Conjugated Linoleic Acid Treatment Attenuates Cancerous features in Hepatocellular Carcinoma Cells

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Background. A growing number of hepatocellular carcinoma (HCC), and recurrence frequency recently have drawn researchers’ attention to alternative approaches. The concept of differentiation therapies (DT) relies on inducing differentiation in HCC cells in order to inhibit recurrence and metastasis. Hepatocyte nuclear factor 4 alpha (HNF4α) is the key hepatogenesis transcription factor and its upregulation may decrease the invasiveness of cancerous cells by suppressing epithelial-mesenchymal transition (EMT). This study aimed to evaluate the effect of conjugated linoleic acid (CLA) treatment, natural ligand of HNF4α, on the proliferation, migration, and invasion capacities of HCC cells in vitro. Materials and Method. Sk-Hep-1 and Hep-3B cells were treated with different doses of CLA or BIM5078 [1-(2′-chloro-5′-nitrobenzenesulfonyl)-2-methylbenzimidazole], an HNF4α antagonist. The expression levels of HNF4α and EMT related genes were evaluated and associated to hepatocytic functionalities, migration, and colony formation capacities, as well as to viability and proliferation rate of HCC cells. Results. In both HCC lines, CLA treatment induced HNF4α expression in parallel to significantly decreased EMT marker levels, migration, colony formation capacity, and proliferation rate, whereas BIM5078 treatment resulted in the opposite effects. Moreover, CLA supplementation also upregulated ALB, ZO1, and HNF4α proteins as well as glycogen storage capacity in the treated HCC cells. Conclusion. CLA treatment can induce a remarkable hepatocytic differentiation in HCC cells and attenuates cancerous features. This could be as a result of HNF4α induction and EMT inhibition.
1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer [1]. The global incidence of HCC is rising, and it is predicted that by 2030, this cancer will be one of the leading causes of cancer death worldwide. Patients with advanced tumors are offered different treatments, including systemic prescription-based therapies like sorafenib, regorafenib, and nivolumab to loco-regional ablation or resection [2–4]. Liver transplantation, immunotherapeutic and radionuclide-based approaches, and targeted molecular and gene therapy interventions are other advanced modalities [5, 6]. However, the high rate of tumor recurrence after treatment has led to a growing interest in developing innovative therapeutic approaches [7].

Liver chronic inflammation results in morphological changes and dedifferentiation of mature hepatocytes [8]. Poorly differentiated carcinoma cells have a worse prognosis and are more aggressive than the well-differentiated cells [9–12]. During epithelial-mesenchymal transition (EMT), epithelial parenchymal cells lose their cell-cell junctions and dissociate from each other and from the surrounding extracellular matrix (ECM) that results in the initiation of their migration and invasion [13, 14]. EMT is also associated with enhanced stem cell properties and drug resistance in cancer cells [15–17]. Recent findings indicated that EMT could be switched to mesenchymal-to-epithelial transition (MET) after modulating the gene expression pattern of EMT-related transcription factors (TFs) such as SNAIL, SLUG, TWIST1, and ZEB1 [18–20]. MET is one of the most essential mechanisms in regulating hepatocyte differentiation from definitive endoderm (DE); such process is orchestrated by hepatocyte nuclear factor 4 alpha (HNF4α) [21]. Understanding the cellular and molecular mechanisms of the hepatocytes dedifferentiation could provide necessary insights into differentiation therapy (DT) as a novel strategy in HCC treatment [22]. DT investigates the feasibility of converting the phenotype of cancerous cells toward a less aggressive and more differentiated one [23, 24]. Various strategies can induce differentiation of cancer cells through alteration of EMT molecular pathways including epigenetic alterations, miRNA-based methods to change the expression pattern, and TF-based mediated directed induction of signaling pathways [20]. Given that cellular differentiation is a continuous process regulated by different TFs, their application can be a practical approach to induce differentiation of cancer cells [25]. HNF4α is the key hepatogenesis TF which drives differentiation of stem and progenitor cells to mature hepatocytes and controls the acquisition of an epithelial phenotype [26–29]. In adult hepatocytes, HNF4α high expression is sustained in order to maintain the hepatocyte functions. HNF4α plays a pivotal role in the maintenance of epithelial/hepatocyte phenotype and regulates dynamic events of EMT by suppressing snail, the master regulator of EMT, and increasing E-cadherin in cancer cells [30, 31]. Downregulation of HNF4α has been demonstrated in HCC and its upregulation might accordingly suppress EMT and inhibit the progression of HCC [32, 33]. Several studies have shown that the induction of HNF4α activates the expression of various hepatocytic genes which enhances the differentiation of hepatocytes [34, 35].

