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ORIGINAL ARTICLE

SAHA induce hippo pathway in CCA cells without increasing cell proliferation

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Abstract

Background Cholangiocarcinoma is a malignant tumor originating from bile duct epithelial cells. Since tumor metastasis is associated with poor prognosis and short-term survival of patients, there is an urgent need for alternative therapeutic approaches for CCA. Because of that reason, we aimed to investigate effect of SAHA which is known as HDAC inhibitor on extrahepatic cholangiocarcinoma cell line (TFK-1).

Methods Cell cycle was measured by Muse Cell Analyzer. YAP, TAZ, TGF-β protein levels were determined by western-blotting method. TEAD (1–3), TIMP2 and TIMP3 genes level were determined by real-time PCR analysis.

Results We have seen the positive effects of SAHA on the TFK-1 cell line as it reduces cell viability and arresting cells in the G0/G1 phase. We also observed the negative effects of SAHA, as it increases the expression levels of YAP, TAZ, TGF-β protein and TEAD (1–3) gene. We also found that SAHA reduced the expression levels of TIMP2 and TIMP3 in TFK-1 cells, but was not statistically significant.

Conclusions Although observing its antiproliferative effects, these negative effects may be related to the cells being resistant to the drug or the remaining cells having a more aggressive phenotype. Therefore, we think that caution should be exercised in the use of this drug for CCA treatment.

Keywords Cholangiocarcinoma · Suberoylanilide hydroxamic acid · Hippo pathway · YAP/TAZ

Introduction

Cholangiocarcinoma (CC) is a type of primary hepatobiliary cancer that can originate from any point of intrahepatic and extrahepatic bile ducts and constitutes approximately 3% of all gastrointestinal malignancies [1]. This epithelial cancer, a high metastatic capacity, has been detected in many organs such as the brain, colon, and stomach. This metastatic spread is associated with short survival and poor prognosis. Therefore, alternative therapeutic approaches are urgently needed for cholangiocarcinoma [2].

Recent studies have highlighted that DNA methylation, histone modifications, and non-coding RNA, called epigenetic modification, play an important role in the development of carcinogenesis [3]. Histone acetylation, one of the histone modifications, is controlled by two families of enzymes: Histone acetyltransferases (HATs) catalyzes the addition of acetyl groups to lysine residues and histone deacetylase (HDAC) removes this acetyl group [4].

Increased histone acetylation by HATs is characterized by a loose chromatin structure and is associated with increased transcription. In the case of hypoacetylation via HDACs, histone proteins are characterized by a tight chromatin structure and are associated with a suppressed transcription and gene silencing. The HDACs, which are responsible for the removal of acetyl groups, change the transcription of tumor suppressors and oncogenes by reversing chromatin

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acetylation [5, 6]. HDACs are divided in to three classes; Class I HDACs (HDACs 1, 2, 3 and 8) located in the nucleus, Class II HDACs located in both the nucleus and cytoplasm (HDACs 4, 5, 6, 7, 9 and 10) and Class III HDACs [7]. The HDACs, which are responsible for the removal of acetyl groups, change the transcription of tumor suppressors and oncogenes by reversing chromatin acetylation. Increased levels of HDAC activity have been implicated in various cancer types such as, gastric, prostate, colon, breast, colorectal and cervical cancer [8]. Histone deacetylase (HDAC)-mediated epigenetic changes are also observed in the pathogenesis of CCA patients. HDAC 1, 2, 3, and 8, members of the class I HDAC family, have been shown to be significantly higher in CCA tissues than in non-tumor tissues and have been associated with increased lymph node metastasis and poor survival. Similarly, class I and II HDACs are overexpressed in various CCA cell lines [9]. HDAC inhibitors (HDACIs) have received attention as a potential approach to cancer therapy [10]. Various HDACIs have been used for therapeutic purposes in Phase I and II clinical cancer studies and most of the positive effects of these HDACIs have been demonstrated to be in hematological malignancies [11]. Suberoylanilide hydroxamic acid (SAHA) is a class I and II HDACI approved by FDA. SAHA is being investigated in other types of cancer as it is effective in CTCL patients [12].

