysis

The culture medium was removed from the TFK-1 cells. The TFK-1 cells were washed with PBS twice and lyzed in lysis buffer. (400 μL RIPA Buffer; 5 μL Phosphatase Inhibitor Cocktail 3; 5 μL Phosphatase Inhibitor Cocktail2, SIGMA Chemical Co). Lysis buffer was performed on ice cold for 5 min. Then, the TFK-1 cells were harvested. A standart curve was occured using albümin (1.35 mg/mL) to determine the protein concentration by Bradford method of TFK-1 cells. Equal amounts of protein (40–45 μg) were loaded, resolved on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% non-fat dry milk for 1 h at room temperature. Then washed three times 5 min each time with tris-buffered saline with Tween 20 (TBS-T). The membrane was incubated overnight at 4°C with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:1000; BIO-RAD cat. no. 170-6515) for 1 h at room temperature. Immunoreactive bands were probed with the enhanced chemiluminiscence (ECL) western blot detection system according to manufacturer's instructions and viewed in gel documentation system. All the bands were quantitated by image software.

Statistical analysis

SPSS 19.0 statistical software was used for statistical analysis. Values are shown as the mean ± SD. Groups were compared by Student's t-test. The values of $*p < 0.05$ were considered significant.

Results

Effect of SAHA on cell viability

We first investigated effective concentration of SAHA on TFK-1 cell line. The cells were treated with SAHA for 48 h at concentrations of 1, 2.5, 5 and 10 μM. We showed that SAHA treatment markedly reduced TFK-1 cell viability in

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a dose-dependent manner. Two micromole SAHA at IC_{50} was selected as application dosage for the study (Figure 1).

Effect of SAHA on colony forming assay

We next examined the effects of SAHA on colony formation in TFK-1 [14]. In this assay, SAHA treated cells are contact inhibited and do form statistically less colonies than non-SAHA treated cells; thus, the assay evaluate the neoplastic tendency of cancer cells. Colony forming assay of TFK-1 cells showed that 48 h treatment with 2 μM SAHA markedly decreased the colony numbers compared with control (Figure 2).

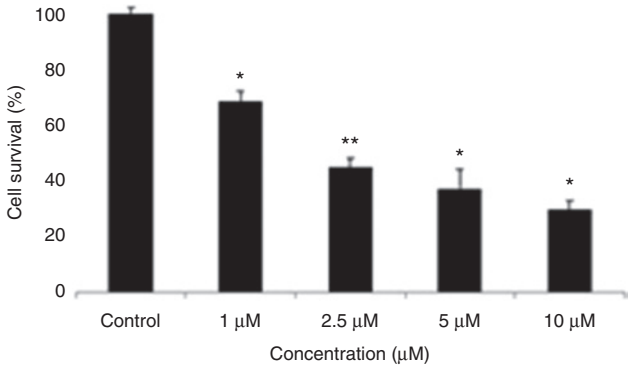


Figure 1: Cells performed with the Count and Viability Assay kit after 48 h of treatment with SAHA.
The effect of SAHA on the viability of TFK-1 cells was determined using the Count and Viability Assay kit. Different concentrations of SAHA (1 μM, 2.5 μM, 5 μM, 10 μM) were treated to TFK-1 cells for 48 h. There was a decrease in the number of TFK-1 cells due to dose dependent in SAHA administration. The IC_{50} value was determined as 2 μM. The asterisks indicate statistical significance *, $p < 0.05$; **, $p < 0.01$.

Effect of SAHA on migration of TFK-1 cells

To assess whether SAHA is involved in cell migration of TFK-1 cells, we performed an in vitro scratch wound healing assay. Two micromole SAHA was used to demonstrate the effect of SAHA on the migration of TFK-1 cells. Images were taken every 24 h with a camera microscope (Figure 3A). Since there was no agent inhibiting cell migration in the control group, it was seen that the wound was closed at the end of 144 h. In the SAHA group, cell migration was inhibited and the wound was not closed. Wound widths were measured and the significant change in wound width with time was plotted in Figure 3B. In conclusion, SAHA has been shown to suppress migration ability of TFK-1 cells.

Effect of SAHA on E-cadherin, N-cadherin and Vim protein levels

As indicated by the data in Figures 3 and 4, SAHA plays a critical role in EMT in TFK-1 cell line. We examined whether inhibition of SAHA leads to inhibition of signaling pathways of EMT in vitro. Cell lysates were collected 48 h after treatment with SAHA. Lysates were subjected to western blot analysis. SAHA, upregulated protein level of E-cad, while downregulated the protein levels of N-cad, Vimentin (Figure 4A–C).

