USP22-dependent accumulation of lipidomes drives hepatocellular carcinoma progression by stabilizing PPAR γ

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Article

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Abstract

Elevated *de novo* lipogenesis (DNL) is considered to be a crucial factor in hepatocellular carcinoma (HCC) development. However, the molecular mechanism for its occurrence in HCC is still unclear. Herein, we identified ubiquitin-specific protease 22 (USP22) as a key regulator for *de novo* fatty acid synthesis, which directly interacts with, deubiquitinates and stabilizes PPAR γ through K48-linked deubiquitination, and in turn, this stabilization increases *ACC* and *ACLY* transcription. In addition, we found that USP22 promoted the *de novo* synthesis of fatty acid labeling from glucose tracers. USP22-dysregulated *de novo* fatty acid synthesis contributes to HCC progression, but USP22 was functionality suppressed by inhibiting the expression of PPAR γ , *ACLY*, or *ACC* in *in vitro* cell proliferation and *in vivo* tumorigenesis experiments. In HCC, USP22 expression positively correlates with PPAR γ expression, and simultaneously, high expression of USP22 and PPAR γ or *USP22*, *ACC* and *ACLY* is associated with a poor prognosis. Taken together, we identified a previously undescribed USP22-regulated lipogenesis molecular mechanism that involves the PPAR γ -*ACLY*/*ACC* axis in HCC tumorigenesis and provide a rationale for therapeutic targeting of lipogenesis via USP22 inhibition.

Introduction

The liver acts as a pivotal organ for the metabolism of the three macronutrients, namely, sugar, lipid, and protein. Various liver diseases, including hepatocellular carcinoma (HCC), are related to metabolic abnormalities¹. Of note, the global incidence of HCC is increasing rapidly, which is partly due to the epidemic of obesity and subsequent development and progression of metabolic-associated fatty liver disease (MAFLD)². More importantly, the lipid-rich state is an important characteristic of obesity- and MAFLD-driven HCC^{1,3}. However, the molecular mechanism of these series of disease processes is still unclear.

Elevated *de novo* lipogenesis (DNL) is a crucial factor in the development of MAFLD⁴ and cancers, including HCC⁵. The abnormal increase in the *de novo* synthesis of fatty acids is the key link of DNL⁶. Fatty acids and their derivatives function as signaling molecules, energy storage molecules, and basic structural units of the cell membrane and play critical roles in cell proliferation. The *de novo* synthesis of the fatty acid pathway converts citrate to acetyl-coenzyme A (CoA), malonyl-CoA and finally palmitic acid by ATP citrate lyase (*ACLY*), acetyl-CoA carboxylase (*ACC*) and fatty acid synthase (*FASN*). Subsequently, after elongation, saturated fatty acids are converted to monounsaturated fatty acids by stearoyl-CoA desaturase (*SCD*), which is the preferred substrate for triglyceride (TG) generation^{6,7}. Importantly, the expression of *ACLY*, *ACC*⁹, *FASN*^{10,11} and *SCD*¹² is upregulated and has been associated with poor prognosis in HCC. Small interfering RNAs (siRNAs) or small-molecule inhibitors targeting these proteins have effectively inhibited cell aggressiveness *in vitro* and *in vivo*^{13,14}. However, to date, there is limited progress on clinical treatments targeting lipid synthesis due to toxicity or complications¹⁵. Therefore, it is particularly important to find new and more effective targets for fatty acid synthesis.

Dysregulation of metabolic enzyme expression or activity is among the key causes of metabolic reprogramming. Ubiquitination and deubiquitination are crucial posttranslational modifications of metabolic enzymes that regulate their degradation, delocalization and activation in cells by tagging or removing ubiquitin from substrate proteins^{16,17}. The dysregulation of ubiquitination and deubiquitination is closely related to cell lipid metabolism and promotes the occurrence and progression of various cancers, including HCC. In recent years, due to the potential roles of the stabilization of oncoproteins, deubiquitinating enzymes (DUBs) have been widely studied in several different cancers. Previously, we conducted in-depth research in the field of deubiquitination and successfully mapped the key deubiquitination molecular interaction networks in different cancers¹⁸. The USP family is a well-defined deubiquitinating enzyme, and its members USP13¹⁹, USP14²⁰ and USP30⁸ play key roles in lipogenesis by directly deubiquitinating and stabilizing the key lipogenesis factors ACLY and FASN in tumorigenesis and high-fat diet-driven HCC. USP22 has been reported to be a cancer signature gene and is highly expressed in a variety of cancers. USP22 mainly plays the role of oncoprotein in chromatin remodeling by removing ubiquitin from histones (H2B and H2A) and subsequently activates or stabilizes transcription factors in cancer progression^{21,22}. Additionally, USP22 participates in cell cycle regulation and apoptosis by deubiquitinating CCND1²³, CCNB1²⁴, and SIRT1²⁵ in cancer cell proliferation. In addition, USP22 has been found to deubiquitinate and stabilize PDL1 and thus cause cancer immune resistance²⁶. However, whether USP22 regulates lipogenesis has not been identified.

Peroxisome proliferator-activated receptor gamma (PPAR γ) is a ligand-activated transcription factor belonging to the nuclear hormone receptor family and promotes lipogenesis by upregulating lipid synthesis enzymes, including ACC, ACLY and FASN²⁷⁻³⁰. However, the upstream role of PPARG in cancer biology is poorly characterized. Recent studies revealed that the phosphorylation of AKT2 promotes the expression of PPAR γ and thereby promotes lipid synthesis and tumorigenesis in HCC³¹. In this study, we found that the abnormally high expression of USP22 is accompanied by a significant upregulation of lipid synthesis in HCC. We further demonstrated that USP22 promotes *de novo* synthesis of fatty acids and tumorigenesis by deubiquitinating PPAR γ in HCC. Our findings provide a new option for targeting fatty acid synthesis that may provide therapeutic benefits to patients with high USP22 expression in HCC.