Interestingly; in recent studies use of HDACI has been disappointing because of inducing EMT pathway which related cancer cell invasion, in solid tumors [13]. The Hippo pathway has a crucial regulator role in organ growth, apoptosis, tissue regeneration, cell proliferation [14]. Hippo signaling pathway components the YES-associated protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ) act via TEA domain transcription factors (TEADs) [15]. Although YAP has an important role in several cancers as an oncogene, in recent years some of the studies are suggesting that YAP also has a role in tumor suppression [16, 17]. In addition, YAP/TAZ interacts with other pathways, especially transforming growth factor (TGF-β), which play an important role in cancer [18]. YAP and TAZ, transcriptional regulators, have been shown to promote TGF-β induced tumorigenic phenotypes in breast cancer cells [19]. TGF-β has attracted attention with its role as a tumor suppressor in the early stages of carcinogenesis, namely in the premalignant period. On the other hand, it plays an oncogenic role in advanced cancer cells and tumor cells eliminate the antitumor effect of this growth factor by an unknown mechanism. Therefore, TGF-β known to plays a dual role in cancer [20]. TGF-β promotes extracellular matrix degradation by down-regulating the expression of matrix-metalloproteinases (MMPs) and upregulating their inhibitors (TIMPs) [21]. Disruption of the extracellular matrix (ECM), causes invasion of cancer cells. The impaired balance between MMP and TIMP activities during carcinogenesis can affect invasion and metastasis and worsen patient outcomes [22].

To our knowledge, there is no study demonstrating the effects of SAHA on the protein level of YAP/TAZ, TEAD1-3, TGF-β, TIMP2-3 and cell cycle in the human extrahepatic cholangiocarcinoma cell line (TFK-1). Therefore, we investigated the effect of SAHA in TFK-1.

Materials and methods

SAHA was purchased from Cayman Chemical and prepared as a 1 mM stock solution in DMSO. All chemicals used in medium preparation were (RPMI 1640, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), trypsin, penicillin/streptomycine and glutamine) purchased from Biological Industries, ISRAEL. Cell Cycle kit was obtained from Merck Millipore. Antibodies were purchased from Proteintecht. Secondary antibodies, non fat dry milk and other buffers of the western blot were purchased from BIO-RAD. RIPA Buffer, Phosphatase Inhibitor Cocktail 2 and 3 were purchased from SIGMA Chemical Co. TRIzol was purchased from (Thermo Fisher Scientific) cDNA Synthesis Kit was purchased ROCHE (GERMANY).

Cell lines and culture conditions

TFK-1 was purchase from DSMZ. TFK-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% Penicillin and L-Glutamine at 37 °C. We used dimethyl sulfoxide (DMSO; BioChemical) for negative control. TFK-1 cells were treated with 2 µg/mL of SAHA and we showed in our previous study [23]. All experiments were done triplicate.

Cell cycle analysis

For the cell cycle analysis, TFK-1 cells were seeded 4.10^5 cell per well in a 6 well-plate and the next day cells were treated with 2 µM SAHA for 48 h. Then the culture medium was removed and the cells were made suspense using trypsin. Then, the cells were centrifuged at 300xg for 5 min. Due to the stability of 70% ethanol, slowly was add ice cold ethanol then stored -20 °C for 3 h and were centrifuged at 300xg for 5 min. Cell cycle analysis was carried out by Cell Cycle Assay Kit (Merck Millipore, Billerica, MA, USA). After, the measurement was performed by Muse Cell Analyzer.

Western blotting analysis

TFK-1 cells were seeded 4.10^5 cell in a flask and the next day cells were treated with 2 µM SAHA for 48 h.

TFK-1 cells were washed and lysed on ice for 5 min in lysis buffer containing 400 µl of RIPA Buffer, 5 µl of Phosphatase Inhibitor Cocktails 2 and 3. Then, the TFK-1 cells were harvested with scraper. A standart curve was occured using albümin (1,35 mg/ml) to determine the protein concentration of TFK-1 cells. This measurement was executed by Promega GloMax. Equal amounts of protein were loaded each well, seperated on a 10% SDS–Polyacrylamide Gel and semidry transferred to a PVDF membrane. 5% non-fat dry milk was used for blocking then washed three times 5 min each time with TBST. The membrane was incubated overnight at 4 °C with specific primary antibody (YAP, TAZ, TGF-β, Tubulin and GAPDH) followed by incubation with secondary antibodies for 1 h at room temperature. For visual chemiluminescence detection system (Biorad ChemiDocTM) was used. All the bands were quantitated by imagej software.

