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Up-regulated HOTAIR induced by fatty acids inhibits PTEN expression and increases triglycerides accumulation in HepG2 cells

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic lipid accumulation unrelated to excess alcohol intake, in which the hepatic expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is inhibited. Long non-coding RNA HOTAIR suppresses PTEN expression in hepatic stellate cells during liver fibrosis, and it is involved in liver lipid dysregulation. In this study, we evaluated whether the PTEN down-regulation and lipid accumulation in hepatic cells might be mediated by HOTAIR during the development of NAFLD. Free fatty acids (FFAs) treatment promoted triglyceride accumulation in HepG2 cells, significantly increasing HOTAIR expression and inhibiting PTEN expression (both at mRNA and protein levels). The effects on HOTAIR and PTEN expressions disappeared after withdrawal of the FFAs treatment. SiRNA-mediated HOTAIR knockdown prevented PTEN down-regulation and triglyceride accumulation in HepG2 cells treated with FFAs; and CAPE (an NF-κBp65 inhibitor) treatment prevented the HOTAIR up-regulation and PTEN down-regulation. FFAs could induce the up-regulation of HOTAIR in HepG2 cells probably dependent on the NF-κB signaling, and consequently suppress PTEN expression and promote triglyceride accumulation. Aberrant up-regulation of HOTAIR mediated by excessive circulating FFAs levels may be a crucial mechanism associated with liver steatosis.

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KEYWORDS

Non-alcoholic fatty liver disease; free fatty acid; triglyceride; HOTAIR; PTEN; NF-κB

Introduction

Non-alcoholic fatty liver diseases (NAFLD) involve macrovesicular fatty changes in liver unrelated to excessive alcohol intake [1]. NAFLD include hepatic steatosis, nonalcoholic steatohepatitis, chronic fibrosis, and cirrhosis [1,2]. Metabolic dysfunction of elevated fatty acid synthesis is associated with NAFLD. The extent of triglyceride accumulation is the basis for grading the severity of steatosis in NAFLD [3]. The prevalence of NAFLD increases with increasing body mass index (BMI) [4] and its incidence is increasing each year [5,6]. And of note, NAFLD is a major risk factor for hepatocellular carcinoma (HCC) [7,8].

Previous studies had examined the pathological mechanisms of NAFLD in NAFLD-related cell models that were established by treating HepG2 cells with free fatty acids (FFAs) (such as palmitic and oleic acids) [9,10]. Indeed, this approach reproduces the mitochondrial dysfunction [9], apoptosis and autophagy [10], and inflammation [11] observed in NAFLD.

NAFLD involves the disruption of many genes, including Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [12]. PTEN is a major tumor suppressor that is down-regulated in the liver of human and rodent models with NAFLD or cancers (including HCC) [13–16]. Hepatocyte-specific null mutation of PTEN in mice leads to massive hepatomegaly and steatohepatitis with triglyceride accumulation, and later to HCC [17,18]. PTEN expression is also down-regulated in cultured hepatocytes exposed to FFAs, and PTEN expression inhibited by FFAs could trigger hepatic steatosis via an NFκB/mTOR-dependent pathway [19]. Dietary unsaturated fatty acids promote the growth ability and metastatic capacity of HepG2 cells by suppressing PTEN, and the effects were prevented by NF-κB inhibitor [20]. These data suggest that alterations of PTEN expression might be a critical factor in the development of NAFLD [16]. Nevertheless, the upstream regulatory mechanisms

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of PTEN expression in NAFLD are still not completely understood.

The abnormal expression of PTEN in hepatocytes may result from the regulation by long non-coding RNAs under certain pathological conditions. The homeobox transcript antisense RNA (HOTAIR) is a long intergenic non-coding RNA that is up-regulated in a variety of cancers [21,22]. A recent study showed that HOTAIR enhances the methylation of PTEN and suppresses its expression in hepatic stellate cells, contributing to liver fibrosis [21]. And another study showed that HOTAIR is involved in lipid metabolism dysfunction in hepatitis C [23]. Taken together, we hypothesized that during the pathological progress of NAFLD, decreased hepatic expression of PTEN in response to FFAs might be mediated by HOTAIR, which has never been studied before.

