

CD36 accelerates the progression of hepatocellular carcinoma by promoting FAs absorption

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Abstract

Background: CD36 is emerging as a potential strategy for cancer treatment because of its function of regulating fatty acid intake. The purpose of this study was to clarify the molecular mechanism of CD36 in the progression of HCC.

Methods: TCGA database was used to analyze the relationship of CD36 with HCC. The expression of CD36 in HCC clinical samples and cell lines was detected by qRT-PCR and western blot. Huh7 cells and HCCLM3 cells were transfected and treated into different group. CCK-8 and clone formation assay were used to detect the cell proliferation ability. Wound healing and transwell experiment were used to detect the metastatic ability. HCC xenografts were constructed in nude mice by subcutaneous injection of stably transfected Huh7 cells. The expression of CD36 in HCC was detected by immunohistochemistry (IHC). The contents of phospholipids and triglycerides in HCC cells were detected by ELISA. And the content of neutral lipids in HCC cells was detected by staining with BODIPY 493/503 and DAPI dye. Then transcriptional sequencing was used to determine the downstream mechanism of CD36 in HCC, and the differentially expressed genes (DEGs) were analyzed.

Results: CD36 was up regulated in HCC. Knockdown of CD36 could suppress the proliferation and metastasis of HCC in vitro and in vivo by regulating FAs intake in HCC. In addition, the expression of AKR1C2 was suppressed by sh-CD36, and which was also involved in the regulation of FAs intake.

Conclusion: The molecular mechanism by which CD36 accelerated the progression of HCC was to promote the expression of AKR1C2 and thus enhance fatty acids (FAs) intake.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, ranks third in cancer-related deaths in the world [1, 2]. Generally speaking, surgical resection or liver transplantation is the potentially curative treatment for early detection of liver cancer [3, 4]. However, most patients are diagnosed at an advanced stage of liver cancer, thus radiotherapy and chemotherapy are either infeasible or ineffective [5]. Consequently, it is of great significance to study and analyze the mechanism of the occurrence and development of HCC for the development of new treatment methods.

Fatty acids (FAs), consist of a terminal carboxyl group and a hydrocarbon chain, are required for energy storage, membrane proliferation, and the generation of signaling molecules [6]. The metabolism of cancer cells frequently exhibit characteristic alterations, in which cell proliferation is a common feature of all cancers [7]. FAs metabolism participates in cell proliferation by synthesizing cell membrane and signal molecules. Therefore, lipid metabolism is considered as one of the most important metabolic hallmarks of cancer cells, and activation of FAs synthesis is required for tumor cell survival [8, 9]. And there is evidence that limiting FAs availability can control cancer cell proliferation [10, 11]. Epidemiological studies have proved that there is a link between the elevated FAs caused by obesity and the occurrence of

HCC and high mortality [12, 13]. In addition, miR-4310 could affect the growth and metastasis of HCC tumor by regulating the FASN/SCD1-mediated lipid synthesis pathways [14].

Cluster of differentiation 36 (CD36) is a kind of intact transmembrane glycoprotein expressed in a variety of tissues. Researchers found that it can participate in the high affinity uptake of long-chain FAs [15]. Previously, CD36 has been reported to promote lipid peroxidation and dysfunction in CD8 + T cells by uptaking of oxidized lipids in tumors, resulting the alteration of the tumor microenvironment (TME) [16]. And CD36 could promote melanoma angiogenesis by regulating adhesion to the extracellular matrix [17]. CD36 or FAs translocase protein mediates FAs intake in many cell types, while its expression level is very low in normal hepatocytes. Nevertheless, its expression level is notably enhanced in hepatocytes of diet induced obese rodent model, which has also been proved to be associated with increased FAs intake [18, 19]. Through the above researches, we speculate that the change of CD36 expression may promote the progression of HCC by enhancing the intake of FAs in HCC cells. Aldo-keto reductases family 1 member C2 (AKR1C2), a member of the aldo/keto reductase superfamily, can metabolize dihydrotestosterone (DHT) into 5 α -androstane-3 α , 17 β -diol (3 α -diol)[20, 21]. Previous studies have shown that it is pivotal in the development of urogenital and gastrointestinal cancers [22, 23].

In the research work, we intended to elucidate the role of CD36 in HCC progression and explore the potential molecular mechanisms. We comprehensively analyzed that CD36 induced fatty acids intake through regulating AKR1C2, and then affects the development of HCC in vivo and in vitro. Based on this study, we confirmed CD36 exerts a novel role in mediating FAs intake and suggest that it might be a therapeutic target for HCC.