Quantitative real-time PCR

TFK-1 cells were seeded 4.10^5 cell in a flask and the next day cells were treated with 2 µM SAHA for 48 h. Total RNAs were removed by TRIzol. cDNA Synthesis Kit was used for cDNA synthesis. The cDNAs were amplified for the mRNA sequence of TEAD1-3, TIMP2-3, and Housekeeping genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the house keeping gen. $2^{-\Delta\Delta CT}$ method was used for quantitative real-time polymerase chain reaction (qPCR) data analysis. Experiments were done triplicate.

Statistical analysis

Statistical analysis was performed with SPSS version 23.0 software (SPSS Inc, Chicago, IL, USA). Student's *t*-test was used for group comparison.

Results

Effect of SAHA on cell cycle

SAHA is a well-established epi-drug of growth arrest, differentiation, apoptosis, and suppressing tumor growth, therefore has received attention as a potential approach to cancer therapy [24]. Since cell proliferation may change with alters in the cell cycle, we examined whether SAHA induced G0/G1 phase in TFK-1 cells. SAHA has been shown to induce G0/G1 cell cycle arrest in TFK-1 cells. At the same time SAHA reduced in G2/M phase (Fig. 1).

Effect of SAHA on YAP/TAZ and TGF- β protein that play role in Hippo pathway

YAP/TAZ is overexpressed in the intrahepatic and extrahepatic parts of the biliary tract and also alters the activity of TGFβ, an oncogenic role in advanced cancer [25]. Therefore, we next examined the effect of SAHA on YAP/TAZ and TGF- β protein that play role in Hippo pathway by western blot. SAHA statistically induced protein level of YAP, TAZ and TGF- β (*$p < 0.001$). GAPDH was used as a loading control (Fig. 2).

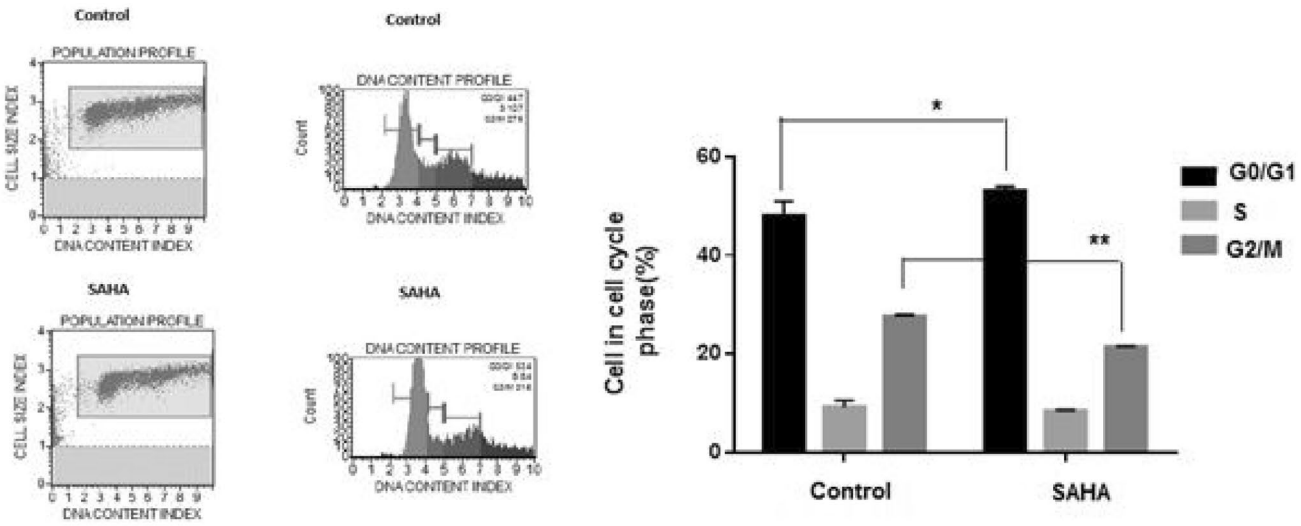

Fig. 1 SAHA statistically increased the percentage of TFK-1 cells in the G0/G1 phase from 44.7% to 53.7% and statistically decreased in the G2/M phase from 28.1 to 21.2. Expreriments were performed triplicate. The asterisks indicate statistical significance (*$p < 0.05$; ** $p < 0.001$)