The present study aimed to investigate whether the down-regulation of PTEN in hepatic cells might be mediated by HOTAIR. To do so, NAFLD cell models were established in HepG2 cells incubated with FFAs (including oleic acid and/or palmitic acid). Knockdown of HOTAIR was performed with siRNA-HOTAIR in order to confirm the regulatory mechanism. And an NF- κ Bp65 inhibitor (caffeic acid phenethyl ester, CAPE) was used to examine whether the NF- κ B pathway might be involved in the regulation.

Materials and methods

Cells

HepG2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) in a humidified 37°C incubator with 5% CO₂.

FFAs (Adamas-beta, Shanghai, China) were used to induce the NAFLD cell models. HepG2 cells were incubated with FFAs [50 μ M oleic acid (OA), linoleic acid (LA), palmitic acid (PA), or PA/OA at a ratio of 1:2 (PA/OA); or 500 μ M PA/OA (PA/OA#)] for 24 h. Particularly, in the experiment concerning ‘metabolic memory’, the medium was replaced once, and the PA/OA treatment was kept or withdrawn in the second half of the cell culture. Control cells were cultured in medium without FFAs.

Besides, HepG2 cells were subjected to siRNA-mediated gene knockdown or inhibitor pretreatment before FFAs were added, so as to investigate the regulatory mechanism. Twenty-four hours after the transfection of siRNA-HOTAIR, HepG2 cells were incubated with 50 μ M PA/

OA or 500 μ M PA/OA# for 24 h; HepG2 cells were incubated for 48 h with 2 μ M CAPE (Selleckchem, Houston, TX, USA), and then for another 24 h with 50 μ M OA/PA.

After FFAs treatment, the cell viability and intracellular triglycerides content of HepG2 cells were determined; PTEN and HOTAIR expression were assessed by qRT-PCR and/or western blotting.

RNA interference

Transfection of the HepG2 cells was performed with HiPerFect Transfection Reagents (Qiagen, Venlo, The Netherlands), according to the manufacturer’s instructions. Briefly, HepG2 cells were seeded in a 6-well plate at a density of 5×10^5 per well, and cultured with RPMI 1640 complete medium to 70 ~ 90% confluence; and then the cells were incubated with 400 μ l siRNA/lipofectamine mixture [the mixture contained 8 μ l siRNA solution, 4 μ l lipofectamine and 400 μ l RPMI 1640 medium (without FBS)] and 1600 μ l RPMI 1640 medium (without FBS) for 24 h.

The sequence of siRNA-HOTAIR: 5'-UUCAAGAGCUUCCAAAGGGCTT-3'.

The sequence of siRNA-NC (control): 5'-CCCAGCCCCAUUUAAAGAAUTT-3'.

All siRNA oligonucleotides were designed and synthesized by GenePharma (Shanghai, China).

Cell viability

Cell viability was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The MTT solution (Sigma, St Louis, MO, USA) was added to each well. After 4 h, the medium was discarded, 100 μ l of dimethyl sulfoxide was added, and the plate was shaken gently. The absorbance at a wavelength of 492 nm was recorded using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Intracellular triglycerides

Intracellular triglycerides levels were determined using a colorimetric quantification kit (Genemed Biotechnologies, Inc., South San Francisco, CA, USA), according to the manufacturer’s instructions. Each sample was assayed in triplicate.

Quantitive reverse transcription (qRT)-PCR

Total RNA was extracted from the cells using TRIZOL (Invitrogen Inc., Carlsbad, CA, USA) and cDNA was synthesized using HiScript II 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd, Nanjing, Jiangsu, China), both according to the manufacturer's instructions. The qRT-PCR was performed with qPCR SYBR Green Master Mix (Vazyme Biotech) using an ABI 7500 instrument (ABI Biosystems, USA), and the results were analyzed with Rotor Gene 3000 application software. The relative HOTAIR expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, with the CT values normalized to those of 18S rRNA as internal control.