Methods

Cell culture and treatments

Hepatocyte cells (LO2) and HCC cell lines (Hep3B, Huh7, HCCLM3 and MHCC-97H), were cultured in DMEM medium (Gibco) containing 10% FBS (Gibco) with 5% CO₂ at 37°C. For gene knockdown or over expression, Huh7 and HCCLM3 cells were grown to confluence and transfected with shRNA (sh-CD36, TTGTACCTATACTGTGGCTAAATGAGAC) or shcontrol using lipo3000 (Life Technologies) for 48 h, then the protein and mRNA were extracted for protein and RNA expression analysis. For induced steatosis, Huh7 cells were cultured in six-well plates at 5 \times 10⁵ cells/well. The cells were treated with 0.4 mM palmitic acid (PA) or 1% bovine serum albumin (BSA)solution for 24 h and used for laboratory analyses.

Clinical samples

To verify the expression of CD36 in HCC, tumor and adjacent normal tissue samples were obtained from 48 HCC patients at the Affiliated Hospital of Yangzhou University. All patients underwent surgical resection and were followed up until December 2020. The median follow-up time was 25.5 months (range, 15–70). All the enrolled patients provided written informed consent. Each tissue sample was diagnosed as HCC by two different professional pathologists.

qRT-PCR analysis

As described above, RNA was isolated and cDNA was obtained using primescrip RT Master Mix (Takara, Tokyo, Japan). Gene expression of CD36 and AKR1C2 was quantified by the StepOnePlus Real-Time PCR System (Life) and ChamQ SYBR Color qPCR Master Mix (Vazyme Biotech, Nanjing, China). Primer pair sequences were as follows: CD36 forward: 5'-GAACCACTGCTTTCAAAAAGTGG-3' and reverse: 5'-GTCCTGAGTTATATTTTC CTTGG-3'; AKR1C2 forward: 5'-CTGTGAGGGAGGAAGAAACATTTGCTAA-3' and reverse: 5'-GGCTTCTATTGCCAATTTGACGGC-3'; β -actin, forward: 5'-GG GAAATCGTGCGTGACAT-3' and reverse: 5'-CTGGAAGG TGGACAGCGAG-3'.

Western blot

Total proteins were extracted from cells and liver tissues with lysis buffer, then quantified by BCA (Pierce, Rockford, IL, USA). Separating proteins via SDS-PAGE and then transferring onto PVDF membranes (Invitrogen, USA) by electroblotting. Subsequently the membranes were blocked and followed by incubation with primary antibodies. Primary antibodies: CD36 (1:5000, Abcam, ab133625), AKR1C2 (1:5000, Abcam, ab179448), FASN (1:10000, Abcam, ab128870), ACC1 (1:5000, Abcam, ab109368), FABP5 (1:1000, Abcam, ab255276) β -actin (1:5000, Abcam, ab8226) and secondary antibody IgG (1:10000, Abcam, ab205718). Protein bands were observed using an enhanced chemiluminescence kit (Pierce, Rockford, USA).

Animal experiment

5–6-week-old nude BALB/c mice (Model Animal Research Center of Nanjing University, Nanjing, China) were raised to study the effect of CD36 on HCC development in vivo. The mice were divided into 3 groups with 8 mice in each group by random numbers. Huh7 cells (2×10^6) were stably transfected with sh-CD36 and sh-control group, then the cells were subcutaneous injected to establish the transplanted tumor model in nude mice. One week after vaccination, the tumor growth was recorded via measuring the tumor volume every week. 5 weeks after inoculation, tumor samples were collected and weighted.

Hematoxylin and eosin (H&E) staining

The liver tissue was fixed at 4°C in 4% paraformaldehyde for 24 h, then dehydrated, embedded in paraffin and sectioned into 4 μ m sections subsequently for H&E staining. The results of stained tissue sections were evaluated by a committee certified pathologist.

Immunohistochemistry (IHC)

HCC tissues were consecutively cut into 4- μ m slices and then mounted on slides. The sections were dewaxed and then washed with Tris-buffered saline (TBS). Antigen retrieval was performed using EDTA antigen repair solution. After soaking and blocking, the sections were incubated with a primary antibody against AKR1C2 (1:500, Abcam, ab96087) overnight at 4°C. The following day, the sections were soaked in TBST, then incubated with an anti-rabbit secondary antibody for 45 min at 37°C, washed, stained with

hematoxylin, and sealed with a sealing agent. After drying, the sections were examined and photographed under an microscope (Olympus, BX63).