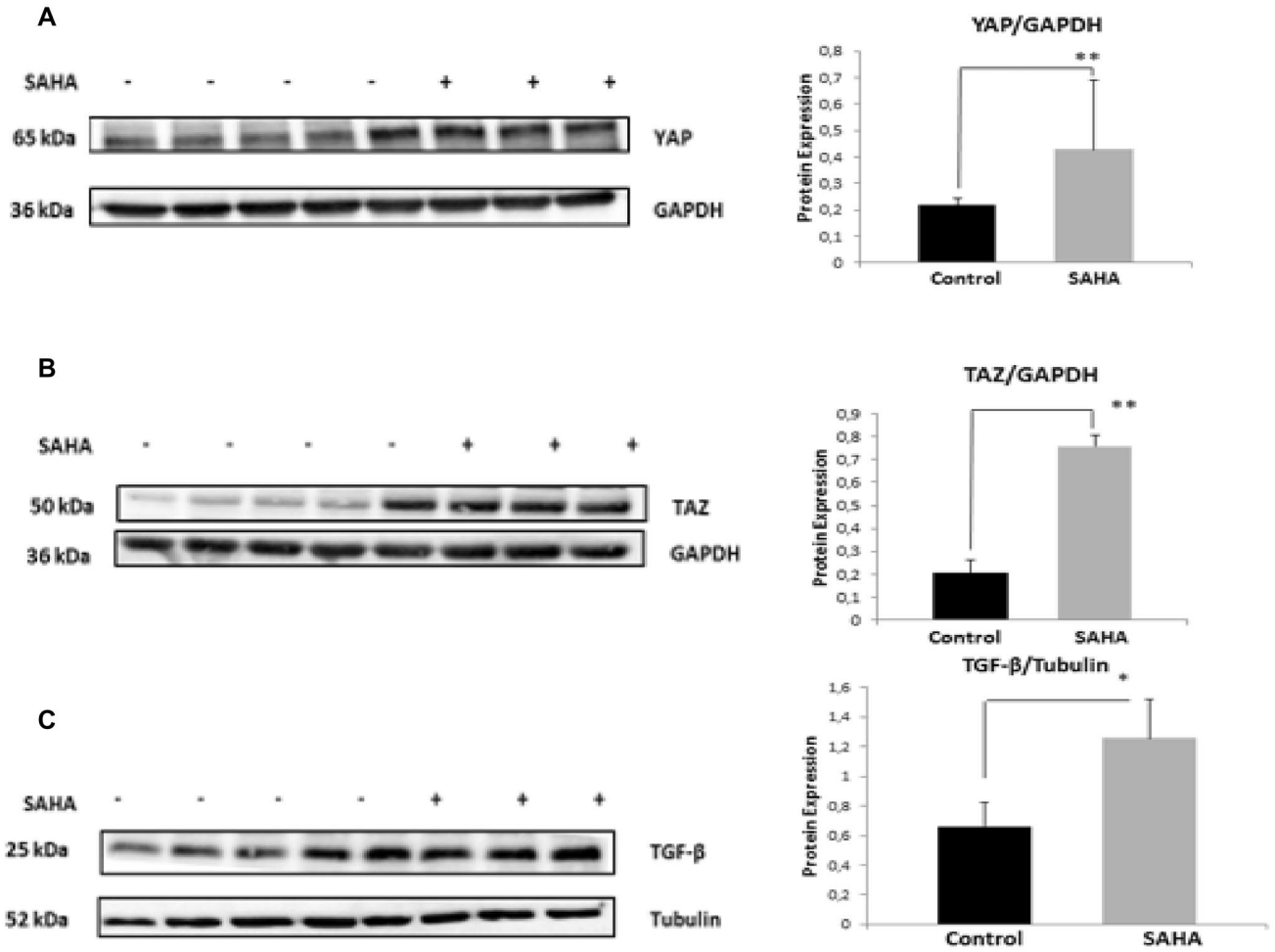

Fig. 2 YAP, TAZ and TGF-β protein levels were determined by Western blot analysis. GAPDH and Tubulin were used as a loading control. SAHA statistically induced protein level of YAP (**A**). SAHA statistically increased protein level of TAZ (**B**). SAHA statistically increased protein level of TGF-β (**C**). The asterisks indicate statistical significance (*$p < 0.05$; **$p < 0.001$)

Effect of SAHA on TEAD1- 3 genes expression levels

Tumor initiation potential of YAP/TAZ occurs by binding to TEAD transcription factors [26]. We examined TEAD (1–3) genes expressions levels after YAP/TAZ inhibition with SAHA. SAHA statistically increased expressions of TEAD1-3 genes (*$p < 0.001$) (Fig. 3).