The primers for HOTAIR were 5'-GTA GAA AAA GCA ACC ACG AAG C-3' (forward) and 5'-ACA TAA ACC TCT GTC TGT GAG TGC C-3' (reverse). The primers for PTEN were 5'-TAG CAT TTG CAG TAT AGA GCG TGC-3' (forward) and 5'-TTG GAG AAA AGT ATC GGT TGG C-3' (reverse).

Western blotting

Cells were harvested, washed, and lysed in cold RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% SDS, and 1% protease inhibitor cocktail). Western blotting was performed according to standard protocols. About 30 µg protein samples were loaded. After SDS-PAGE electrophoresis, blotting and blocking, the PVDF membranes were incubated with anti-PTEN antibody [Y184] (ab32199; Abcam, Cambridge, MA, USA) at 4°C overnight. For the loading control, anti-β-actin monoclonal antibody (Sigma, St Louis, MO, USA) was used. Then the membranes were incubated with secondary antibodies at 37°C for 1 h, and finally visualized with electrochemiluminescence western blot detection kit.

Statistical analysis

All analyses were performed using SPSS 13.0. (SPSS Inc., Chicago, IL, USA). Results were expressed as means \pm standard deviation, and analyzed with one-way ANOVA and the SNK post hoc test. Two-sided P-values <0.05 were considered statistically significant.

Results

FFA treatment promotes triglyceride accumulation in HepG2 cells, increasing HOTAIR expression and inhibiting PTEN expression

HepG2 cells were incubated for 24 h with 50 µM oleic acid (OA), 50 µM linoleic acid (LA), 50 µM palmitic

acid (PA), or the mixture of PA and OA (1:2) at 50 µM (PA/OA) or 500 µM (PA/OA#). After 24 h, we observed that all groups had increased triglyceride accumulation (all $P < 0.05$) (Figure 1(b)), without affecting cells viability (Figure 1(a)). We then investigated whether the FFAs treatments affect HOTAIR and PTEN expression. The RNA level of HOTAIR was upregulated in all groups of cells treated with FFAs (all $P < 0.05$) (Figure 1(c)). And consistent with previous studies [19], PTEN mRNA and protein levels were decreased in the cells treated with 50 µM OA or LA; in addition, PA, PA/OA, or PA/OA# also resulted in down-regulation of PTEN mRNA and protein levels (all $P < 0.05$) (Figure 1(c,d)).

FFAs treatment does not establish a 'metabolic memory' on HOTAIR and PTEN expressions in HepG2 cells

Metabolic memory is the phenomenon whereby hyperglycemia-induced complications persist and progress after glycemic recovery is achieved in diabetes [24,25]. Certain epigenetic changes may provide new and interesting clues towards explaining the pathogenesis of metabolic memory. It is unclear whether there is a metabolic memory for FFAs-induced changes in the expressions of HOTAIR and PTEN in hepatocytes.

In the present study, HepG2 cells were incubated with 50 µM PA/OA for 12 h or 24 h. After that, the cells were incubated for 12 or 24 h in medium without or with PA/OA. The results showed that there were significant up-regulation of HOTAIR and down-regulation of PTEN in cells treated with 50 µM PA/OA for 12 and 24 h (all $P < 0.05$), but these changes disappeared when the cells were cultured in medium without PA/OA for another 12 or 24 h (Figure 2(a,b)). These results suggest that the effects of FFAs on HOTAIR and PTEN expression do not form a metabolic memory.

HOTAIR knockdown by siRNA prevents PTEN down-regulation and triglyceride accumulation in HepG2 cells upon FFAs treatment

HepG2 cells were transfected for 24 h with siRNA-HOTAIR, and then incubated for 24 h with PA/OA or PA/OA#, respectively. The results showed that when HOTAIR was inhibited (Figure 3(a)), the PTEN expression was elevated (Figure 3(a,b)) and triglyceride accumulation was reduced (Figure 3(c)) in HepG2 cells treated with PA/OA or PA/OA# ($P < 0.05$). The siRNA-HOTAIR did not affect HOTAIR expression in control HepG2 cells. The results suggest that

