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Research Article

Suberoylanilide hydroxamic acid and 3-deazaneplocin a decrease lncRNA hox transcript antisense RNA in liver fibrosis

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Abstract

Objectives: Liver fibrosis stimulates the abnormal wound repair response of chronic tissue damage, leading to the emergence of many chronic liver diseases such as cirrhosis. In addition, this process is characterized by excessive accumulation of extracellular matrix (ECM) components. The activation of silent hepatic stellate cells (HSCs) in liver fibrosis leads to the release of more ECM. HOX transcript antisense RNA (HOTAIR) is a long non-coding RNAs (lncRNAs) that are overexpressed in numerous types of cancer and are also associated with a number of various fibrosis processes. Recent studies have shown that lncRNAs are important in epigenetic modification. In our study, we aimed to evaluate the effect of the epigenetic drugs suberoylanilide hydroxamic acid (SAHA) and 3-deazaneplanocin A (DZNep) on HOTAIR expression in HSC line (LX2).

Methods: LX2 cells were scraped with TRIzol using a scraper and total RNAs were taken into ependorphs. After cDNA synthesis from RNA was obtained with the appropriate kit, cDNAs were amplified with FAM-labeled primer probes specific to the mRNA sequence of the genes HOTAIR expression levels which were calculated $2^{(-\Delta\Delta)(CT)}$ method and GAPDH was used for control gene.

Results: Both SAHA and DZNep statistically decreased HOTAIR gene levels in LX2 cells ($p<0.001$; $p<0.001$, respectively).

Conclusion: Due to the fact that both DZNep and SAHA reduced HOTAIR expression, it can be thought that the combined use of both drugs synergistically could be an important approach in preventing hepatic fibrosis. However, further mechanisms related to HOTAIR inhibition should be investigated in hepatic fibrosis.

Keywords: DZNep, hepatic stellate cell, HOTAIR, SAHA

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Hepatic fibrosis stimulates the abnormal wound repair response of chronic tissue damage, leading to the emergence of liver diseases such as cirrhosis and liver, excessive accumulation of extracellular matrix components [1]. Hepatic stellate cells (HSCs), known as main cell type responsible for liver fibrosis, play an important role in liver processes. During liver fibrosis, HSCs transform into a myofibroblast-like cell phenotype mainly by excessive secretion of collagen, alpha-smooth muscle actin, which enhances proliferative ability, and proinflammatory and pro-fibrogenic growth factors [2].

Epigenetics is a condition that results from changes in phenotype (appearance) or gene expression rather than the primary structure of DNA [3].

Epigenetic mechanisms generally include processes mediated by DNA methylation, modifications of histone proteins, and non-coding RNAs (ncRNAs) [4]. Acetylation and methylation at specific sites and residues are known as most common modifications of histone tails, and they control gene expression by regulating transcription factors. Histone acetyltransferase enzymes are responsible for adding acetyl groups to histone tails, while histone deacetylase (HDACs) enzymes on the contrary are responsible for removing acetyl groups [5]. HDAC activity plays a critical role in fibrosis, cancers, metabolic homeostasis, and various biological processes [6]. HDACs are overexpressed in many cancers, particularly in tissue fibrosis in many organs, including the kidney,

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heart, and lung, and therefore, inhibitors of HDAC modify the disturbed balance toward chromatin and restore the expression of genes [7]. Suberoylanilide hydroxamic acid (SAHA) is an FDA-approved small molecule HDAC inhibitor in cutaneous T-cell lymphoma [8]. Similar to acetylation, histone methyltransferases introduce methyl groups into histone tails, while histone demethylases remove methyl groups. 3-deazaneplanocin A (DZNep) is an inhibitor of histone H3 lysine 27 trimethylation and known as a enhancer of zeste homolog 2 (EZH2) inhibitor [9]. DZNep has been reported to alleviate fibrotic states due to EZH2 inhibition [10].

Recently, various studies demonstrated that ncRNAs play crucial role in regulating gene expression in the course of carcinogenesis and liver fibrogenesis [11]. Long non-coding RNAs (lncRNAs) have diverse functions in governing cellular processes and HOX transcript antisense RNA (HOTAIR) suppresses gene expression through chromatin [12]. It has been reported that HOTAIR upregulated in CCl4-induced human fibrotic livers [13].