Effect of SAHA on TIMP 2–3 genes expression levels

Disruption of balance between MMP and TIMPs that play a role in ECM causes increased metastasis and invasion ability in cancer cells. Although many studies report a decreased TIMPs expression in metastatic disease, a few experimental studies show that TIMPs increase cancer cell invasion [27]. Therefore, we also investigated effect of SAHA on TIMP2-3 genes expression levels. SAHA decreased expressions of TIMP2-3 genes, however were not statistically significant (Fig. 4).

Discussion

Many studies, on the anti-cancer effects of HDACIs, have shown to decrease the expression of cancer-related genes via increasing the histone acetylation level. Therefore, clinical studies have been focused on HDACIs. HDACIs are designed for therapeutic use in clinical and are being tested in Phase I, II and III clinical studies [28, 29]. HDACIs have a positive effect on hematological malignancies and the results are exciting [30]. In contrast, recent studies have reported that the effect of HDACIs was disappointing on solid tumors and little is known about the mechanism of anti-tumor in CCA [31].

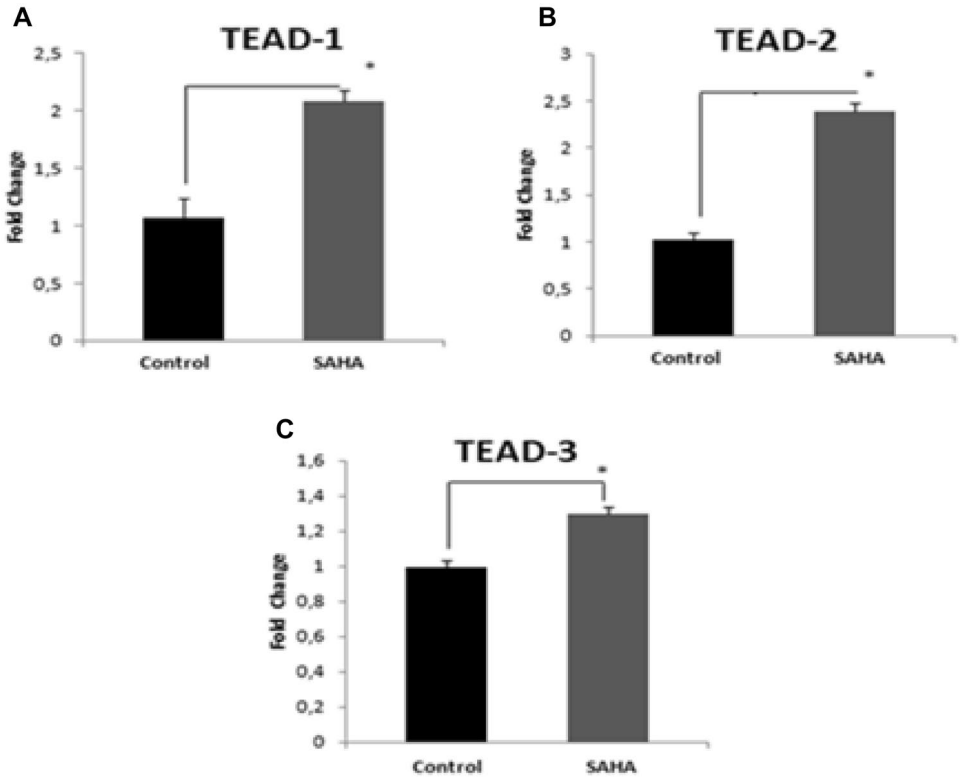

Fig. 3 TFK-1 cells assessed for expression of TEAD1-3 by Real-Time PCR. SAHA statistically increased expression of TEAD1 (**A**). SAHA statistically increased expression of TEAD2 (**B**). SAHA statistically increased expression of TEAD3 (**C**). The asterisks indicate statistical significance (*$p < 0.001$)