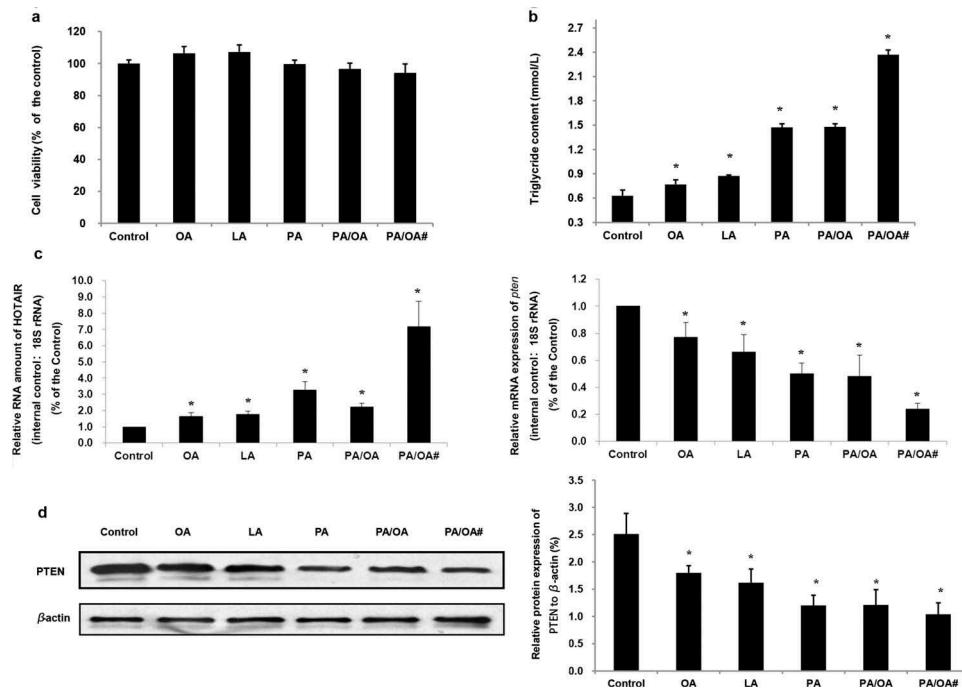


Figure 1. FFAs treatment promotes triglyceride accumulation in HepG2 cells, increasing HOTAIR expression and inhibiting PTEN expression. HepG2 cells were incubated with 50 µM of FFAs [oleic acid (OA), linoleic acid (LA), palmitic acid (PA), or palmitic acid/oleic acid at a ratio of 1:2 (PA/OA)], or 500 µM PA/OA (PA/OA#) for 24 h. Control cells received no FFAs treatment. (a) The viability of HepG2 cells was estimated by the MTT assay. (b) Intracellular triglyceride content was analyzed using a commercial kit. (c) RNA expression of HOTAIR and mRNA expression of PTEN were detected by quantitative reverse transcription (qRT)-PCR. (d) Protein expression of PTEN was measured by western blotting. Results are shown as mean ± standard deviation (SD) of three independent experiments. *P < 0.05 vs. Control HepG2 cells.