Effect of SAHA on EZH2

It has been reported that SAHA suppressed EZH2 protein level in human cancer cells [15]. EZH2 enhances

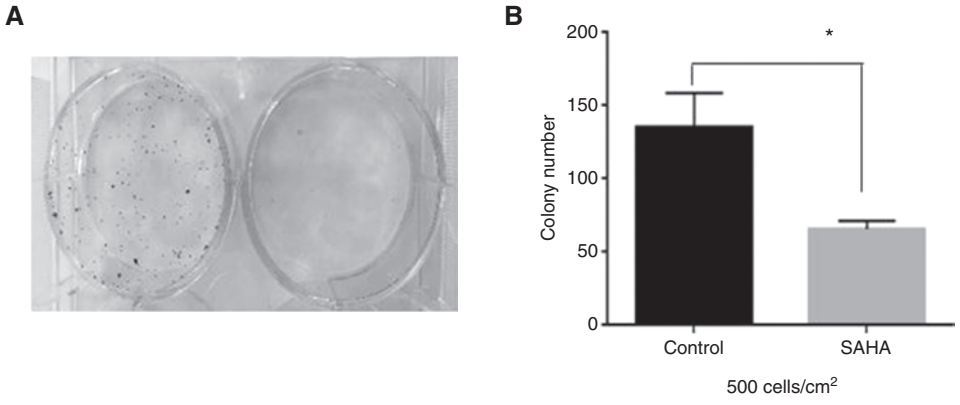


Figure 2: The effect of SAHA on colony formation of TFK-1 cell line.
(A) Colony forming assay of TFK-1 cells showed that 48 h treatment with 2 μM SAHA markedly decreased the colony numbers compared with control. Experiment was done in triplicate. (B) Statistical analysis of colony formation in TFK-1 cell line. The asterisk indicates statistical significance *, $p < 0.01$ ($p < 0.008$).

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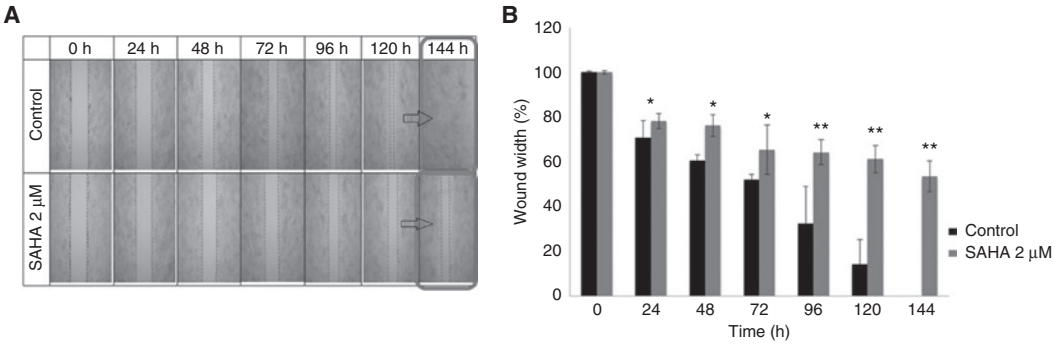


Figure 3: Demonstration of SAHA's effect on migration in TFK-1 cells.
(A) Cells were visualized with Phase-Contrast Microscopy (X4) and follow up until the wound in the control group was closed. (B) The wound width was measured and compared with the initial wound width and the wound healing percentage was quantified. Values were found statistically significant with respect to control. Experiment was done in triplicate. The asterisks indicate statistical significance*, $p < 0.05$; **, $p < 0.01$ (24 h $p < 0.002$, 48 h $p < 0.003$, 72 h $p < 0.009$, 96 h $p < 0.001$, 120 h $p < 0.001$, 144 h $p < 0.000$).