Results

Abnormal lipid metabolism in human HCC with high USP22 expression.

Metabolic reprogramming is a characteristic event in the occurrence of HCC³². To determine disordered metabolism in HCC, high-throughput metabolomics was performed to detect the differentially altered metabolites in 10 paired HCC and adjacent normal tissues (Fig. 1a). Compared with adjacent normal tissues, metabolites that are significantly upregulated in cancer tissues are almost all lipids and lipid-like metabolites (Fig. 1b), which are mainly composed of fatty acids (FAs), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), and sphingomyelin (SM) (Fig. 1c). Unexpectedly, further pathway enrichment analysis showed that the

biosynthesis of PC, cardiolipin, phospholipids, PE and triacylglycerol was enriched in cancer tissues (Fig. 1d). These results confirmed that lipid synthesis was significantly upregulated in HCC tissues, but what caused the abnormal upregulation of lipid synthesis in HCC tissues remains unclear.

Previously, we conducted an analysis of USPs by bioinformatics and found that the abnormal expression of USPs was highly associated with poor prognosis in HCC (Extended Data Fig. 1b), suggesting that USPs might play an important role in HCC. To determine whether the abnormally upregulated lipid synthesis in HCC was related to the expression of USPs, we examined the expression of USPs in the above 10 pairs of cancerous and paracancerous tissues and found that USP22 was the only USP member with a significantly high expression (Extended Data Fig. 1a and Fig. 1e,f). Subsequently, pathway enrichment analysis based on the TCGA database revealed that USP22 was significantly negatively associated with FA degradation in HCC (Extended Data Fig. 1D). Notably, USP22 was highly expressed in HCC and cholangiocarcinoma but not in other cancer types (Extended Data Fig. 1C). Moreover, Kaplan–Meier Plotter data revealed that high USP22 expression is correlated with poor overall survival (OS) in patients with HCC (Extended Data Fig. 1E). Altogether, these data demonstrate that high expression of USP22 might be associated with the abnormal upregulation of lipid and lipid-like metabolites in HCC. Nonetheless, the specific role of USP22 in lipid metabolism in HCC is still unclear.

USP22 promotes *de novo* synthesis of fatty acids.

To define the specific role of USP22 in HCC lipid metabolism, we generated USP22 stably knocked down and transduced overexpression cell lines in MHCC-97H and MHCC-97L HCC cells, respectively (Extended Data Fig. 2A). We found that 31 metabolites were significantly altered in USP22-engineered HCC cells by metabolomics analysis (Fig. 2a). Among them, fatty acid metabolites were significantly decreased in USP22-knockdown MHCC-97H cells and increased in USP22-transduced MHCC-97L cells (Fig. 2b). Indeed, lipid-related metabolites were altered in total, 70.6% (Extended Data Fig. 2b) and 59.4% (Extended Data Fig. 2c) in USP22 stably knocked down and overexpressing cell lines, respectively. Furthermore, pathway enrichment analysis showed that USP22 was abnormally related to the fatty acid synthesis pathway (Fig. 2c). Glucose is an important synthetic source for the *de novo* synthesis of fatty acids (Extended Data Fig. 2d). Subsequently, we used [U-¹³C₆] glucose to trace fatty acid synthesis and observed that USP22 shRNA knockdown cells had significantly decreased palmitate and palmitoleic acid labeling from glucose tracers (Fig. 2d). Conversely, USP22-overexpressing MHCC-97L cells exhibited significantly increased palmitate and palmitoleic labeling from glucose (Fig. 2e). To avoid cell line specificity, we performed similar experiments in the Bel-7402 HCC cell line. We observed that a large number of lipid metabolites were significantly upregulated in USP22-overexpressing Bel-7402 cells, and fatty acid biosynthesis was enriched by pathway enrichment analysis (Fig. 2f,g and Extended Data Fig. 2a,e). Taken together, these results provide strong evidence supporting that USP22 promotes the *de novo* synthesis of fatty acids in HCC cells.

USP22 deubiquitinates and stabilizes the lipid metabolism key transcription factor of PPAR γ .

Next, to identify the specific targeting deubiquitinating substrate of USP22, we isolated the USP22-associated protein complex in HEK293T cells through tandem affinity purification followed by mass spectrometry analysis. Interestingly, PPAR γ was identified on the prey list (Fig. 3a). The specific interaction between USP22 and PPAR γ was confirmed by pulldown and coimmunoprecipitation assays (Fig. 3b,c and Extended Data Fig. 3b). The pathway enrichment analysis found that USP22 was significantly correlated with the PPAR pathway in HCC (Extended Data Fig. 3a). Furthermore, we found that PPAR γ is the only counterpart among several lipid metabolism-related transcription factors, including PPAR α , PPAR δ and SREBF1 (Extended Data Fig. 3b). The immunofluorescent staining assay showed that USP22 and PPAR γ were mainly colocalized in the nucleus (Fig. 3d and Extended Data Fig. 3c). Moreover, endogenous PPAR γ was present in endogenous USP22 immunoprecipitates from MHCC-97H, MHCC-97L, Bel-7402 and SNU449 cells (Fig. 3e). It has been reported that PPAR γ contains three structural domains: AF-1, DBD and Hinge-LBD (Extended Data Fig. 3d). Pull-down assays showed that USP22 strongly associated with the DBD fragment and weakly interacted with the Hinge-LBD fragment of PPAR γ (Extended Data Fig. 3d).