The use of natural compounds as medications has gained significant attention [36, 37]. In 2009, a study showed that conjugated linoleic acid (CLA) acts as a natural intracellular ligand of HNF4α [38]. CLA is an 18-carbon essential free fatty acid with two double bonds, which were separated by a single bond that is why it is called conjugated. Natural forms of CLA are often found in ruminant products such as milk or cheese. The cis-9,trans-11 (c9,t11) is the common CLA isomer [39] and has shown its potential in treating some malignancies. Medical evidences have proved that the c9,t11 CLA isomer exerts its anticancer function by acting on apoptotic genes [40]. Despite many studies on the significance of HNF4α as an important target in preventing and treating liver malignancies, further investigations are still required to understand the mechanism and the correlation between CLA and HNF4α in regulating and inhibiting EMT. This study aimed to induce HNF4α expression by using c9,t11 isomer of CLA in order to reduce the cancerous phenotype (invasion and migration capacity) and proliferation rate of HCC cells.

2. Materials and Methods

2.1. Preparation of Chemical Compounds. The total amount of 50 mg conjugated linoleic acid (CLA, Sigma-Aldrich Co. Missouri, USA) was dissolved in 1780 μl absolute ethanol to prepare 0.1 M CLA stock. To prepare 3.9 μM BIM5078 and HNF4α antagonist (Sigma-Aldrich Co. Missouri, USA), 25 mg BIM5078 was dissolved in 1529 μl DMSO. All stocks were stored at −20 °C in dark.

2.2. Treatment of Hep-3B and Sk-Hep-1 cells with CLA. Two HCC cell lines taken from the Royan Institute cell bank were used in this study: Sk-Hep-1, an invasive endothelial hepatic carcinoma cell line, and Hep-3B, poorly differentiated primary liver cancer cells. No mutation in the HNF4α gene has been reported in both cell lines according to Broad Institute and CCLE databases.

Both cell lines were cultivated in Dulbecco’s modified Eagle’s medium (DMEM, high glucose, Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 1% GlutaMAX™ (Life Technologies), 1% non-essential amino acid (Life Technologies), 1% penicillin-streptomycin (Life Technologies), and 0.1% 2-Mercapto ethanol (Sigma-Aldrich), at 37 °C and 5% CO2. The medium was changed every day, and passing the cells was performed when 90% confluency was reached. The effects of treatments were evaluated twenty-four hours after cells plating, by using different concentrations of CLA or BIM in FBS free media for 48 h. The media were renewed every 24 h. After 48 h the samples were collected for analyses.

2.3. Cell Proliferation Assay. Sk-Hep-1 and Hep-3B cells were plated in 96-well plate (3 × 10³ cells/well) in 100 μl culture medium and incubated overnight. The dose escalation data for both compounds at different time points were presented in Supplementary Figure 1(b). Accordingly, The cells were treated with different concentrations of CLA (Sk-Hep-1: 30 and 60 μM, Hep-3B: 80 and 100 μM) or...
BIM (Sk-Hep-1: 390 and 780 nM, Hep-3B: 780 and 1170 nM). To assess the cell proliferation rate in those different experimental conditions, Orangu™ kit (Cell Guidance Systems, Cambridge, UK) was used at 24 and 48 h after treatment, while metabolic activity was quantified by measuring light absorbance at 450 nm [41] and compared to the control nontreated group.