Quantification of phospholipids and triglycerides

According to the procedure provided in the kit, we measured the contents of intracellular phospholipids and triglycerides content were assayed by EnzyChrom™ phospholipids assay kit and EnzyChrom™ triglycerides assay kit from Hayward (BioAssay Systems, CA, USA) respectively.

Quantification of neutral lipid

The lipophilic fluorescence dye BODIPY 493/503 (Invitrogen) was employed for monitoring the neutral lipid accumulation in A549 cells according to the manufacturer's instructions. Briefly, cells were washed in Phosphate Buffered Saline (PBS), fixed with 4% paraformaldehyde and stained with BODIPY 493/503 (1 µg/mL) for 45 min at room temperature, and then nuclei were counterstained with Hoechst for 15 min. The results of immune staining were detected using a fluorescence microscope (Olympus, Tokyo, Japan).

CCK-8 assay

Cells were cultured in 96 well plates, CCK-8 analysis was used to evaluate cell viability of Hun7 cells and HCCLM3 cells. We added Cell counting kit-8 (CCK-8) solution (10 µL of CCK-8, Dojindo, Japan) to each well, and then cultured for 1–3 h at 37°C. The absorbance at 450 nm was measured by a microplate reader (Thermo,USA), the obtained data were sorted and analyzed.

Clone formation assay

Hun7 cells and HCCLM3 cells were seeded in 6-well plates and further transfection or treatment was carried out as required by the experiments (800 cells/well). After cultured for about 10 days, Hun7 cells and HCCLM3 cells were fixed with 4% paraformaldehyde. Further, the plates were washed and dried. Finally, the number of clones were counted (> 50 cells/clone).

Wound healing assay

Post 48 h transfection, Hun7 cells and HCCLM3 cells were reseeded in 6-well plates. Subsequently, a straight scratch wound was created in the center of each well with a micropipette tip. Scratch width was monitored to the length at 0 h or after 48 h with inverted microscope (Olympus, Japan). The migration of the cells was assessed by measuring and comparing the gap area caused by the scratch in the well.

Cell invasion assay

For the cell invasion assay, matrigel (BD Biosciences, 354480, USA) was used to precoat the chamber (Corning, 3422, USA) and incubated for 1 h. Then Hun7 cells and HCCLM3 cells (5×10^5 cells) were seeded on the upper chamber, and DMEM medium containing 20% FBS was added to the lower chamber. After 12 h incubation, all cells were fixed and stained, then staining the invaded cells with Calcein-AM and counting under a microscope (200 × magnification) in at least five random fields.

Transcriptome sequencing

For whole transcriptome sequencing, RNA from 1×10^7 Huh7 cells of different transfection groups was extracted using the Trizol reagent kit (Invitrogen, Waltham, MA, USA) and then quantified using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The whole transcriptome RNA-seq was performed using the Ion Total RNA-Seq kit, the Ion PI™ Chip kit, the Ion PI™ Template OT2 200 kit, and the Ion PI™ Sequencing 200 kit based on the protocols of Life Technologies Corp (Waltham, MA, USA). In brief, 100 µg of total RNAs for each sample was purified using oligo-dT beads and then fragmented. Reverse-transcribing the cleaved RNA fragments into first-strand cDNA, followed by second-strand cDNA synthesis. After that, a single 'A' base was added to the cDNA fragments at the 3' end. PCR amplification was used to generate the final cDNA library. Finally, RNA-seq was performed on the sequencing template according to the standard protocol with the ion proton system (Life Technologies).

Statistical analysis

All graphs were statistically analyzed using Graphpad Prism 8.0 (GraphPad Software, La Jolla, USA), and the results of three experiments were used for SEM detection. The differences between two groups were compared with Student's *t*-test. Data of this study were shown as mean \pm SD. $P < 0.05$ was considered statistically significant.

Result

CD36 was up regulated in HCC and associated with poor prognosis of HCC patients

We first analyzed the expression of CD36 in HCC by using TCGA database. The results showed that CD36 was significantly over expressed in HCC (Fig. 1A). Further, the level of CD36 expression was determined in HCC clinical samples by qRT-PCR. Among them, 48 biopsy HCC tissues and the paracancerous tissues were collected from patients before chemotherapy at the Affiliated Hospital of Yangzhou University. CD36 Expression was significantly up-regulated in HCC tissues compared with non-tumoral liver tissues (Fig. 1B). Western blot showed that the expression of CD36 in 5 pairs of cancer tissues was higher than that in adjacent tissues (Fig. 1C). Immunohistochemical results again confirmed the high expression of CD36 in HCC (Fig. 1D). Moreover, consistent with the results of HCC tissues, the detection results of qRT-PCR and western blot show that CD36 was significantly over expressed in HCC cell lines compared with normal hepatocytes (Fig. 1E-F). These results suggest that CD36 plays a potential role as a novel biomarker of HCC progression.