Next, to explore whether USP22 regulates the stability of the PPAR γ protein, we examined PPAR γ protein levels in the presence of cycloheximide (CHX), an inhibitor of protein translation. Notably, overexpression of USP22, but not the enzyme-dead mutant (USP22 C185S), led to a prominent increase in the stability of overexpressed PPAR γ protein, whereas the stability of vinculin was not affected (Fig. 3f). Additionally, we found that USP22 stabilized PPAR γ protein in a dose-dependent manner, whereas the C185S mutation did not considerably affect PPAR γ protein levels (Fig. 3g). Next, we examined the deubiquitination of PPAR γ in USP22-knockdown cells. The polyubiquitylation level of PPAR γ was markedly increased in two independent USP22-shRNA knockdown cells (Fig. 3h). In contrast, overexpression of USP22 inhibited polyubiquitylation of PPAR γ but not the enzyme-dead mutant (Fig. 3i). Moreover, we found that USP22 significantly inhibited the Lys-48-linked polyubiquitylation of PPAR γ (Fig. 3j). Taken together, these results indicate that USP22 directly interacts with PPAR γ and functions as a *bona fide* PPAR γ deubiquitylase in cells.

USP22 upregulates ACC and ACLY expression by stabilizing PPAR γ .

Abnormal upregulation of fatty acid anabolism is closely related to the key metabolic enzymes of ACLY, ACC, FASN and SCD⁷. The expressions of *ACC* and *ACLY* transcripts are regulated by lipid metabolism-related transcription factors, such as PPAR γ . Due to the USP22 localization in the nucleus (Extended Data Fig. 3c), we hypothesized that USP22 may regulate PPAR γ that control *ACLY* and *ACC* expression by deubiquitinating in cells. To investigate whether these key enzymes in fatty acid synthesis were affected by USP22 in HCC cells, we performed high-throughput RNA sequencing analysis. In the list of the top 30 differentially expressed genes, previously reported FA biosynthesis-related genes, such as *ACLY*, emerged

as responsive genes sensitive to USP22 depletion (Fig. 4a). Follow-up gene set enrichment analysis (GSEA) identified the gene sets of fatty acid biosynthesis that were significantly enriched in both two independent USP22_shRNA-transduced MUCC-97H cells (Fig. 4b). To confirm that USP22 regulates fatty acid metabolic genes, we performed qPCR and immunoblot analysis of USP22-knockdown and USP22-overexpressing HCC cells (Fig. 4c,d). The expression of ACC and ACLY was decreased in USP22-knockdown MHCC-97H cells but increased in USP22-overexpressing MHCC-97L and Bel-7402 cells. However, the expression of FASN and SCD was not affected by USP22 expression in HCC cells (Fig. 4c,d).

We next sought to explore the correlation between USP22 and key fatty acid metabolic enzymes in HCC tissues. *USP22* was significantly positively correlated with *ACLY* and *ACC* but not with *FASN* and *SCD* in The Cancer Genome Atlas (TCGA) HCC database (Fig. 4e), which was consistent with the expression results. In addition, IHC analysis of the HCC tissue microarray (TMA) revealed that USP22 protein expression was positively correlated with ACLY and ACC protein expression (Fig. 4f). Collectively, these results suggested that upregulated USP22 expression increases the expression of ACC and ACLY in HCC.

USP22 promotes HCC cell tumorigenesis.

Next, to determine whether USP22 promotes tumorigenesis in HCC we performed *in vivo* xenograft assay. First, we analyzed the Cancer Cell Line Encyclopedia and revealed the ninth highest expression of USP22 in liver cancer cells among 1,457 human cancer cell lines from 40 tumor types (Extended Data Fig. 4a). Additionally, USP22 protein expression was more abundant in the HCC cell line than in normal liver epithelial cells (Extended Data Fig. 4b). The IHC analysis in HCC TMA revealed that USP22 expression was significantly increased in cancer tissues compared to adjacent normal tissues (Fig. 5a,b). Similarly, the *USP22* transcripts were upregulated in the TCGA HCC database, which was consistent with the IHC results (Extended Data Fig. 4c). Of note, patients with high levels of USP22 had much shorter overall survival than did patients with low levels of USP22 in the HCC TMA assay and TCGA HCC dataset (Fig. 5c and Extended Data Fig. 4d).

Next, to verify the tumorigenesis function of USP22, we performed both loss-of-function and gain-of-function analyses in HCC cell lines. Two independent USP22 short hairpin RNAs (shRNAs) both decreased the colony-forming ability in soft agar and the proliferation of MHCC-97H cells (Fig. 5d,e). In contrast, overexpression of USP22 in MHCC-97L, Bel-7402 and SUN449 cells significantly increased soft agar colony formation and cell proliferation (Extended Data Fig. 5a-f). To explore the function of USP22 in HCC cells *in vivo*, we subcutaneously implanted USP22-depleted MHCC-97H and USP22-overexpressing Bel-7402 cells into nude mice. Hosts of USP22 shRNA-expressing MHCC-97H cells had smaller tumor volumes and weights than mice implanted with control shRNA-infected cells (Fig. 5f,g). Conversely, overexpression of USP22 increased the tumorigenic ability of Bel-7402 cells (Fig. 5f and Extended Data Fig. 5g).