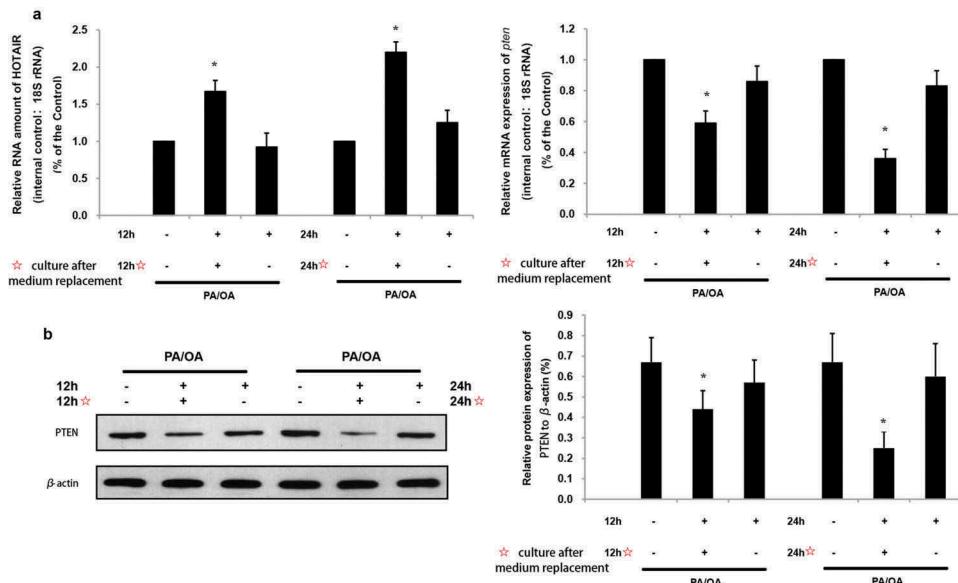


Figure 2. FFAs treatment does not establish a 'metabolic memory' on HOTAIR and PTEN expressions in HepG2 cells. HepG2 cells were incubated with PA/OA for 24 h or 48h. The medium was replaced once, and the PA/OA treatment was kept or withdrawn in the second half of the cell culture; Control cells received no FFAs treatment all the time. (a) RNA expression of HOTAIR and mRNA expression of PTEN were detected by qRT-PCR. (b) Protein expression of PTEN was measured by western blotting. Results are shown as mean ± SD of three independent experiments. *P < 0.05 vs. Control HepG2 cells.

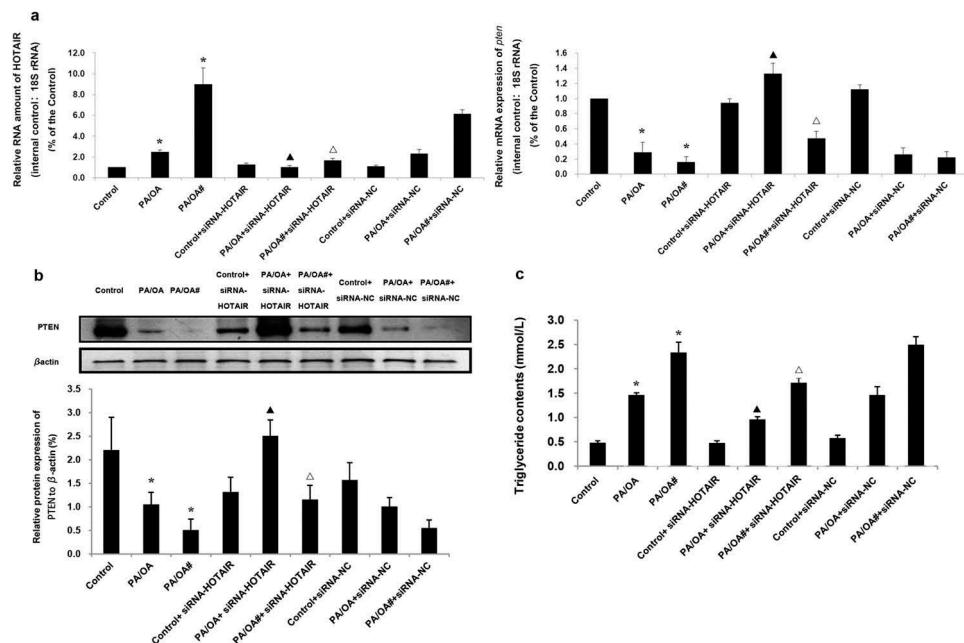


Figure 3. HOTAIR knockdown by siRNA prevents PTEN down-regulation and triglyceride accumulation in HepG2 cells upon FFAs treatment. Twenty-four hours after the transfection of siRNA-HOTAIR or siRNA-NC, HepG2 cells were incubated with PA/OA, PA/OA# or medium without FFAs for 24 h, respectively. Control cells received neither siRNA transfection nor FFAs treatment. (a) RNA expression of HOTAIR and mRNA expression of PTEN were detected by qRT-PCR. (b) Protein expression of PTEN was measured by western blotting. (c) Intracellular triglyceride content was analyzed using a commercial kit. Results are shown as mean \pm SD of three independent experiments. * P < 0.05 vs. Control HepG2 cells; ^ P < 0.05 vs. HepG2 cells transfected with siRNA-NC and treated with PA/OA; △ P < 0.05 vs. HepG2 cells transfected with siRNA-NC and treated with PA/OA#.