CD36 promotes the proliferation and metastasis of HCC in vitro

To determine whether CD36 can affect the function of HCC cells. First, We knockdown CD36 expression in HCC cell line, Hun7 and HCCLM3, and qRT-PCR was used to detect the transfection efficiency (Fig. 2A). The result of western blot showed the expression of CD36 protein in sh-CD36 group was significantly

lower than that in sh-control group(Fig. 2B). CCK-8 and clone formation assay were used to detect the cell proliferation ability of each transfection group. The results showed that the OD450 value and colony forming efficiency of CD36 knockdown group were significantly lower either in Hun7 cell or in HCCLM3 cell compared with the sh-control groups (Fig. 2C-D). Wound healing and transwell experiment were used to detect the metastatic ability of HCC cells. We observed that knockdown of CD36 significantly inhibited the migration and invasion of Hun7 cell and HCCLM3 cell (Fig. 2E-F). The above data showed that knockdown of CD36 could suppress the proliferation and metastasis of HCC cells in vitro.

CD36 promotes the proliferation and metastasis of HCC in vivo

In order to further verify the regulatory role of CD36 on HCC in vivo, we designed a tumorigenesis experiment in nude mice. We constructed HCC xenografts in nude mice by subcutaneous injection of stably transfected Huh7 cells. Then the tumor tissues of nude mice in each group were dissected (Fig. 3A). After subcutaneous injection for 5 weeks, the mean tumor volume for the NC groups were more and more apparently larger than their CD36 knockdown treating groups, respectively (Fig. 3B). As expected, the tumor weight statistic of excised tumor showed a similar trend to that of tumor volume (Fig. 3C). HE staining showed that knockdown of CD36 apparently repressed the tumor growth (Fig. 3D).The results of IHC detection showed that the expression of CD36 and Ki-67 decreased prominently after CD36 knockdown (Fig. 3E). The above results showed that CD36 could affect the occurrence and development of HCC in vivo.

CD36 regulates FAs metabolism to enhance proliferation and metastasis of HCC cells

Studies have shown that CD36 could promote the intake of FAs. We speculate that CD36 may promote the proliferation and metastasis of HCC by helping to improve the absorption of FAs in HCC cells. In order to verify this hypothesis, we first detected the contents of phospholipids and triglycerides in HCC cell lines by ELISA. The result substantiated that sh-CD36 treatment decreased nearly three folds of cellular phospholipids compared with control Hun7 cells or HCCLM3 cells (Fig. 4A). And cellular content of triglycerides was apparently reduced in sh-CD36 group compared with control in Hun7 cells or HCCLM3 cells (Fig. 4B). Furthermore, the content of neutral lipids in HCC cells was detected by staining with BODIPY 493/503 and DAPI dye. The result showed that knockdown of CD36 prominently decreased the neutral lipids content in Hun7 cells or HCCLM3 cells (Fig. 4C). In addition, to further explore the act on of CD36 on FAs intake, the protein expression levels of key lipid metabolic enzymes FASN, ACC1 and FABP5 were analyzed by western blot and the result demonstrated that sh-CD36 treatment obviously inhibited the expression levels of key lipid metabolic enzymes in Huh7 cells (Fig. 4D). The above results revealed that CD36 could affect the FAs intake in HCC cells, and the content of FAs decreased dramatically after knockdown of CD36 in HCC cells.

Further, to prove that CD36 promotes the proliferation and metastasis of HCC cells by promoting the intake of FAs in cells, we designed different treatment groups, and then tested the proliferation and metastasis of Huh7 cells in these groups. The results of CCK-8 and clone formation assay showed that compared with the sh-control group, there was no significant difference in the cell proliferation ability of BSA + sh-control treatment group, the cell proliferation ability of PA + sh-control treatment group was significantly increased and was the highest in all groups, the cell proliferation ability of sh-CD36 group was greatly decreased and was the lowest in all groups, compared with sh-CD36 group, the cell viability in PA + sh-CD36 treatment group was slightly increased, but it was significantly lower than that of PA + sh-control group (Fig. 4E-F). Wound healing and transwell experiment were used to detect the metastatic level of HCC cells. The result trend of each group is consistent with the cell proliferation ability, we found that compared with sh-control group, there was no significant change in cell metastasis in BSA + sh-control group, the cell metastasis level of PA + sh-control was the highest, sh-CD36 group was the lowest, and the cell viability in PA + sh-CD36 treatment group was significantly lower than that of PA + sh-control group (Fig. 4G-H). These results revealed that CD36 could participate in the occurrence and metastasis of HCC by regulating the intake of FAs.