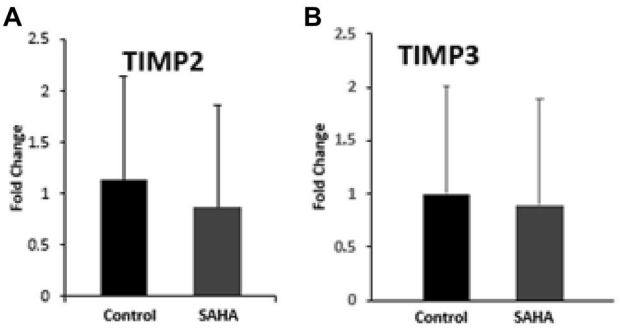

Fig. 4 TFK-1 cells treated with 2 µM SAHA and assessed for expression of TIMP 2–3 by Real-Time PCR. SAHA decreased expression of TIMP2 (**A**). SAHA decreased expression of TIMP3 (**B**). TIMP2-3 expression levels were not statistically significant (respectively, $p > 0.05$, $p > 0.05$)

In our previous published article, we determined the drug concentration of SAHA as 2 µM in the TFK-1 cell line. We also showed that SAHA dose-dependently reduces cell proliferation and inhibits migration [23].

Therefore; firstly we investigated the effect of an HDACI SAHA treatment on cell cycle in CCA cell line (TFK-1).

Furthermore, studies have shown that SAHA arrest the cell cycle by inhibiting cell proliferation and induce apoptosis in cancer cells [32, 33]. In many studies, it has been shown that HDAC inhibitors arrest G2/M phase by reducing cyclin B, cyclin A2 and inducing cyclin-dependent kinase (CDK) inhibitors p21, in tumor cells [7].

In the present study, SAHA arrested G0/G1 phase and reduced cell viability in CCA.

In the human uterine sarcoma cell line (MES-SA), SAHA treatment has been shown to increase the expression of the CDK inhibitor $p21^{WAF1}$, thereby reducing tumor cell proliferation by cell cycle arrest in the G1/S phase [32].

In another study, similar effects of SAHA on CCA (TFK-1 and HuCCT-1) cell lines have been observed and SAHA treatment shown to decrease cell proliferation, arrest G0/G1 phase via CDK1 and cyclin B1 levels. [31]. Likewise, in the hepatocellular carcinoma cell line (HepG2), SAHA have been shown to decrease the rate of cell proliferation depending on time and dose, increase the early cell apoptotic rate and the G0/G1 phase [34].

Lastly; in prostate cancer cell line (DU145 and PC-3), SAHA treatment has been shown to induce apoptosis via activating the Akt/FOXO3a pathway, reduce cell proliferation depending on the dose and arrest the G2/M phase. In addition, SAHA decreased the levels of cyclin B and cyclin A2 proteins, which play a role in the cell cycle in DU145 and PC-3 cells [33].

In our study, SAHA may have arrest cancer cells in the G0/G1 phase by decreasing CDK levels or increasing the levels of CDK inhibitors.

YAP/TAZ, a role in the Hippo pathway, effects in various oncogenic processes and is a targeted mechanism

for cancer treatment in these processes [35]. It has been reported that YAP/TAZ overexpress in the intrahepatic and extrahepatic parts of the biliary tract, as in many human cancers [25]. Tumor initiation potential of YAP/TAZ, occurs by connecting to TEAD transcription factors. TEADs expression has been shown to increase in many types of cancer, such as stomach, colorectal, breast, prostate, and cholangiocarcinoma cancers, which are associated with poor prognosis [26]. It has been also reported that YAP promotes CCA growth, proliferation, chemoresistance and angiogenesis through TEAD transcription factors [36]. YAP/TAZ also alters the activity of various transcription factors, including TGFβ-mediated SMAD complexes. TGF-β, which has a dual effect, shows oncogenic properties in tumoral tissues and causes uncontrolled proliferation, epithelial-mesenchymal transition, invasion and metastasis [37]. Interestingly, recent studies have reported that HDACIs are not effective for cancer, especially in solid tumors. HDACIs have shown to induce EMT related to YAP/TAZ pathway, indicating that HDACIs increase the invasion of cancer cells [13, 38, 39]. For this reason, secondly, we also investigated the effect of SAHA on YAP, TAZ, TEADs and TGF-β genes that play a role in the Hippo pathway.

We observed the unexpected effects of SAHA on TFK-1. In our study, we found that SAHA increased the levels of YAP, TAZ, TGF-β protein and TEAD (1–3) gene expression in TFK-1 cells.