2.4. Colony Formation Assay. To evaluate the impact of CLA treatment on colony formation ability of Sk-Hep-1 and Hep-3B cells, about 10 cells/cm² were seeded in a 6-well plate and cultured for four days in DMEM supplemented with 10% FBS. Then, Sk-Hep-1 and Hep-3B cells were, respectively, treated with 60 and 100 µM CLA or BIM and 780 nM and 1170 nM in serum free medium. After 14 days, the emerged colonies were fixed by using 4% formaldehyde and stained with 0.5% crystal violet. Imaging was performed by inverted microscope (Olympus CKX41). The total number and the surface area of each colony were measured by ImageJ software (version 1.46).

2.5. RNA Extraction and Quantitative Real-Time PCR. Total RNA was extracted using RNA extraction kit (Macherey Nagel, KG, Duren, Germany), and the RNA quality was checked by gel electrophoresis (1.5 × 10⁶ cells per test). Synthesis of cDNA was performed by reverse transcription of 1 µg total RNA using the cDNA synthesis kit (Life Technologies), according to the manufacturer’s instructions. Quantitative real-time PCR assays were carried out using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Data analysis was performed with the Applied Biosystems StepOne software v2.3. The C_i values of target genes were normalized vs the corresponding control groups. The primer sequences used are listed in the Supplementary Table S1.

2.6. Migration Assay. The scratch assay was used to determine the impact of CLA treatment on the migration ability of Sk-Hep-1 and Hep-3B cells. A total number of the cells/cm² were seeded in a 6-well plate and cultured for four days in DMEM supplemented with 10% FBS. Then, Sk-Hep-1 and Hep-3B cells were, respectively, treated with 60 and 100 µM CLA or BIM and 780 nM and 1170 nM in serum free medium. After 14 days, the emerged colonies were fixed by using 4% formaldehyde and stained with 0.5% crystal violet. Imaging was performed by inverted microscope (Olympus CKX41). The total number and the surface area of each colony were measured by ImageJ software (version 1.46).

2.7. Immunoﬂuorescence Staining. To evaluate the quality of hepatocytic differentiation, immunofluorescence analysis was performed to visualize the expression of liver speciﬁc proteins in both HCC cell lines after treatment with 60 or 100 µM CLA (Sk-Hep-1 and Hep-3B, respectively). Next, the samples were ﬁxed in 4% paraformaldehyde, permeabilized (Triton™ X-100, Merck, Burlington MA, USA, 108634), blocked using BSA 1%, and incubated with primary antibodies against ALB (Abcam Cat NO: ab106582, 1:200) overnight at 4°C in a moist chamber, followed by secondary antibody incubation for one h at 37°C (Antigoat, Alexa Flour 488, Invitrogen, 1:1000). Then, the nuclei were counterstained with DAPI, washed with PBS, and visualized using a fluorescence microscope (Olympus, IX7).

2.8. ALB Secretion Assessment. The impact of CLA treatment on ALB secretion in Sk-Hep-1 and Hep-3B cells was measured by enzyme-linked immunosorbent assay (ELISA) (Bethyl Laboratories, Montgomery, TX, USA). Sk-Hep-1 and Hep-3B cells were treated with 60 or 100 µM CLA in 6-well plates, respectively. The medium was refreshed every day. The supernatant was collected and ALB content measured by the ELISA kit according to the manufacturer’s instructions.

2.9. Periodic Acid-Schiff (PAS) staining. The glycogen storage in Hep3B cells, after eight days of treatment with 100 µM CLA, was visualized using periodic acid-Schiff (PAS) kit (Sigma-Aldrich Co. Missouri, USA) staining protocol. In brief, the cells were fixed in 4% paraformaldehyde for 20 min and then treated with periodic acid for 15 min. After washing with ddH₂O, cells were incubated with Schiff’s reagent for 5-20 min. The stored glycogen was visualized under a light microscope.

2.10. In Silico Data Analysis. The expression of HNF4α and its association with the EMT signatures [42] and differentiation markers were analyzed using TCGA-Bioblink package under R software across 372 live carcinoma samples deposited in The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/). The protein-protein interaction was performed and analyzed using online version of STRING software (https://string-db.org/).