Impact of CD36 silencing on gene expression profile in Huh7 cells

To further determine the downstream mechanism of CD36 in HCC, we explored the effect of silencing CD36 in HCC cells. Huh7 cells treated with shRNA (sh-CD36) or control shRNA (sh-control) were then subjected to comparative transcriptional sequencing. A total of 1121 differentially expressed genes (DEGs) were obtained by transcriptome sequencing, of which 412 were down-regulated and 709 were up-regulated. Then DEGs were analyzed by volcanic plot to characterize the distribution of fold changes and the *P* values of all genes (Fig. 5A). The 50 genes with the highest differential expression were analyzed by heat map of expression hierarchical cluster analysis to study the probable downstream mechanism of CD36 (Fig. 5B). Combined with the previous literature, AKR1C2 in the top 50 genes was reported to be related to FAs metabolism [24]. Then we verified the regulatory relationship between CD36 with AKR1C2 in vitro via transfecting Huh7 cells with knockdown or overexpression of CD36. qRT-PCR and western blot analysis showed that CD36 could promote the mRNA and protein expression of AKR1C2 (Fig. 5C-D). From the above data, we speculated that CD36/NF axis affect the FAs metabolism of HCC cells, thus participate in the progression of HCC.

CD36 enhance the proliferation and metastasis of HCC cells by increasing FAs intake through AKR1C2

We designed rescue experiment to confirm that CD36 and AKR1C2 jointly affect FAs metabolism of HCC cells. Huh7 cells were transfected with over expressed CD36 and silenced AKR1C2, then transfection efficiency was detected (Fig. 6A). The result reflected that over expressed CD36 prominently elevate the contents of phospholipids, triglycerides and the neutral lipids in Huh7 cells. While silencing AKR1C2 apparently reversed the effect of CD36 on cellular FAs intake, the contents of phospholipids, triglycerides

and the neutral lipids depressed significantly (Fig. 6B-D). Whatsmore, we proved that oe-CD36 treatment markedly enhanced the expression levels of FASN, ACC1 and FABP5, the key lipid metabolic enzymes, in Huh7 cells. Correspondingly, silencing AKR1C2 reversed the effect of oe-CD36 on the expression of these key proteins (Fig. 6E).

In order to further explore the mechanism of CD36 accelerating the progression of HCC, we designed rescuing experiment to detect the effect of CD36 / AKR1C2 axis. The Huh7 cells were treated with control or PA, then transfected with sh-AKR1C2, oe-CD36 or negative control. CCK-8, clone formation assay, wound healing assay and Transwell experiment were used to detect the proliferation and metastasis of HCC cells, respectively. The results demonstrated that overexpression of CD36 greatly increased the level of cell proliferation and metastasis of Huh7 cells, while silencing AKR1C2 could dramatically reverse and neutralize the high level of cell proliferation and metastasis caused by PA treatment or oe-CD36 transfection (Fig. 6F-H).

Discussion

CD36 is expressed in many cell types, which mediates immune recognition, inflammation, molecular adhesion, apoptosis and lipid uptake as a scavenger receptor [25–27]. It has been demonstrated that CD36 could regulate the proliferation, metastasis, and angiogenesis of different tumor types, such as Esophageal squamous cell carcinoma [28], gastric cancer [29], and leukemia [30]. The role of CD36 in HCC is usually related to the tumor microenvironment, and the procedure is to participate in the progression of HCC mediated by tumor metabolic reprogramming [31, 32]. Luo et al. found that CD36 exerts a stimulatory effect on the progression of HCC, and its molecular mechanism is mediating aerobic glycolysis by the Src/PI3K/AKT/mTOR signaling pathway, thus promote the proliferation, migration and invasion of HCC cells [33]. The mechanism of CD36 in NAFLD-associated HCC has also been reported, CD36-Nogo-B-YAP pathway consequently reprograms metabolism and induces carcinogenetic signaling for NAFLD-associated HCC [34]. Moreover, CD36 cooperates with hepatic stellate cells (HSCs)-derived COMP and subsequently played an considerable role in MEK/ERK and PI3K/AKT pathway mediated HCC progression [32]. In our study, we first confirmed that CD36 was up regulated in HCC and associated with poor prognosis of HCC patients. Then, cell experiment and tumor bearing nude mice experiment showed that CD36 could reinforce the ability of proliferation and metastasis of HCC in vitro and in vivo.