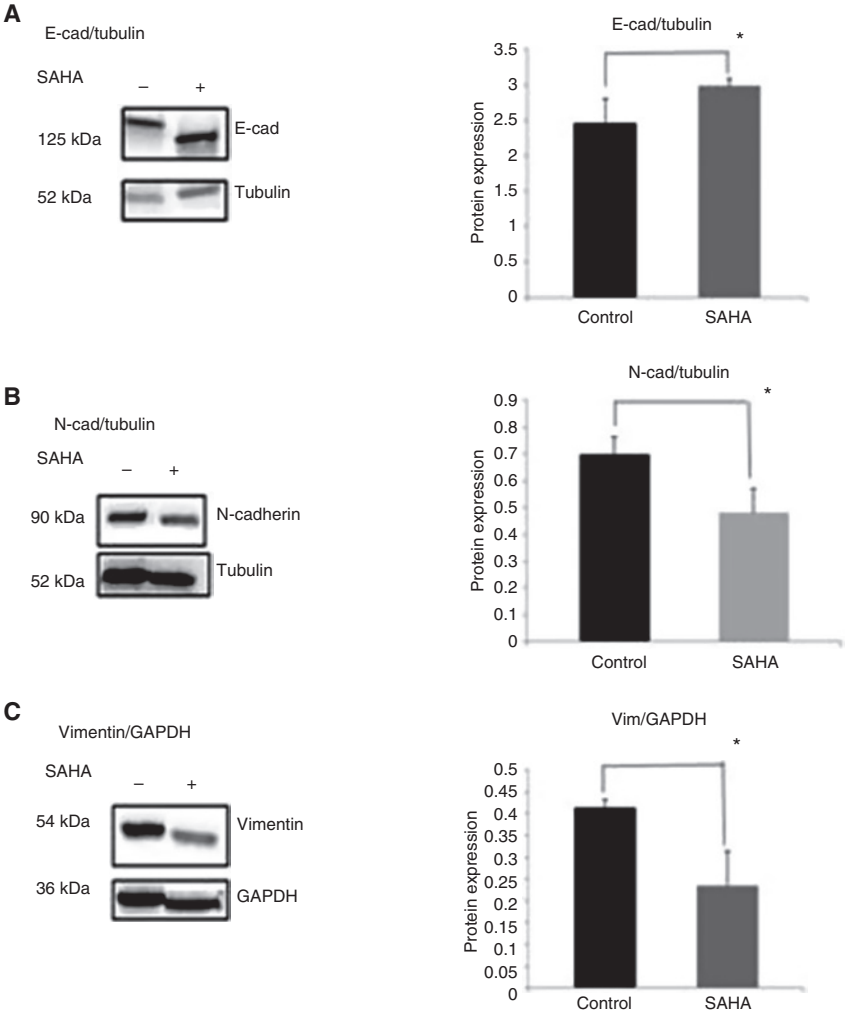


Figure 4: E-cad, N-cad and Vim protein levels were determined by Western blot analysis.
(A) Tubulin and GAPDH were used as a loading control. SAHA upregulated protein level of E-cad. Experiment was done in triplicate. The asterisks indicate statistical significance*, $p < 0.05$; (E-cad $p < 0.026$). (B) N-cad protein level was determined by Western blot analysis. Tubulin and GAPDH were used as a loading control. SAHA down regulated N-cad protein level. Experiment was done in triplicate. The asterisks indicate statistical*, $p < 0.05$; (N-cad $p < 0.008$). (C) Vim protein level was determined by Western blot analysis. Tubulin and GAPDH were used as a loading control. SAHA down regulated protein level of Vim. Experiment was done in triplicate. The asterisks indicate statistical significance*, $p < 0.05$; (Vim $p < 0.004$).

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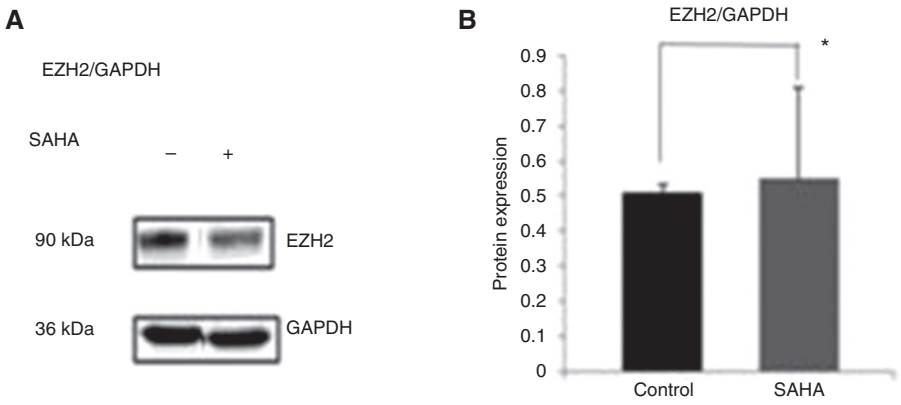


Figure 5: Protein level of EZH2 was determined by Western blot analysis.
(A) GAPDH was used as a loading control. Experiment was done in triplicate. (B) SAHA did not change to EZH2 protein level (*$p > 0.05$).

tumorigenesis and is commonly overexpressed in several types of cancer [16]. So, we further wondered the effects of SAHA on EZH2 protein level in TFK-1 cell line. SAHA did not affect EZH2 protein level (Figure 5).