2.11. Data Analysis. All experiments were performed at least in three biological replicates. The data were analyzed using Prism software (version 6.07; GraphPad Software, San Diego, CA, United States). Since the distribution of the quantitative data was normal, comparison between the groups was evaluated using the ANOVA test and Fisher’s least significant difference (LSD). The p ≤ 0.05 was considered as statistically significant.

3. Results

3.1. CLA Treatment Reduces Cell Proliferation in Liver Cancer Cell Lines. The expression status of HNF4α in Hep-3B and Sk-Hep-1 cell lines was initially evaluated by RT-qPCR, and data showed downregulation of HNF4α in both cell lines as compared to primary hepatocytes in 2D culture (Supplementary Figure 1(a)). Then, cell proliferation in Sk-Hep-1 and Hep-3B cells after treatment with CLA or BIM for 24 and 48 h was quantified. The cell proliferation rate for both cell lines significantly decreased in CLA treated groups as compared to the control groups in both time points in a dose-dependent manner by 30 and 55% for Sk-Hep-1 and 20 and 25% for Hep3B cells (Figure 1(a)), whereas BIM treatment...
Figure 1: Continued.
significantly increased cell proliferation rate in both cell lines. Furthermore, Sk-Hep-1 and Hep-3B cells which were treated with 60 and 100 μM CLA, respectively, showed a marked reduction in cell proliferation rate compared to the 30 and 80 μM CLA after 48 h. This data indicated that CLA treatment reduced the proliferation rate in both HCC cell lines in a dose- and time-dependent manner and at both time points (Figure 1(a) and Supplementary Figure 1(b)).

3.2. CLA Treatment Attenuates Colony Formation Capacity in HCC Cell Lines. To evaluate colony formation capacity of both cell lines in terms of number and total surface area of emerged colonies, the plated cells were treated with an optimized concentration of CLA and BIM for each cell line (Figures 1(b) and 1(c)). Data showed that the number of colonies in CLA treated groups significantly decreased in both cell lines, while BIM treatment augmented colony formation capacity compared to the control groups in both cell lines (Figure 1(d)). Moreover, CLA treated colonies had a smaller surface area compared to the control and BIM treated groups in both cell lines (Figure 1(e)). BIM treatment in both cell lines resulted in more extended colonies in comparison with control groups, respectively. Altogether, CLA treatment considerably changed the colony-forming capacity of both HCC cell lines, and the number and surface area of the colonies were decreased remarkably.

3.3. CLA Treatment Induced Downregulation of EMT-Related Genes. The expression of HNF4a, a central regulator of hepatocytic differentiation, SNAIL2, ZEB1, ZEB2, and CDH2 (N-CAD), EMT-related genes, and MMP14, a cell invasion marker, was assessed in both Sk-Hep-1 and Hep-3B cells after 48 h treatment with CLA and BIM at optimized concentration for each cell line. CLA treatment enhanced the expression of HNF4a in a dose-dependent manner in both cell lines as compared to the control groups. The upregulation of HNF4a was more than 2- to 6-fold in Sk-Hep-1 and Hep-3B cells, respectively, after treatment compared to the control groups. Regarding EMT-related genes, CLA treatment decreased the expression levels of SNAIL2, ZEB1&2, MMP14, and N-CAD in a dose-dependent manner, whereas BIM treatment induced the opposite in Sk-Hep-1 and Hep-3B cells (Figure 2). The expression of ZEB2 was reduced in CLA treated SK-Hep-1 cells but was essentially undetectable in Hep3-B cells (Figure 2). Protein-protein interaction (PPI) between HNF4a and EMT-related proteins highlighted strong association between HNF4a and CDH1, SNAI1, SNAI2, and ZEB2. Correlation coefficient analysis between expression of the HNF4a and top 50 epithelial and mesenchymal genes across liver carcinoma samples was performed [42]. These results indicate a strong negative association (R = −0.4) between HNF4a upregulation and reduction of mesenchymal-related genes involved in EMT program. These results highlighted that induction of HNF4a might suppress mesenchymal phenotype and reduce metastatic capacity of liver carcinoma cells.