In recent years, HOTAIR suppression has been cited as a potential therapeutic target to inhibit liver fibrosis. In the present study, we investigated effect of epigenetic drugs SAHA and DZNep on HOTAIR expression in LX2 cell line.

Materials and Methods

LX2 cells culture and treatment

LX2 cell used as hepatic fibrosis cell line was provided kindly as a gift by Dr. Scott L. Friedman (Mount Sinai School of Medicine, New York). LX2 cells, isolated from human HSCs (8th passage), were cultured in DMEM-high glucose, in which medium was supplemented with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin. We formed two groups; control group and treated group with SAHA (Cayman Chemical, Michigan, USA) and DZNep (Cayman Chemical, Michigan, USA). In our previously published article, we determined the drug concentration of both SAHA and DZNep in LX2 cells. In LX2 cells, IC_{50} values of SAHA and DZNep were determined as 2.5 μM and 5 μM, respectively [14, 15]. All reagents required for cell culture medium were obtained from Biological Industries (USA). All experiments were done triplicate. Ethics committee approval is not needed because it is a cell culture study. All experiments were conducted in Betül-Ziya Eren Genome and stem cell center.

Real-time PCR

For the real-time PCR analysis, LX2 cells were seeded 5.105 cell per well in a T75 flask and next day cells were treated with 2.5 μM SAHA for 48 h and 5 μM DZNep for 72 h. Then, T75 flasks were scraped with TRIzol (Thermo Fisher Scientific; USA, cat. No. 15596026) using a scraper and total RNAs were taken into eppendorph. cDNA Synthesis Kit was used for cDNA synthesis (Roche, Germany). The cDNAs were amplified for the mRNA sequence of HOTAIR and GAPDH (Table 1). HOTAIR and GAPDH expression levels were calculated $2^{(-\Delta\Delta)(CT)}$ method.

Table 1. Human primers for semiquantitative RT-PCR analysis

Genes	Forward primer	Reverse primer
HOTAIR	AGCACGCCCACAAAGAAC	GATGAAGATGGTGGACATTGC
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

RT-PCR: Reverse transcription polymerase chain reaction; HOTAIR: HOX transcript antisense RNA

Statistical analysis

SPSS 23.0 program was used for statistical analysis. Shapiro-Wilk's test was analyzed to assess the data normality. Student's t-test values are shown as mean±SD differences between groups. $P<0.05$ was considered statistically significant.

Results

Effect of SAHA and DZNep on HOTAIR gene expression

HOTAIR is associated with a number of fibrosis processes, so we evaluated whether DZNep and SAHA reduce HOTAIR gene expression in LX2 cells. SAHA and DZNep statistically decreased level of HOTAIR expression in LX2 cells ($p<0.001$, $p<0.001$; respectively) (Fig. 1a, b).

Discussion

Recently, most studies have shown that lncRNAs, although expressed in lower amounts than mRNA, have great biological importance as they are involved in almost every step of gene expression in both physiological and pathological cellular conditions [16]. The clinical utility of HOTAIR in predicting hepatocellular carcinoma (HCC) and predicting tumor stage has been investigated, and HOTAIR levels have been shown to be higher in early and late stage liver cancer than controls. Based on the data obtained from the same study, it was thought that circulating HOTAIR could be a potential biomarker that can be used alone or preferably in combination with AFP to aid in the detection of HCC in the cirrhotic liver and to preform tumor stage [17]. In the renal interstitial fibrosis model, significantly higher alfa smooth muscle actin (α-SMA) and HOTAIR as well as decreased E-cadherin as well as decreased miR-124 levels were observed in UUO rats and transforming growth factor beta 1 (TGF-β1)-induced HK-2 cells [18]. In *in vitro* and *in vivo* model of AF, mRNA expression (HOTAIR, Wnt5a, and PTBP1), and protein levels (Wnt5a, collagen I/III, α-SMA, CTGF, p-ERK, ERK, p-JNK, and JNK) were evaluated and HOTAIR knockdown, significantly inhibited fibrosis in heart tissues of AF mice through regulation of Wnt signaling [19].