HOTAIR is involved in the regulation of PTEN expression and triglyceride accumulation in hepatic cells upon FFAs treatment.

FFAs treatment induces HOTAIR up-regulation in HepG2 cells dependent on the NF-κB signaling

HepG2 cells were incubated for 48 h with different concentrations of CAPE, and for another 24 h with 50 μM of PA/OA. High concentrations of CAPE (20–100 μM) significantly decreased cell viability (Supplementary Figure S1) (P < 0.05), while 2–10 μM CAPE was well tolerated by HepG2 cells. CAPE at 2 μM prevented HOTAIR up-regulation (Figure 4(a)) and PTEN down-regulation (Figure 4(a,b)) induced by FFAs treatment (P < 0.05).

Discussion

NAFLD with early liver steatosis is a high-risk factor for HCC [7,8]. Hepatocyte-specific PTEN deficiency results in liver steatosis [13–16]. HOTAIR could regulate PTEN expression and modulate the risk of NAFLD [21,22]. Therefore, this study investigated whether the down-regulation of PTEN in hepatic cells might be mediated by HOTAIR. The results suggest

that FFAs induce the up-regulation of HOTAIR in HepG2 cells probably dependent on the NF-κB signaling, and consequently suppressed PTEN expression and increased triglyceride accumulation.

NAFLD occurs when triglycerides accumulate in the liver [3,7]. When obesity causes triglycerides failure to be stored in the adipose tissue, triglycerides are transformed into FFAs that are transported to non-adipose tissues [26]. Saturated palmitic (PA; C16:0) and monounsaturated oleic (OA; C18:1) acids are the most abundant FFAs in the liver triglycerides of patients with NAFLD [27,28]. Previous studies showed that *in vitro* NAFLD models induced by FFAs mixture (PA/OA, 1:2 ratio) could lead to triglyceride accumulation in hepatocytes similar to that of patients with NAFLD, with minor toxic and apoptotic effects [29,30]. The present study used FFAs to establish a cell model of human NAFLD. The results showed triglyceride accumulation in HepG2 cells after treatment with FFAs, consistent with previous studies [27–30].

PTEN is a negative regulator of insulin signaling and is associated with cellular growth and metabolic signaling. It is usually down-regulated in patients and rodent models with NAFLD and HCC [13–16]. Vinciguerra et al. [19,20] showed that PTEN down-regulation