3.4. CLA Treatment Reduced the Migration Capacity of Both HCC Cell Lines. Sk-Hep-1 and Hep-3B cells migration capacity was assessed after treatment with CLA and BIM for 48 h. The migration velocity of both cell lines was significantly reduced in CLA treated groups compared to control groups (Figures 3(a), 3(b), and 3(d)). Moreover, comparison of the total scratch area revealed that in CLA treated cells, the vacant area in the dish after 48 h was larger compared to the control groups in both mitomycin treated (+Mit) and non-treated (-Mit) cells (Figures 3(c) and 3(e)). In contrast, BIM treatment notably enhanced the migration capacity of the both cell lines as compared to the control groups (Figure 3).
3.5. CLA Improved Hepatocytic Differentiation of Hep-3B Cells through HNF4α Activation. Since the Sk-Hep-1 cells are stromal hepatic cancer cells and originated from endothelial cells, hepatocytic differentiation analysis and functional evaluations were presented only on Hep-3B cells in the manuscript. To assess the effects of CLA on the
Figure 3: Continued.
induction of differentiated phenotype of Hep-3B cell line, expression levels of albumin in CLA treated cells were analyzed eight days post treatment.

Significantly enhanced expression of ALB was shown in CLA treated Hep-3B cells, while BIM treatment decreased the expression level of this protein (Figure 4(a)). ELISA assay confirmed that CLA treated Hep-3B cells significantly secreted more ALB rather than BIM treated cells and respective control group (Figure 4(b)). In line with ALB protein expression level, ALB gene expression increased after treatment with 100 μM CLA compared to the control group (Figure 4(c)).

To perform more assessments in terms of differentiation evaluation in CLA treated Hep-3B cells, glycogen storage was also evaluated. Glycogen storage was notably enhanced following CLA treatment as shown by using PAS staining method. On the contrary, BIM treatment decreased glycogen storage compared with the control group (Figure 4(d)). Supplementary Figure 2 presents the hepatocytic differentiation analysis for Sk-Hep-1 cells after treatment with CLA. This treatment improved ALB expression based on IF staining. Moreover, CLA treatment significantly upregulated ALB and Cyp3A4 mRNA expression in Sk-Hep-1 cells.

3.6. In Silico Data Analysis Showed Strong Association between HNF4α and Specific Genes. Protein-protein interaction (PPI) between HNF4α and classical EMT-related proteins highlighted a strong association between HNF4α and CDH1, SNAI1, SNAI2, and ZEB2 (Figure 5(a)). Correlation coefficient analysis between expression of HNF4α and top 50 epithelial and mesenchymal genes derived from (PMID: 25214461) across liver carcinoma samples (n = 372) indicated a strong negative association (R = −0.4) between the upregulation of HNF4α expression and the reduction of mesenchymal-related genes involved in EMT program (Figure 5(b)). Compared to the mesenchymal genes, a weak negative association (R = −0.036) was observed between HNF4α expression and epithelial genes. These results highlighted that induction of HNF4α might suppress the mesenchymal phenotype and deteriorate the metastatic capacity of liver carcinoma cells. The scatter plot depicts positive association between HNF4α and the expression levels of ZO1 and ALB across 372 liver carcinoma samples from TCGA (Figure 5(c)).