To further determine the critical role of USP22 in fatty acid anabolism, we observed ACC and ACLY expression and metabolic alterations in the above xenograft tissues (Fig. 5f). Compared with the shControl group, tumors formed by USP22 shRNA-expressing MHCC-97H cells exhibited downregulation

of ACC and ACLY compared to tumors formed by control shRNA-expressing MHCC-97H cells (Fig. 5h). This downregulation of ACC and ACLY decreased a large number of lipid metabolites, including fatty acids (Fig. 5i). Taken together, these data indicated that USP22 regulates HCC tumorigenesis through lipid disorders by regulating ACC and ACLY expression.

USP22 participates in HCC tumorigenesis through ACC and ACLY regulated by PPAR γ .

To determine whether the enhanced *de novo* synthesis of fatty acids regulates the growth-promoting effect of USP22-knockdown or ectopically expressed cells, we transduced PPAR γ in USP22-depleted MHCC-97H cells and expressed PPAR γ shRNAs in USP22-overexpressing MHCC-97L cells (Fig. 6a-d). Transduction of PPAR γ rescued ACC and ACLY expression (Fig. 6a,c); however, knockdown of PPAR γ decreased the expression of ACC and ACLY (Fig. 6b,d).

In line with this, we observed glucose oxidation to fatty acid synthesis by culturing cells with uniformly labeled [U- $^{13}\text{C}_6$] glucose in PPAR γ -transduced USP22-knockdown MHCC-97H cells (Fig. 6e). PPAR γ transduction significantly increased fatty acid labeling from glucose tracers (Fig. 6e). Conversely, knockdown of PPAR γ or ACC in USP22-overexpressing MHCC-97L cells significantly decreased fatty acid labeling from glucose (Fig. 6f and Extended Data Fig. 6a). These results suggested that USP22 regulates fatty acid biosynthesis through PPAR γ stabilization.

To verify that USP22 regulates tumorigenesis by activating fatty acid synthesis signaling, we conducted colony formation assays and xenograft tumor experiments using the above cell lines. As expected, knockdown of PPAR γ , ACC and ACLY in USP22-overexpressing cells inhibited *in vitro* clone formation (Extended Data Fig. 6c-e); however, transduction of PPAR γ increased the clone-forming ability in USP22-knockdown cells (Extended Data Fig. 6f). Then, we implanted these cells subcutaneously into mice. Notably, mice implanted with either shControl MHCC-97H cells or MHCC-97H cells with simultaneous USP22 knockdown and PPAR γ overexpression showed similar tumor growth rates, whereas mice bearing USP22-knockdown MHCC-97H cells showed markedly inhibited tumorigenesis (Fig. 6g,i). Furthermore, mice implanted with USP22-overexpressing MHCC-97L cells formed large tumors compared to control MHCC-97L cells; however, MHCC-97L cells with simultaneous USP22 overexpression and PPAR γ , ACC or ACLY knockdown showed smaller tumor growth rates (Fig. 6h,j). Indeed, we also observed that compared with the tumors formed, USP22 overexpression upregulated PPAR γ , ACC and ACLY; however, this upregulation was inhibited by knockdown of PPAR γ in USP22-overexpressing MHCC-97L cells (Fig. 6k) or MHCC-97L cells with simultaneous overexpression of USP22 and knockdown of PPAR γ tumor growth rates, whereas mice bearing USP22-knockdown MHCC-97H cells showed markedly inhibited tumorigenesis (Fig. 6g,i). PPAR γ overexpression restored the downregulation of ACC and ACLY in USP22-knockdown MHCC-97H cells (Extended Data Fig. 6g). Collectively, these results reveal that overexpression of USP22 can lead to activation of *de novo* synthesis of fatty acid signaling *in vivo*.

The USP22–PPAR γ /ACC/ACLY axis controlled HCC prognosis

To investigate the relevance of our findings to human HCC, we analyzed the expression of USP22 and PPAR γ in TCGA and HCC TMA by IHC. We found a significant positive correlation between *USP22* and *PPAR γ* transcript levels in the TCGA HCC database (Extended Data Fig. 7a). Additionally, USP22 protein expression was strongly correlated with PPAR γ expression (Fig. 7a,b). Next, we evaluated the prognostic value of USP22 and PPAR γ in the TMA dataset of HCC. Of note, patients with high levels of PPAR γ had much shorter overall survival than patients with low levels of PPAR γ among patients with HCC (Fig. 7c and Extended Data Fig. 7b). In addition, patients with simultaneously high levels of USP22 and PPAR γ had significantly poor overall survival (Fig. 7d and Extended Data Fig. 7c).

To reveal the contribution of ACC and ACLY to the prognosis of USP22-positive patients with HCC, we examined the prognostic value of *USP22*, *ACC* and *ACLY* in the TCGA dataset of HCC tumors from 332 patients. The patients with simultaneously high expression levels of *USP22*, *ACC* and *ACLY* had short overall survival in HCC cohorts (Fig. 7e). On the other hand, when *USP22* was highly positively expressed with either *ACLY* or *ACC* in patients with HCC, they had a much shorter overall survival curve (Extended Data Fig. 7d-h). Collectively, we revealed a previously undescribed pathogenic relationship between USP22 and the *de novo* fatty acid synthesis factors of PPAR γ , ACC and ACLY in patients with HCC.