Although the functional mechanisms of lncRNAs in liver fibrosis are not totally clear, they may have important roles in regulating in the epigenetic process. In addition, due to fundamental roles of ncRNAs, they may be attractive new prognostic markers and therapeutic targets for liver fibrosis. Increasing evidence has shown that the ncRNA, HOTAIR, is increased in HSCS and contributes to liver fibrosis [10].

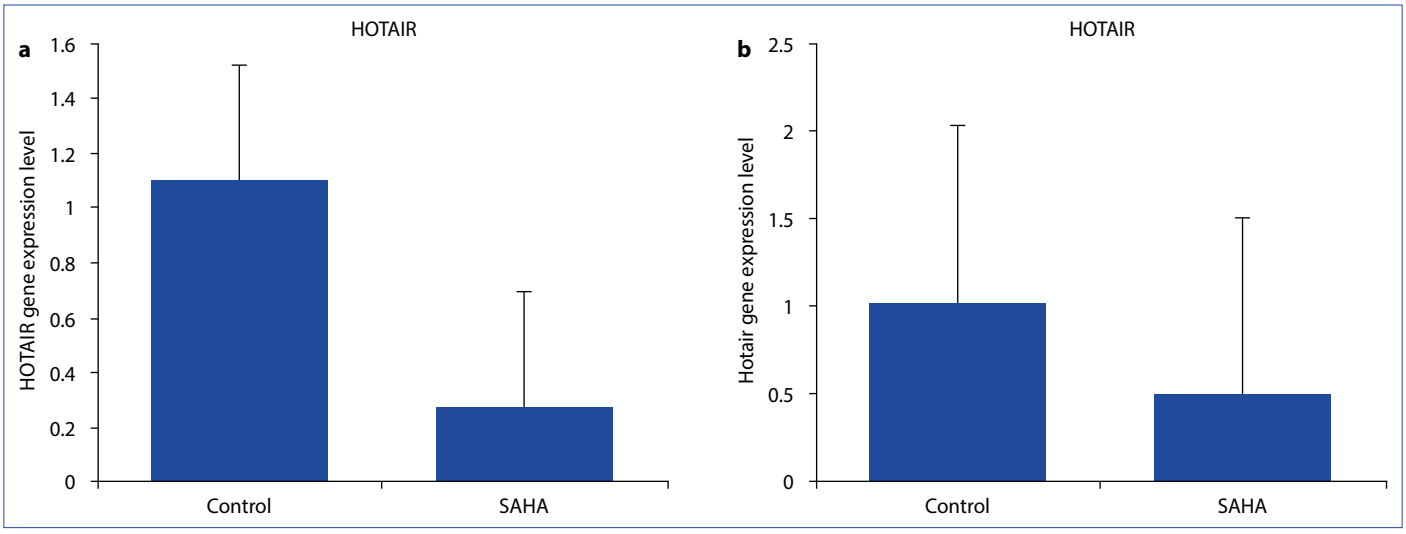

Figure 1. (a) SAHA statistically decreased HOTAIR gene expression level in LX2 cells ($p<0.001$). (b) DZNep statistically decreased HOTAIR gene expression level in LX2 cells ($p<0.001$).

HOTAIR: HOX transcript antisense RNA; SAHA: Suberoylanilide hydroxamic acid; DZNep: 3-deazaneplanocin A.

To the best our knowledge, there is no study present evaluating the effect of DZNep and SAHA on HOTAIR levels during fibrosis so far. This is the first study to evaluate effect of DZNep and SAHA on HOTAIR gene expression levels in LX2 cells. In our previous published articles, we determined the IC_{50} concentrations of SAHA and DZNep are 2.5 μM, 5 μM, respectively [14, 15]. In our present study, we found that SAHA and DZNep significantly reduced HOTAIR expression levels in LX2 cells.