4. Discussion

HNF4α is a liver-enriched TF that plays important roles including in gluconeogenesis and lipid metabolism [43, 44]. Numerous studies have shown that HNF4α expression is reduced in HCC patients in a stage dependent manner. Interestingly, the upregulation of HNF4α in cancer cells has been shown to be strongly associated with tumorigenesis suppression via induction of differentiation [45–47]. Overexpression of HNF4α is associated with a reduced proliferation rate and regulated expression of genes involved in the control of hepatocyte cell cycle [48]. Therefore, restoring the expression of HNF4α could be an influential milestone to reverse the HCC phenotype. Various molecular mechanisms control the expression of this TF at different levels, including epigenetic, transcriptional and posttranscriptional modifications [45, 49, 50]. Thus, several strategies have been employed to induce HNF4α overexpression in cancer cells using long non-coding RNAs, premade DNA vectors for HNF4α, miRNAs, small molecules, recombinant proteins, and growth factors [47, 51–60]. Recently, natural compounds have drawn much attention in the discovery and development of novel anticancer agents [61]. Natural compounds are bioactive ingredients produced by living organisms like animals, plants, fungi, and microorganisms that can selectively regulate signal transduction pathways and epigenetic mechanisms to modulate gene
expression [62]. Studies have shown that CLA as a natural
compound mainly found in ruminant products induces a
decreased proliferation rate of cancer cells [63]. The multiple
anticancerous effects of CLA were shown in a variety of can-
cers including HCC1. The literature was found that CLA
exerts anticancerous features though different mechanisms
including ER stress, autophagy, and PPAR γ. Our
finding revealed that CLA could reduce cancerous phenotypes such
as proliferation and colony formation, which were discussed
in the following section [64–66].

Considering the association between the overexpression of HNF4α and reduction in cancerous phenotype of HCC
cells, in the present study through a differentiation therapy
approach, we investigated the effect of HNF4α induced
eexpression in HCC cell lines after treatment with CLA, the
natural ligand of HNF4α. Hep-3B as primary and Sk-Hep-
1 as stromal liver cancer cell lines were assessed to show
whether CLA treatment can reduce various cancerous fea-
tures including proliferation rate, colony formation, and
migration capacity. Our results showed that CLA treatment
reduced proliferation rate, colony formation capacity, and
migration of cancerous cells, whereas the expression of
EMT-related genes was downregulated in a dose-
dependent manner, while ALB production and glycogen
storage capacity significantly increased. Results of a study
demonstrated that the two isomers of CLA, trans10, cis12
(t10, c12), and c9,t11 have essential roles in growth inhibition
in colon and prostate cancers. Treatment of Caco-2 cells
Figure 5: In silico data analysis. (a) Analysis of protein-protein interaction (PPI) between HNF4a and classical EMT-related proteins highlighting a strong association between HNF4a and CDH1, SNAI1, SNAI2, and ZEB2. (b) Correlation coefficient analysis between expression of HNF4a and top 50 epithelial and mesenchymal genes derived from (PMID: 25214461) across liver carcinoma samples (n = 372). These results indicate a strong negative association (R = −0.4) between the upregulation of HNF4a expression and the reduction of mesenchymal-related genes involved in EMT program. Compared to the mesenchymal genes, a weak negative association (R = −0.036) was observed between HNF4a expression and epithelial genes. These results highlighted that induction of HNF4a might suppress the mesenchymal phenotype and deteriorate the metastatic capacity of liver carcinoma cells. (c) The scatter plot depicts positive association between HNF4a and the expression levels of ZO1 and ALB across 372 liver carcinoma samples from TCGA.
with c9,t11 and t10,c12 isomers changed the expression pattern of lipid metabolism-related genes. Moreover, CLA treatment inhibited cell proliferation in breast cancer cells, and at the cytostatic concentration, CLA treatment caused cell cycle arrest in G1 [67]. Furthermore, in another study, the expression of HNF4a was upregulated after treatment with t10,c12 CLA [28]. However, the mechanism of CLA action on cell proliferation in various cancer cells was not clearly defined [68]. Correlation coefficient analysis between the expression of HNF4a and top 50 epithelial and mesenchymal genes across liver carcinoma samples demonstrated a strong negative association between the upregulation of HNF4a expression and reduction of mesenchymal-related genes involved in EMT process. Our data were also supported by in silico data and were in line with other studies [42].

The results suggested that CLA treatment may significantly reduce invasiveness capacity of cells through the reduction of EMT markers at the transcription level (Figure 2). On the other hand, substantial experimental evidence supports the contribution of hepatocytes that undergo EMT that form myofibroblasts in the injured liver. Therefore, it seems that CLA treatment can also prevent progression of liver fibrosis through EMT suppression [69].