Discussion

Dysregulation of cellular metabolism is a hallmark of cancer³³. In addition to elevated glycolysis, *de novo* fatty acid synthesis is a common feature that needs to meet the biosynthetic requirements of growing tumors¹⁴. Fatty acid synthesis occurs at a lower rate in nondividing cells, which mainly absorb lipids from the extracellular circulation. In contrast, DNL, especially *de novo* fatty acid synthesis, is an important lipid source for cancer cells¹². Here, we have identified USP22 as a deubiquitinating enzyme that regulates *de novo* fatty acid synthesis, which directly deubiquitinates and stabilizes PPAR γ , and this stabilization in turn activates ACC and ACLY transcription (Fig. 7f).

HCC is a major cause of cancer-related death worldwide. MAFLD is an increasingly important risk factor for HCC, especially in developed countries¹. MAFLD is characterized in part by the excessive accumulation of TGs in hepatocytes, which is due to elevated hepatic *de novo* fatty acid synthesis⁴. The resulting fatty acids are either stored in the form of TGs or used to synthesize sphingomyelin and glycerol phospholipid and eventually used as signal molecules or membrane building blocks. In this study, we observed that a variety of lipids in HCC tissues were significantly increased, including fatty acids, phospholipids and sphingomyelin, accounting for 70.83% of all differential metabolites (Fig. 1c). These results are similar to our previous study³⁴ but more extreme, which further increases our interest in the sample set and exploring the specific reasons for this drastic change.

Metabolic alterations are caused by abnormal expression or activation of related enzymes^{35,36}. The ubiquitin-proteasome system (UPS), including ubiquitination and deubiquitination^{36,37}, is responsible for most enzymes of regulated proteolysis and has a nondegradation function. Recently, due to the potential roles of the stabilization of oncoproteins, DUBs have been widely studied in cancer progression.

Approximately 100 DUBs are encoded in the human genome, and USP is the largest subfamily with nearly 60 members³⁸. Several lines of evidence indicate that USPs play critical roles in lipogenesis. USP13 is abnormally highly expressed in ovarian cancer and participates in lipid synthesis and tumorigenesis through deubiquitination of ACLY¹⁹. USP14 directly interacts and stabilizes FASN, followed by elevated triglyceride accumulation²⁰. Additionally, USP30 deubiquitinates and stabilizes ACLY and FASN and plays important roles in lipogenesis and HFD-driven HCC⁸. Interestingly, we also observed the differential expression of USP13 and USP14 in HCC tissues, but the difference in USP22 expression was more significant than that in USP13 and USP14 (Fig. 1f). USP22 functions as an oncoprotein during tumorigenesis. It is highly expressed in a variety of cancers, including HCC²⁶. USP22 participates in biological processes such as transcriptional regulation, the cell cycle, and embryonic differentiation by deubiquitinating histones H2A and H2B²², SIRT1²⁵, CCNB1²⁴, and CCND1²³. Previous studies have shown that USP22 promotes fatty acid oxidation by stabilizing SIRT1 in the liver³⁹. However, we found that USP22 promotes the *de novo* synthesis of fatty acids in HCC rather than fatty acid oxidation. Moreover, the aberrant lipid accumulation caused by USP22 depended on the increased expression of ACC and ACLY. ACC, as a central enzyme controlling DNL, augments HCC development, and the ACC small molecule allosteric inhibitor ND-654 inhibits HCC⁴⁰. The upregulated expression of ACLY increases fatty acid synthesis and promotes tumorigenesis⁴¹. In this study, we observed that individual knockdown of ACC and ACLY obviously inhibited the increase in tumorigenesis and fatty acid synthesis augmented by USP22 overexpression.

PPAR γ is a key transcription factor that regulates lipid synthesis by upregulating the transcription of lipid synthesis enzymes, including ACLY, ACC and FASN. It is highly expressed in adipocytes and is involved in lipid uptake, synthesis, and storage⁴². Notably, upregulated PPAR γ promotes lipid synthesis and tumorigenesis in HCC⁴³. In addition, PPAR γ is degraded by the UPS pathway in mammalian cells^{44,45}. In this study, we demonstrate that USP22 stabilizes PPAR γ through K48-linked deubiquitination. PPAR γ knockdown abolished the upregulation of ACC and ACLY expression and dramatically decreased tumorigenesis and fatty acid synthesis caused by USP22 overexpression in HCC cells and xenograft tissues. In addition, patients with HCC, whether USP22 is simultaneously highly expressed with PPAR γ or ACC or ACLY, have poor prognosis and overall survival. In summary, we identified a previously undescribed signaling pathway of the USP22-PPAR γ -ACLY/ACC axis that plays an important role in lipogenesis and HCC tumorigenesis and provide a new option for cancer therapy targeting fatty acid synthesis.

Declarations

Acknowledgments

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Author Contributions

Z.N, G.X., G.T. and H-I.P. devised and coordinated the project. H-I.P. and G.X. supervised the project. Z.N., X.G., X.L, and C.L performed most of experiments, Z.N., X.G., X.L, C.L and H-I.P. analyzed data. A.W., X.W., H.C., W.Q., X.L., C.M., J.D., H.L., W.O., H.Q., D.H., T.X. and J.L. provided significant intellectual input. Z.N, G.T., G.X. and H-I.P. wrote the manuscript with input from all other authors.

Declaration of Interests

All authors declare no competing interest.

Methods

Reagents

Please see Extended Methods.

Clinical Specimens

10 pairs of HCC samples were obtained from the first affiliated hospital of Dalian Medical University (Dalian, China). All samples were collected with the informed consent of the patients and the experiments were approved by Research ethics committee at the first affiliated hospital of Dalian Medical University. Human HCC Tissue Microarrays were obtained from Shanghai Outdo Biotech Company or Shanxi ChaoYing Biotechnology Company.