Although the above studies support that CD36 does significant in tumors, the most well-known function of CD36 remains as a FAs receptor. Previous studies have proved that CD36 was of great importance in FAs metabolism [35, 36]. And CD36 deficiency can reduce the uptake of FAs in a variety of tissues, including heart, skeletal muscle, and adipose, in humans and mice [36]. CD36 can also form a complex with other proteins to maintain FAs β -oxidation by regulating the activation of AMPK signaling pathway [37]. CD36 palmitoylation disrupts free FAs metabolism and promotes liver tissue inflammation, thus leading to the progression of nonalcoholic fatty liver disease (NAFLD) [38]. CD36 is a negative regulator of autophagy, and the induction of lipophagy by ameliorating CD36 expression can be a potential therapeutic strategy for the treatment of fatty liver diseases through attenuating lipid over accumulation

[39]. Moreover, studies indicated that loss of CD36 impaired insulin action and activated inflammatory response of the liver [40–42]. These above data indicate CD36 is an important regulator of lipid homeostasis. As a result, we further explored the regulatory effect of CD36 on fatty acid metabolism in HCC cells. The results showed that knockdown of CD36 markedly repressed the content of phospholipids, triglycerides and neutral lipids in HCC cells, and the expression of key lipid metabolic enzymes FASN, ACC1 and FABP5. Then in the follow-up study, we also proved that CD36 regulates cell proliferation and metastasis by affecting the intake of fatty acids in HCC cells.

What's more, we transfected Huh7 cells with shRNA (sh-CD36) or control shRNA (sh-control), then the DEGs after silencing CD36 were sorted and analyzed. Among them, AKR1C2 showed significantly low expression, suggesting a positive regulatory relationship between AKR1C2 and CD36. AKR1C2 is one of four human hydroxysteroid dehydrogenases in the aldo-keto reductase (AKR) superfamily, which is involved in the metabolism of steroids and other carbonyl compounds including drugs, and AKR superfamily is related to the pathogenesis of several extrahepatic cancers. Besides, the effective competitive inhibitor of AKR enzyme is unsaturated FAs [43]. Notwithstanding, some former research indicates the AKR1C2 expression level is reduced in breast cancer [44, 45], thyroid carcinoma [46], prostate cancer [47], which seems to contradict our results. However, there are still many studies have confirmed the identity of AKR1C2 as a cancer promoting factor, which supports our results. A recent study suggested that both AKR1C2 and AKR1C3 mediate similar PGD2 conversion toward the accumulation of proliferative signals through FP and PI3K/Akt signaling pathways to promote prostate cell proliferation [48]. Most important of all, the role of AKR1C2 in HCC was considered to be a cancer promoting factor. Cong Li et al. used high-throughput sequencing to screen potential genes related to metastasis of HCC and found that AKR1C2 is a positive regulator of liver cancer metastasis. Subsequently, further clinical results confirm its role in promoting liver cancer [49]. In addition, They have also identified AKR1C2 (positive factor), as the AEG-1 downstream players, accelerates the progression of liver cancer by regulating cell migration, cell invasion, cell proliferation, and EMT [50]. The dual identity of AKR1C2 in cancer progression may be due to the specificity of tumor type, stage and tissue expression.

In conclusion, the molecular mechanism of CD36 involved in the development of HCC may be through positively regulating the expression of AKR1C2 and jointly affecting fatty acid metabolism, so as to promote the proliferation and metastasis of HCC. In addition, the expression of CD36 is of great value in the diagnosis, evaluation and prognosis of HCC.

Declarations

Acknowledgments

No potential conflicts of interest are disclosed.

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Figures

Figure 1

CD36 was up regulated in HCC

Bioinformatics websites and databases were used to predict and analyze the role of CD36 in HCC, then clinical tissue samples of HCC patients were collected, hepatocyte cells and HCC cell lines were cultured, and the expression of CD36 in HCC tissues and cell lines was detected

(A) TCGA database was used to analyze the expression level of CD36 in HCC. (B) qRT-PCR was used to detect the expression of CD36 in 48 clinical samples of HCC. (C) The expression of CD36 in 5 clinical samples was detected by Western blot. (D) The expression of CD36 in HCC samples was detected by IHC. (E) qRT-PCR and (F) western blot were used to detect the expression of CD36 in normal hepatocytes and HCC cell lines. Data are shown as mean \pm SEM (n=3). Asterisks indicate significant differences from the control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 2

CD36 affects the proliferation and metastasis of HCC in vitro

Hun7 cells and HCCLM3 cells were transfected with sh-CD36 and sh-control, then the effect of CD36 differential expression on the proliferation and metastasis of HCC cells were detected. NC means normal control.