In like our data, it has been shown to belinostat, an HDACI, increase the TAZ expression level in human melanoma, breast cancer and colon cancer cell lines. In addition, in kidney cells exposed to DNA damage both belinostat and TSA, have been shown to induce TEAD activation. In contrast, phosphorylation of YAP has been shown to decrease in response to increased belinostat concentrations [40].

In addition, it has been reported that the EMT in which is the epithelial cells gain metastatic properties, is induced by the increase of YAP and TAZ expression levels in colorectal, liver and gastric cancer [26]. TGF-β is one of the important factors playing a role in EMT [41].

Recent studies have been shown to HDACIs induce the EMT process in cancers such as lung, prostate and adenocarcinoma. For example, in lung cancer A549 cells, It has been reported that SAHA decreased epithelial marker E-cadherin's expression and increased the expression of mesenchymal marker Vim which was associated with increased migration capability, in the EMT process [42]. In another study, it has been shown that HDACIs Trichostatin A (TSA) and SAHA induced EMT pathway via increasing of transcription factors ZEB1, ZEB2 and Slug [13].

In many types of cancer, TGF-β has been shown to stimulate to EMT pathway. TGFβ and HIPPO pathways are associated with CCA progression and it has been reported that increase in TGFβ2 and TGFβ3 levels due to activation of YAP/TAZ in CCA [43].

In our study, the increase of TGF-β expression level may be associated with increased expression level of YAP and TAZ.

Although many studies report a decreased TIMPs expression in metastatic disease, a few experimental studies show that TIMPs increase cancer cell invasion [27].

Lastly, we investigated the effect of SAHA on TIMP2 and TIMP3 genes that play a role in ECM. Overexpression of TIMP-1 has been reported to suppress metastasis and tumor growth in human gastric, oral squamous, and melanoma cancer cells. It has also been reported that bile TIMP-1 concentration is lower in the malignant group and inhibits metastasis in cancer by the activity of TIMP-2 expression level in Hilar cholangiocarcinoma [44]. In addition, exogenous treatment with recombinant Ala + TIMP-2 has been reported to block down-regulation of EGF-induced E-cadherin in A549 lung cancer cells. Thus, it has been reported that cancer cells gain invasion and metastatic properties [45].

In hepatocellular carcinoma, it has been reported that TIMP1 expression level increases with decreasing TGF-β expression, thus preventing invasion related EMT process [46]. TIMP3 gene expression has been shown to be significantly higher than CCA tissues [47].

In our study, we found that SAHA reduced TIMP2 and TIMP3 expression levels in TFK-1 cells but it was not statistically significant. Decreased TIMP expression levels may be associated with increased TGF-β and YAP/TAZ.

In conclusion, we have seen positive effects of SAHA because it reduces cell viability, and arrest cells in the G0/G1 phase. In addition, we observed its negative effects as it increases the levels of YAP/TAZ, TEADs and TGF-β.

In another study similar to the results of our study, it has been shown that another HDACI of TSA treatment reduced proliferation, migration and arrested the cell cycle at G1 phases. However, interestingly, although the proliferation of nasopharyngeal carcinoma (NPC) cells is decreased with TSA treatment, it has been reported that remaining cells acquire aggressive phenotype and induce the EMT process [48].

We can say that although SAHA reduces the proliferation of TFK-1 cells, the remaining cells show resistance to the drug, or they may acquire an aggressive phenotype, inducing the hippo pathway with similar effects to the above study.

Therefore, we think that the mechanisms related to the effects of SAHA in the TFK-1 cell line should be investigated and caution should be attended to the use of SAHA.

Author contributions Concept – GB; Design – GB, MB, MÖ; Supervision – GB; Resource – GB; Materials – GB, MÖ, HA, FG, ÇU, BND; Data Collection and/or Processing – GB, MÖ, HA; Analysis and/or

Interpretation – GB, HA, MÖ, FG, ÇU, BND; Literature Search – GB, MB, MÖ; Writing – GB, MÖ; Critical Reviews – GB, MB.

Declarations

Conflict of interest No conflict of interest was declared by the authors. The authors declared that this study had received no financial support.

Ethical approval Ethics committee approval is not needed since it is a cell culture study.

Informed consent No informed consent is needed since it is a cell culture study.

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