Cell Culture Experiments

Human HCC cell lines MHCC-97H, HUH7, BEL-7402, Hep3B, HepG2 and SMMC-7721 were purchased from the cell bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (CTCC, Shanghai, China). THLE-2 and SNU-449 were obtained from ATCC. MHCC-97H, SNU-449 and MHCC-97L cell lines were maintained in RPMI 1640 medium (GIBCO, USA). 293T, HUH7, BEL-7402, Hep3B, HepG2 and SMMC-7721 cells were cultured in DMEM (GIBCO, USA). Cell lines were maintained in culture supplemented with 10% FBS (GIBCO, USA) and 1% penicillin/streptomycin (Thermo) at 37°C with 5% CO₂ in a humidified incubator (Thermo).

Plasmids

shRNA

Lentiviral shRNAs were cloned in pLKO.1 within the AgeI/EcoRI sites at the 3' end of the human U6 promoter. The targeted sequences were shown in the table above.

All the expression vectors used in this study (including SFB-PPAR γ , Myc-USP22, pLoc-USP22 and Lenti-PPAR γ) were constructed using Gateway Technology (Invitrogen). Briefly, cDNAs with attB homologous sequence were generated by PCR and then subcloned into pDONR221 vector as the entry clones. Subsequently, the entry clones were recombined into gateway destination vectors with various tags (SFB and Myc) or lentiviral vectors (pLoc and Lenti). For Myc-USP22C185S vector, mutation in the USP22 cDNA was generated by overlap extension PCR and then cloned into Myc vector as above.

Generation of cell lines expressing shRNAs, Ploc-USP22 or Lenti-PPAR γ .

HEK293T cells were used to package virus. $2-3 \times 10^7$ cells were plated in 10 cm culture dish and the plasmid was co-transfected with psPAX2 and pVSVG by Lipofectamine 2000 according to the manufacturer's protocol. Conditioned medium containing recombinant lentiviruses was collected 48 hours after transfection and filtered through non-pyrogenic filters with a pore size of 0.45 μ m (Merck Millipore, Billerica, MA, USA). Samples of these supernatants were applied immediately to target cells together with Polybrene (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 8 mg/ml, and supernatants were incubated with cells for 12 hr. After infection, cells were placed in fresh growth medium and cultured as usual. Selection with 2 μ g/ml puromycin (InvivoGen) or 8 μ g/ml Blasticidin (InvivoGen) was initiated 48 h after infection.

Cell proliferation and cloning formation assay

For the clone assay, cells were seeded at a density of 500 cells/well in 12-well plates in complete RPMI 1640 medium. And the medium was changed every three days. After 14 days, cells were fixed and stained with crystal violet.

Click-It EdU Alexa Fluor Imaging Kit was used for the EdU Cell Proliferation Assay. All reactions were performed using this kit in accordance with the manufacturer's instructions.

Immunohistochemistry

The samples were then fixed with 4% PFA, and embedded with paraffin. Standard IHC staining procedures were performed according to the instructions of IHC Kit. USP22 (1:100), ACC (1:750), ACLY (1:750) and PPAR γ (1:50) were used as the primary antibodies. EDTA and Citrate solution were used for antigen retrieval depend on antibody instruction. H-score was used to assess the staining intensity.

Xenograft Tumor Model

Pathogen-free male athymic nude mice (4-5 weeks old, 18-22 g) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). All the mice were housed in specific pathogen-free (SPF) environments at the Institute of Genome Engineered Animal Models for Human

Disease of Dalian Medical University. During the tumor formation assay, BEL-7402 (5×10^6) MHCC97L (5×10^6) MHCC97H cells (1×10^6) were injected into the flank of the mice. The tumor volumes were measured using a caliper every 3 days. The mice were sacrificed after four weeks, and tumor volumes were then measured.

Immunoprecipitation and S-Protein pull down assay

Immunoprecipitation and SFB pull-down experiment was performed as described previously. Cells were lysed in E1A lysis buffer (250 mM NaCl, 50 mM HEPES [pH 7.5], 0.1% NP-40, 5 mM EDTA, protease inhibitor cocktail [Sigma]). The antibodies to USP22 and PPAR γ were used for immunoprecipitation. The SFB pull-down experiment was done as described previously. Briefly, 293T cells were transfected with SFB-tagged protein and lysed in NETN buffer (200 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.05% NP-40, 1 mM EDTA, protease inhibitor cocktail [Sigma]) for 20 min at 4°C. Crude lysates were subjected to centrifugation at 14,000 rpm for 15 min at 4°C. Supernatants were incubated with S-Protein Agarose for 4 h (Millipore, USA). The agaroses were washed three times with NETN buffer. Proteins were eluted by boiling in 1× SDS running buffer and subjected to SDS-PAGE for immunoblotting.

In vivo deubiquitination assay

HA-UB and SFB-PPAR γ were co-transfected with MYC-USP22 into HEK-293T cells, after 36h cells were treated with 10 μ M MG132 for 6 h. SFB-PPAR γ was purified with S-Protein Agarose and western blotted with antibodies against HA, MYC and FLAG.

Immunofluorescence

Cells were cultured and fixed on the 8-chamber slide. Cells were permeabilized by 0.1% Triton X-100 and blocked by 10% goat serum before incubation with primary antibodies. Secondary antibodies (goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 555) were obtained from Invitrogen. Cell nucleus were counterstained with DAPI. Images were acquired under a point-scanning confocal microscope on an upright stand.