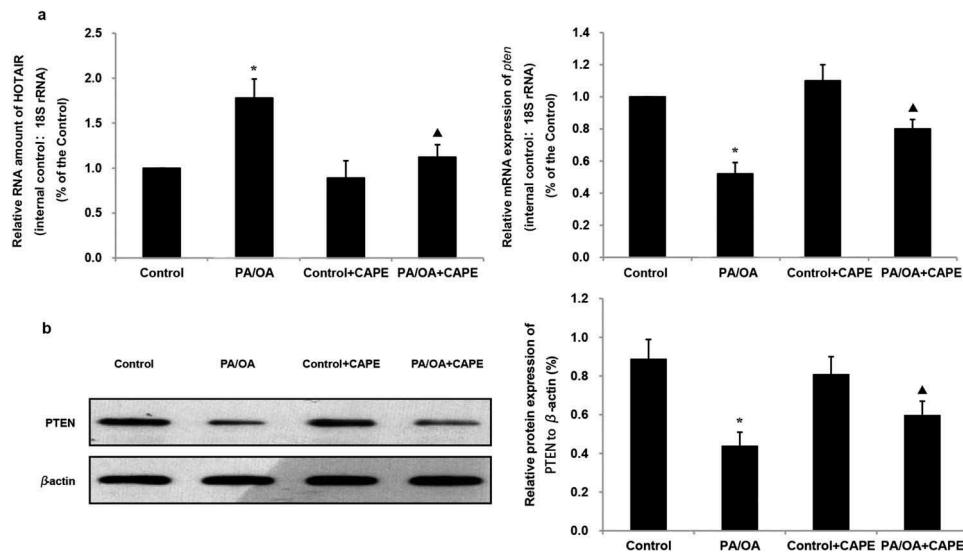


Figure 4. FFAs treatment induces HOTAIR up-regulation in HepG2 cells dependent on the NF-κB signaling. HepG2 cells were incubated for 48 h with 2 μM caffeic acid phenethyl ester (CAPE), an NFκBp65 inhibitor, and then for another 24 h with PA/OA. Control cells received neither CAPE nor FFAs treatment. (a) RNA expression of HOTAIR and mRNA expression of PTEN were detected by qRT-PCR. (b) Protein expression of PTEN was measured by western blotting. Results are shown as mean ± SD of three independent experiments. *P < 0.05 vs. Control HepG2 cells; ▲P < 0.05 vs. HepG2 cells treated with PA/OA.

induced by unsaturated FFAs could accelerate triglyceride accumulation in hepatocytes, and promotes hepatoma proliferation and progression. Accordingly, the present study showed that PTEN was down-regulated in HepG2 cells exposed to FFAs, further supporting the modeling of HepG2 cells into a model of NAFLD upon treatment of PA and OA.

HOTAIR is a long intergenic non-coding RNA regulating chromatin dynamics and silencing many genes through its interaction with histone methylase and demethylase [31]. Although the expression of HOTAIR has not been explored in NAFLD, some studies underlined its role in other kinds of liver disease and HCC. Zhang et al. [32] showed that HOTAIR accelerated carcinogenesis in hepatitis B-infected liver. Ding et al. [33] showed that HOTAIR promote the migration and invasion of HCC cells, while Ishibashi et al. [34] showed that HOTAIR expression is associated with HCC progression. A recent study showed that HOTAIR enhances the methylation of PTEN and contributes to liver fibrosis [21], providing some additional clues about the regulatory effect of HOTAIR associated with PTEN. The present study suggests that HOTAIR might be involved in the negative regulation of hepatic PTEN expression which contributes to the triglyceride accumulation in hepatic cells. Notably, HOTAIR siRNA did not affect the expression of HOTAIR in HepG2 cells without FFAs. It was possibly due to the low levels of expression of HOTAIR in HepG2 cells at baseline. In addition, the present study

found that NF-κBp65 inhibitor could prevent the up-regulation of HOTAIR in hepatic cells upon FFAs treatment, suggesting that FFAs may induce HOTAIR expression dependent on the NF-κB signaling. Taken together, these results imply the involvement of HOTAIR in the pathogenesis of NAFLD. And based on previous studies concerning the roles of HOTAIR in HCC [33,34], it could be inferred that HOTAIR may play an important role in the disease progression from NAFLD to HCC. Nevertheless, additional studies are necessary to confirm the results and the hypothesis.

The metabolic memory phenomenon has recently been described and states that some metabolic effects such as diabetes complications persist and continue to progress unimpeded even when the faulty metabolic pathways are controlled [24]. The metabolic memory is associated with several epigenetic processes that allow for altered gene expression including histone modification, (de)methylation of cytosine residues within DNA, and controlled by non-coding RNAs [35,36]. Notably, the effects on HOTAIR and PTEN expressions disappeared after withdrawal of the FFAs treatment. These results suggest that the negative regulation of PTEN by HOTAIR may act through means other than epigenetic modification in hepatic cells with FFAs-induced abnormality, possibly different from the regulatory mechanism revealed in liver fibrosis [21].

In conclusion, the present work suggests that FFAs could induce the up-regulation of HOTAIR, probably dependent on the NF-κB signaling, and consequently

suppress PTEN expression and increase triglyceride accumulation in HepG2 cells. Aberrant up-regulation of HOTAIR mediated by excessive circulating FFAs levels may be a crucial mechanism associated with liver steatosis.

Disclosure statement

No potential conflict of interest was reported by the authors.

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