DZNep (an inhibitor of histone methyltransferase EZH2) has been shown to be inhibited various fibrosis processes such as hepatic fibrosis [20], chronic tubulointerstitial fibrosis [21], and pulmonary and renal fibrosis [22]. DZNep, exhausts cellular levels of polycomb repressive complex 2 (PRC2) components, notably EZH2 and inhibits trimethylation of H3K27me3 and H4K20me3. Various studies have shown that DZNep inhibits myofibroblast cell formation and biochemical changes that occur with the differentiation of HSCs in the hepatic fibrosis process, together with various fibrosis mechanisms due to EZH2 inhibition [20]. Zeybel et al. [23] have demonstrated an anti-fibrotic effects of DZNep in murine model of liver fibrosis and in TGF-β1-activated HSCs. Ding et al. [20] established a hepatic fibrosis model in CCl4-treated rats and showed that EZH2 level was increased in HSCs and EZH2 expression was significantly inhibited by DZNep treatment. In addition, both *in vivo* and *in vitro* studies have shown that DZNep suppresses fibrosis by reducing hepatic fibrosis markers α-SMA and collagen I.

In our previous study, we showed that DZNep inhibits EZH2 in LX2 cells [15]. Therefore, since EZH2, which belongs to the subunit of the PRC2 complex, contains a SET domain that catalyzes the trimethylation of histone H3, and HOTAIR is known to interact with PRC2, HOTAIR expression may be suppressed due to DZNep inhibiting EZH2. Besides, in cancer studies, DZNep has been shown to inhibit HOTAIR levels. Li et al. [24], have demonstrated that DZNep reduces HOTAIR expression and protein levels in NMIBC and MGH-U4 non-invasive cells in a time-dependent manner.

Mayr et al. [25], have evaluated the mRNA levels of EED, EZH2, and SUZ12, which are core components of PRC2 in biliary tract cancer cell line (EGI-1). It has been shown that DZNep treatment inhibits all three core components, but also reduces HOTAIR mRNA levels in EGI-1 cells. In the light of all these data, the use of DZNep may be beneficial in the treatment approaches of liver fibrosis.

To date, there has been no study evaluating the effect of SAHA on HOTAIR expression in relation to the mechanism of fibrosis. In addition, cancer studies evaluating HOTAIR expression of SAHA are also extremely limited. In the present study, we evaluated effect of another epigenetic agent, SAHA in LX2 cells. It is under investigation in a series of clinical trials as an inhibitor of class I and II histone deacetylases [8].

In our study, we demonstrated that SAHA reduced HOTAIR levels, similar to DZNep.

Strickertsson et al. [26] reported that HOTAIR expression in resected gastric and esophageal adenocarcinomas may be a marker of poor prognosis. However, HOTAIR was transfected into normal gastric cells and showed that overexpression of HOTAIR doubled proliferation in HFE145 gastric cells. They reported that SAHA reduced the expression of HOTAIR in HFE145 cells; therefore, it played key role in regulation of HOTAIR expression.

Besides, it has been reported that HOTAIR inhibition in cancer cells increases E-cadherin expression and decreases vimentin and beta-catenin levels, thereby inhibiting epithelial mesenchymal transition (EMT), which contributes to cancer progression [27]. It has been reported that the EMT process not only plays a role in cancer progression but also plays an important role in organ fibrosis. In conclusion, we could easily say that HOTAIR lncRNA may be associated with fibrosis-related diseases.

In our previous study, we already evaluated effect of SAHA on EMT markers in liver fibrosis and showed that SAHA increases E-cadherin and decreases N-cadherin and vimentin levels, showing that the role of SAHA with EMT inhibition [14]. As a result, taken together all our data relating to the SAHA, we observed that SAHA prevents liver fibrosis. In other words, SAHA can be used as a therapeutic agent in the prevention of hepatic fibrosis.

Conclusion

Since multiple epigenetic mechanisms play a role in silencing genes in cancer, it is important to develop combination therapies that target these pathways [28]. The most important point to be emphasized is that HDAC inhibitors, such as SAHA, have been shown to act synergistically with either 5-Aza-CdR or DZNep [29]. The efficacy of 5-Aza-CdR or SAHA and their combination has been evaluated *in vitro* pancreatic cancer model, and treatment with 5-Aza-CdR or SAHA has been observed to inhibit pancreatic cancer cell proliferation, migration, and induced cell arrest. The combination of the two agents has also been shown to reduce proliferation and migration and increase the rate of cell apoptosis. In our study, although it is important that both DZNep and SAHA individually reduce HOTAIR expression, the synergistic combination of both drugs may be a better treatment in preventing hepatic fibrosis. However, further mechanisms related to HOTAIR inhibition in hepatic fibrosis should be investigated.

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