(A) qRT-PCR was used to detect the transfection efficiency of CD36 in Hun7 cells and HCCLM3 cells. (B) Western blot was used to detect the protein expression of CD36 in Hun7 cells and HCCLM3 cells. (C) CCK-8 and (D) clone formation assay were used to detect the cell proliferation ability in each transfection group. (E) Wound healing assay was used to detect the cell migration ability in each transfection group. (F) Transwell experiment was used to detect the cell invasion ability in each transfection group. Data are shown as mean \pm SEM (n=3). Asterisks indicate significant differences from the control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Fig.3 CD36 affects the proliferation and metastasis of HCC in vivo

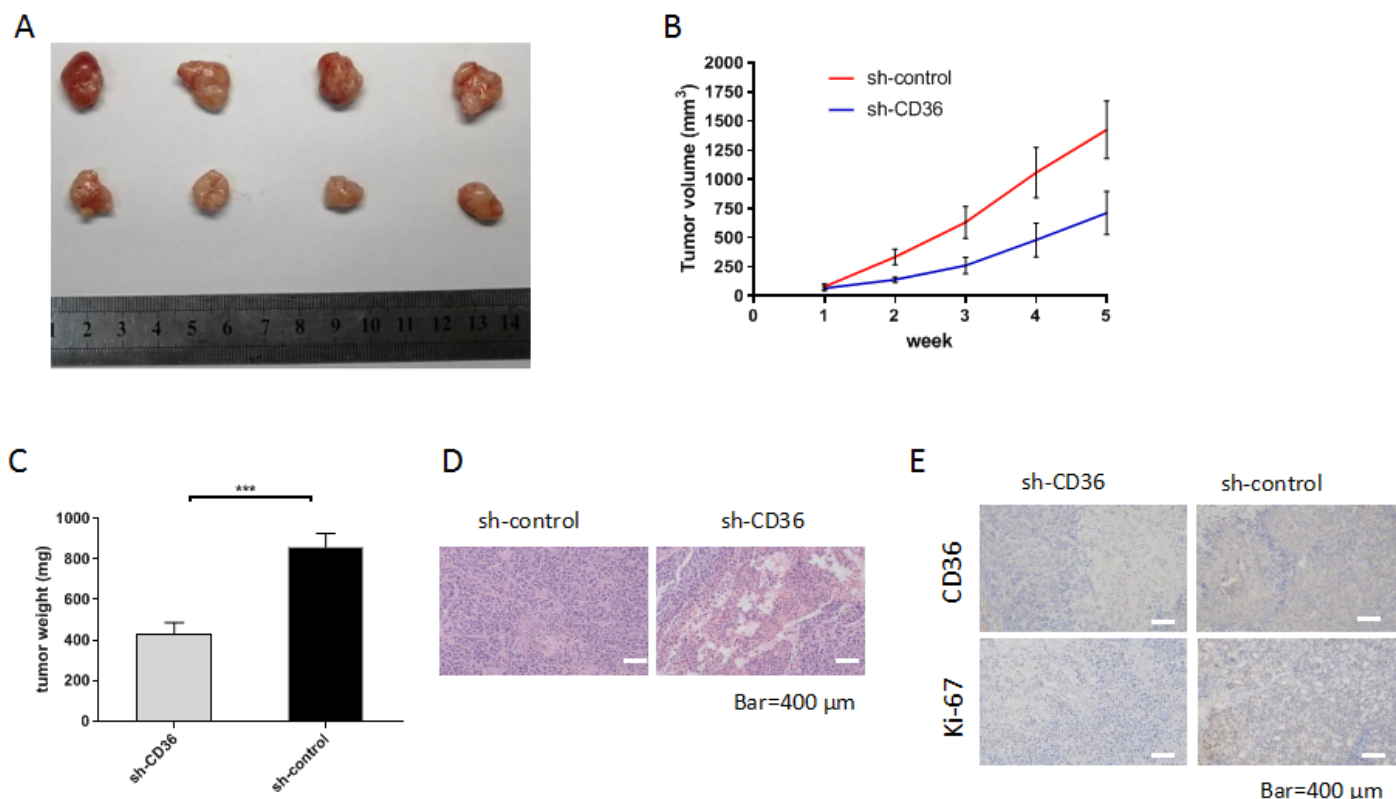


Figure 3

CD36 affects the proliferation and metastasis of HCC in vivo

The Huh7 cells were stably transfected with sh-CD36 and sh-control, then injected into nude mice to construct HCC subcutaneous transplantation tumor. The tumor volume was recorded every week, and after five weeks, the nude mice were killed and the tumor tissue was dissected, weighed and the related indexes were detected.