RNA isolation and quantitative real-time PCR

RNA was isolated from tumor cells using Trizol reagent. Reverse transcription PCR was performed using the Revert Aid First Strand cDNA synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the protocol. Quantitative real-time PCR was performed using StepOnePlus and the DNA double-strand-specific reagent SYBR-Green I for detection (Roche Applied Science, Penzberg, Germany). Fold changes were calculated using the Cq method. The results were normalized to β -Actin levels. The primer sequences were as follows:

USP22

F 5'-AGCAGCGGATTCACCATCTC-3'

R 5'- TGATGTATGCGATCACCAGTGT-3'

PPAR γ

F 5'- GATGCCAGCGACTTTGACTC-3'

R 5'- ACCCACGTCATCTTCAGGGA-3'

ACC

F 5'- ATGTCTGGCTTGCACCTAGTA-3'

R 5'- CCCCAAAGCGAGTAACAAATTCT-3'

ACLY

F 5'- TCGGCCAAGGCAATTCAGAG-3'

R 5'- CGAGCATACTTGAACCGATTCT-3'

FASN

F 5'- AAGGACCTGTCTAGGTTTGATGC-3'

R 5'-TGGCTTCATAGGTGACTTCCA-3'

SCD

F 5'- TCTAGCTCCTATACCACCACCA-3'

R 5'-TCGTCTCCAACTTATCTCCTCC-3'

RNA Sequencing

RNA was extracted from MHCC-97H cells using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked with 4200 TapeStation (Agilent Technologies, Palo Alto, CA, USA). RNA sequencing library preparation used the NEBNext Ultra RNA Library Prep Kit for Illumina followed by manufacturer's instructions (NEB, Ipswich, MA, USA). Sequencing was done on the Illumina HiSeq instrument using a 2x150 Paired End (PE) configuration with 30–40 million reads per sample by GENEWIZ, LLC. (South Plainfield, NJ, USA). Sequencing libraries were constructed from total RNA using SMART-RNAseq Library Prep Kit (Hangzhou KaiTai, AT4201). In briefly, the mRNA were isolated from total RNA with Sera-Mag Magnetic Oligo(dT) particles, and then chemically fragmented. The fragmented RNA was reverse-transcribed into cDNA using random primer containing a tagging sequence at their 3'ends. And the cDNA libraries were subsequently amplified using the KAPA high-fidelity DNA polymerase. Quality of the libraries was validated by the 2100 Bioanalyzer (Agilent Technologies).

Subsequently, High-throughput sequencing was performed using a NovaSeq 6000 (Illumina). After sequences were mapped using hisat2 (version 4.8.2) against the Mus_musculus.GRCm38.dna.primary_assembly.fa, the reads for each library were converted to FPKM (fragments per kilobase of exon per million fragments mapped) by running Cuffdiff 2.1.137 to determine gene expression. Biological pathway analysis was performed using clusterProfiler. RNA sequencing and library construction were performed by technical staff at Hangzhou KaiTai Bio-lab.

Tracing with $^{13}\text{C6}$ -Glucose

Medium was changed to RPMI 1640 containing $^{13}\text{C6}$ -Glucose (2g/L) when the cell density was about 80%, and 24 hours later cell culture plates were washed with PBS and snap-frozen in liquid nitrogen and stored at -80°C .

Sample preparation for metabolomics analysis

The lyophilized powder was redissolved in 30 μL organic solvent (Chloroform/Methanol=2/1) by 30 second vortex, then added 60 μL organic solvent (Acetonitrile/Isopropanol/H₂O=65/30/5). After centrifugation, the supernatant can be directed for UPLC (Waters, USA) -Q Exactive HF MS (Thermo Fisher Scientific, USA) analysis.

UPLC-MS based information acquisition

Waters BEH C8 column (100 mm $\times$ 2.1 mm, 1.7 μm) was used for separation. The Mobile phases were Acetonitrile/H₂O (60:40, v/v) for phase A and Isopropanol/Acetonitrile (90:10, v/v) for phase B, both containing 10 mM ammonium acetate. The flow rate was 0.26 mL/min. The column temperature was at 55°C . The gradient started with 32% B and maintained for 1.5min, then increased to 85% B within 14 min, arrived to 97% at 15.6 min. After maintaining for 2.4 min, it returned to the initial 32% B. The MS capillary temperature was 320°C with the auxiliary air heating temperature set at 350°C . The sheath gas and auxiliary gas flow rate were set as 45 and 10 units. Full scan resolution was set as 120K and the negative mode was used, m/z scan range was 70-1100 Dalton and the spray voltage was 3 kV.

Statistical Analysis

Pearson Correlation Coefficient was used to evaluate the relationship between USP22 and PPAR γ , ACC, ACLY and FASN protein and mRNA expression levels in human HCC tissues and TCGA database. ANOVA-post-hoc pairwise comparison analysis was used to compare the means of more than two groups. Student's t-test (unpaired, two-tailed) and Wilcoxon-test (paired two-samples) was used to compare the mean value of two groups. The differences in survival were calculated using the Kaplan-Meier test. Survival analysis in mouse model was performed by log-rank test. Data representative of two or more independent experiments. Bars and error represent mean $\pm$ standard deviations (SD) of replicate measurements. Statistical significance was defined as *p < 0.05, **p < 0.01. All of the relative protein

expression was normalized by ImageJ (version no.: 1.8.0_112; <https://imagej.nih.gov/ij/>). Statistical analysis was performed using the SPSS 21.0 software package (SPSS, Inc., Chicago, IL, USA).