The expression of HNF4a isoforms is tissue specific, and the liver expression pattern of HNF4a was remarkably changed during HCC progression. In this study, c9,t11 CLA isomer is used to induce HNF4a expression. Our results showed that CLA treatment of Sk-Hep-1 as the most invasive and endothelial tumor, and Hep-3B as the most undifferentiated HCC cell line, could reduce the proliferation rate as well as the number and size of colonies in a dose- and time-dependent manners. Some studies have shown a negative feedback loop between HNF4a and EMT-related genes [9, 70]. In our study, CLA treatment significantly increased the expression of HNF4a while inducing a significant down-regulation of EMT-related genes such as Snail in both cell lines. Furthermore, CLA treatment reduced the invasiveness of HCC cells and improved their hepatocytic differentiation phenotype, i.e., ALB secretion and glycogen storage.

The reduction of CLA in NAFLD and diabetic obese patients demonstrated the correlation between CLA and regulation of energy metabolism and maintenance of metabolic homeostasis in the liver [71]. CLA can also induce significant changes in the fatty acid profile of the liver [72]. In our study, we found that CLA treatment can also regulate the metabolic activity of cancerous cells and induce the expression of ALB and improve glycogen storage capacity. Altogether, our results, in correlation with our in silico findings, indicated the importance of HNF4a in mediating the EMT and MET in HCC cells as supported by the effects of CLA treatment on hepatocytic differentiation of HCC cells and the reduction of their cancerous features. Altogether, these results suggested that CLA might be used as a novel and natural differentiation inducing component for liver carcinoma cells. Our findings were acquired from in vitro experiments, and in near future we will evaluate such inhibitory effects of CLA on animal models to provide more reliable evidence to further clinical investigation.

### Data Availability

Data is available on request.

### Conflicts of Interest

The authors declare no conflict of interest.

### Authors’ Contributions

Z.M.L. performed the experiments and data collection. S.T. drafting the manuscript, data collection, and drafting the results. B.S., R.S, and P.A. contributed in the development of concept, study design, and critical reviewing the manuscript. Z.H., A.H., and E.Z. contributed in study design and set up for experiments, data analysis, and reviewing the manuscript. A.P., Z.F., and M.H. contributed in study design, data collection, data analysis, and drafting the results. H.A.E and A.N. developed the concept and contributed in critical reviewing and final approval of the manuscript. M.N. and M.V. developed the concept and contributed in data analysis, critical reviewing, and approval of the manuscript. M.V. contributed to the financial support of the project. Zohre Miri-Lavasani and Shukoofeh Torabi contributed equally to this work.

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### Supplementary Materials

Supplementary Figure 1: (a) Relative mRNA expression of HNF4a in both HCC cell lines, Sk-Hep-1, and Hep-3B cells. The transcription level of HNF4a is very low in Hep-3B and Sk-Hep-1 cells. The expression of HNF4a was normalized to primary hepatocytes. (b) The proliferation rate of Sk-Hep-1 and Hep-3B cells was measured after treatment with different concentrations of CLA and BIM in two time points 24h and 48h. Treatment with CLA reduced the proliferation rate in a dose dependent manner in Sk-Hep-1 cells at both time points. Hep-3B cells also showed significant decrease in proliferation rate after treatment with CLA at both time points. BIM treatment induced proliferation rate in both cell lines at both time points. Data are presented as the mean ± SD, n = 3 (∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001, and ∗∗∗∗p < 0.0001). Supplementary Figure 2: (a) Immunofluorescence staining induced the expression of ALB in CLA treated Sk-Hep-1cells as compared to the BIM treated and control groups. ALB expression is suppressed after treatment with BIM. (b) The relative mRNA expression of the ALB, E-Cad, and CYP3A4 genes was increased after treatment with CLA in Sk-Hep-1cells as compared to the control group. BIM treatment resulted in significant decrease in the E-Cad and ALB genes. Data are presented as the mean ± SD, n = 3 (∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001, and ∗∗∗∗p < 0.0001). Supplementary Table 1. The list of primers used in this study. (Supplementary Materials)
References