Discussion

HDACs have been reported as one of the epigenetic mechanisms associated with tumorigenesis. Many of signaling pathways which regulate critical processes such as cell growth, survival, differentiation and migration are altered in cancer cells. Understanding of the molecular changes that accompany cell transformation led to the development of anticancer targeted therapy [10–12].

SAHA is used in clinical research for cancer treatment. The ability of SAHA to inhibit HDAC activity and thereby regulate gene transcription has been linked to its ability to inhibit growth and induce apoptosis in several types of malignant cells in vitro. However, many of the mechanisms underlying the effects of this agent on cell growth, differentiation, and death have not been fully clarified [12].

Metastatic potential of several carcinomas including breast [17], pancreatic [18], gastric [19], colorectal [20], and lung cancer [21] have been related with expression of EMT features [22].

Effect of SAHA treatment on EMT features of cholangiocarcinoma has not been studied yet. We researched the effects of SAHA on CCA cell viability, proliferation and migration in vitro. The molecular mechanisms underlying SAHA activities were also explored. It was found that SAHA could significantly inhibit cell viability, proliferation and migration of TFK-1 cells, accompanied by reversing of EMT markers.

The ability of tumor cells to express some mesenchymal properties at different levels is largely reported [23–26]. Clinical studies correlated the expression of EMT features with an increased metastatic potential and a poor clinical outcome in many cancers, including colorectal [27], and gastric cancer [28]. Effect of SAHA on EMT is contraversial in different cancer types. It has been reported that SAHA promotes the epithelial mesenchymal transition of triple negative breast cancer cells via HDAC8/FOXA1 signals [29]. In another study on this topic, it has been published that HDAC inhibitors induce epithelial-to-mesenchymal transition in prostate cancer cells [30]. In addition, It has been suggested that HDAC inhibitor induction of epithelial-mesenchymal transitions via up-regulation of Snail facilitates cancer progression [31]. We found that SAHA changed protein pattern of EMT markers by decreasing level of mesenchymal-associated molecules such as Vimentin and N-cadherin [24, 32]. In contrast, SAHA increased the level of epithelial junctional protein E-cadherin. Thus, these results suggest that SAHA treatment may reverse EMT.

The CFU assay has been used extensively to determine the proliferation of cancer cells and to determine the effects of drugs [33, 34]. CFU assay was used to investigate the effect of SAHA on TFK-1 cell colonization. CFU assay revealed that SAHA treatment clearly decreased colony formation of TFK-1 cells. We do not know how SAHA repressed colony formation of cancer cells. In the literature EZH2 inhibition of SAHA has been accepted as SAHA's anti-proliferative mechanism [15]. However, in this study, SAHA treatment did not affect EZH2 protein levels.

Migration capability of TFK-1 cells was showed by wound healing experiment. SAHA treatment clearly decreased migration capability of TFK-1 cells as reported

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in different cancer lines. Suppression or prevention of EMT process by SAHA treatment may be the factor in stopping migration of cancer cells.

Decreasing cancer cells viability, stopping cancer cells proliferation and preventing cancer cell migration are important steps in cancer treatment. SAHA is an epigenetic drug which shows antitumor affect by inhibiting HDAC. HDAC inhibition by SAHA express those positive affects in different cancer models.

In this study, SAHA treatment clearly decreased migration and proliferation of CCA, which is highly metastatic. SAHA also leads to inhibition of signaling pathways of EMT in vitro. Thus, our findings suggest that SAHA treatment may be beneficial for CCA patients and SAHA might be a potential therapeutic agent for the treatment of CCA. However, future studies are needed to evaluate the clinical applicability of SAHA as a part of the chemotherapeutic regimen for CCA.

Ethics committee approval: Ethics committee approval is not needed.
Informed consent: No informed consent is needed.
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