(A) The tumor tissues of nude mice were dissected in each group. (B) Tumor volume and (C) weight were measured. (D) HE staining was used to observe the tumor tissue of nude mice in each group. (E) The expression of CD36 and Ki-67 in tumor tissues were detected by IHC. Data are shown as mean ± SEM (n=3). Asterisks indicate significant differences from the control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

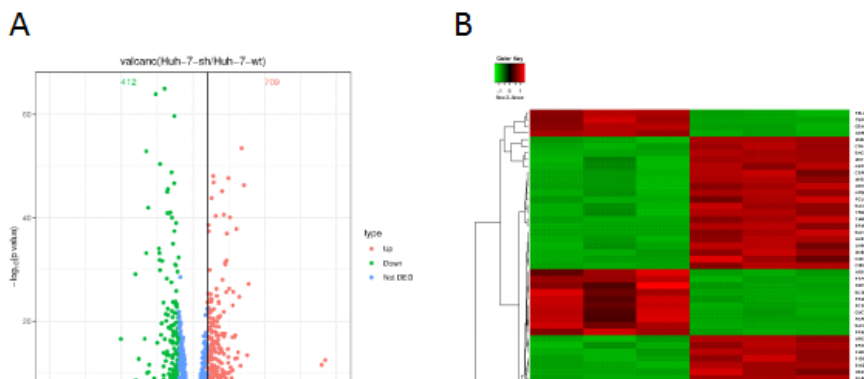
Figure 4

CD36 regulates FAs metabolism to enhance proliferation and metastasis of HCC cells

Huh7 cells and HCCLM3 cells were transfected with sh-CD36 and sh-control, FAs related indexes were detected. Then according to different transfection and treatment, Huh7 cells were divided into 5 groups: sh-control, BSA+sh-control, PA+sh-control, sh-CD36 and PA+sh-CD36. Then the proliferation, migration and invasion of cells in each group were detected.

(A) Phospholipids and (B) triglycerides in Huh7 cells and HCCLM3 cells were detected by ELISA. (C) The content of neutral lipids in Huh7 cells and HCCLM3 cells were detected by staining with BODIPY 493/503 dye and DAPI. (D) The expression of FASN, ACC1 and FABP5 in Huh7 cells and HCCLM3 cells were detected by western blot in Huh7 cells and HCCLM3 cells. (E) CCK-8 and (F) clone formation assay were used to detect the cell proliferation ability in Huh7 cells of each group. (G) Wound healing assay was used to detect the cell migration ability in Huh7 cells of each group. (H) Transwell experiment was used to detect the cell invasion ability in of each group. Data are shown as mean \pm SEM (n=3). Asterisks indicate significant differences from the control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Fig.5 Impact of CD36 silencing on gene expression profile in HCC cells



Impact of CD36 silencing on gene expression profile in HCC cells

The Huh7 cells were transfected with sh-CD36 and sh-control. Then the cells were sequenced by transcriptome, and the obtained data analyze.

(A) Volcanic plot was used to analyze DEGs. (B) Heat map of expression hierarchical clustering analysis for top 50 DEGs. (C) qRT-PCR and (D) western blot were used to detect the mRNA and protein expression of AKR1C2 in each group. Data are shown as mean \pm SEM (n=3). Asterisks indicate significant differences from the control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 6

CD36 enhance the proliferation and metastasis of HCC cells by increasing FAs intake through AKR1C2

Huh7 cells were transfected with following groups: oe-NC+sh-control, oe-CD36+sh-control and oe-CD36+sh-AKR1C2, and FAs related indexes were detected. Then according to different transfection and treatment, Huh7 cells were divided into 5 groups: sh-control, PA+sh-control, sh-AKR1C2, PA+sh-AKR1C2 and PA+sh-AKR1C2+oe-CD36. Then the proliferation, migration and invasion of cells in each group were detected.

(A) Phospholipids and (B) triglycerides in Huh7 cells were detected by ELISA. (C) The content of neutral lipids in Huh7 cells were detected by staining with BODIPY 493/503 dye and DAPI. (D) The expression of FASN, ACC1 and FABP5 in Huh7 cells and Huh7 cells were detected by western blot in Huh7 cells. (E) CCK-8 and (F) clone formation assay were used to detect the cell proliferation ability in Huh7 cells of each group. (G) Wound healing assay and (H) Transwell experiment were used to detect the cell migration and invasion ability in Huh7 cells of each group. Data are shown as mean \pm SEM (n=3). Asterisks indicate significant differences from the control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)