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Figures

Figure 1

Abnormal lipid metabolism in human HCC with high USP22 expression. a, LC-MS–based nontargeted metabolomic analysis detecting differential metabolites between the tumor tissues and normal adjacent tissues of patients with HCC (n=10). b, Volcano plots of metabolites in HCC and normal adjacent tissue. Red represents lipids and lipid-like molecules. c, Heatmap analysis of significantly changed metabolites in cancer tissues compared to paired normal adjacent tissue. $p < 0.05$, paired two-sample Wilcoxon test. d,

USP22 promotes de novo synthesis of fatty acids. a, Venn diagram of the overlap between USP22-knockdown (shUSP22-1 vs shControl) and USP22-overexpression (USP22 vs Control)-induced differential metabolites. The red number 31 refers to the number of metabolites with the opposite trend. b, Heatmap analysis of these 31 significantly changed metabolites. $p < 0.05$, unpaired two-tailed Student's t test. c, Significantly enriched pathways identified by pathway analysis based on these 31 differential metabolites. (<https://www.metaboanalyst.ca/>) d and e, The percentages of various isotopomers of palmitate (FA 16:0) and palmitoleic acid (FA 16:1) after trace to [U-13C] glucose in MHCC-97H-shUSP22-1 (d) and MHCC-97L-USP22 (e) cells. * $p < 0.05$, ** $p < 0.01$, unpaired two-tailed Student's t test. f, Heatmap analysis of significantly altered lipids and lipid-like metabolites in Bel-7402-USP22 cells. $p < 0.05$, unpaired two-tailed Student's t test. g, Significantly enriched pathways identified by pathway analysis based on these significantly changed lipids and lipid-like metabolites from (F) (<https://www.metaboanalyst.ca/>).

Figure 3

USP22 deubiquitinates and stabilizes PPAR γ . a, Tandem affinity purification–mass spectrometry detection of USP22-interacting proteins after HEK293T cells were transfected with SFB-USP22 for 24 hours. b and c, HEK293T cells were transfected for 24 hours with plasmids encoding either Flag-PPAR γ or Myc-USP22 alone or in combination. Cell lysates were immunoprecipitated with anti-Flag (b) and anti-Myc (c) antibodies, and immunoblotting was performed using anti-Myc or anti-Flag antibodies. d, Triple

immunofluorescence (IF) staining for USP22 (green), PPAR γ (red), and nuclei (DAPI, blue) was performed in HEK293T cells that were transfected for 24 hours with plasmids encoding Flag-PPAR γ and Myc-USP22 in combination. Scale bars, 10 μ m. e, Cell lysates of MHCC-97H, MHCC-97L, Bel-7402 and SNU-449 cells were immunoprecipitated with IgG or USP22 antibodies, and immunoblot assays were performed using anti-USP22 or anti-PPAR γ antibodies. f, HEK293T cells were transfected for 24 hours with Flag-PPAR γ alone or together with Myc-USP22 or its catalytically inactive mutant (C185S) and treated with 40 μ M cycloheximide for the indicated times. Cell lysates were immunoblotted with anti-Myc and anti-Flag antibodies. Relative levels of Flag-PPAR γ were quantified by ImageJ. * p < 0.05, ** p < 0.01, ANOVA test. g, HEK293T cells were transfected with Flag-PPAR γ alone or together with increasing doses (0, 0.5, 1.0, 1.5, and 2.0 μ g) of Myc-USP22 or Myc-USP22C185S. Cell lysates were immunoblotted with anti-Myc and anti-Flag antibodies. h, HEK293T cells were transfected for 24 hours with HA-Ub, SFB-PPAR γ , shControl or shUSP22-1/2 alone or in combination and treated for 6 hours with 10 μ M MG132. Immunoprecipitation of cell lysates was performed with S-beads, and lysates were immunoblotted with anti-HA, anti-Flag and anti-USP22 antibodies. i, HEK293T cells were transfected for 24 hours with HA-Ub, SFB-PPAR γ , Myc-USP22 or Myc-USP22C185S alone or in combination and treated for 6 hours with 10 μ M MG132. Pulldown assays of cell lysates was performed with S-beads, and lysates were immunoblotted with anti-HA, anti-Flag and anti-Myc antibodies. j, 293T cells were transfected for 24 h with HA-K48Ub, HA-K63Ub, SFB-PPAR γ , or Myc-USP22 alone or in combination and treated for 6 h with 10 μ M MG132. Pulldown assays of cell lysates was performed with S-beads, and lysates were immunoblotted with anti-HA, anti-Flag and anti-Myc antibodies.

Figure 4

USP22 upregulates ACC and ACLY expression. a, Heatmap depicting the top 30 down and upregulated genes in MHCC-97H cells transduced with USP22 shRNA ($Q < 0.05$). b, Gene set enrichment analysis of fatty acid biosynthesis gene sets in the expression profiles of MHCC-97H cells transduced with two independent USP22 shRNAs. (<http://software.broadinstitute.org/gsea>). c and d, qRT-PCR analysis (c) and western blotting (d) of key metabolic enzymes (ACC, ACLY, FASN and SCD1) in MHCC-97H cells transduced with two independent USP22 shRNAs, and MHCC-97L and Bel-7402 cells transduced with

USP22. The data shown represent the means ($\pm$ SD) of biological triplicates. * $p < 0.05$, ** $p < 0.01$, ANOVA test or unpaired two-tailed Student's t test. e, Correlation analysis between USP22 and ACC, ACLY, and FASN based on the TCGA HCC database. R represents the Pearson correlation coefficient. f, Representative IHC staining of USP22, ACC and ACLY in HCC tissues. Scale bars, 50 μ m. g, Correlation analysis between USP22 and ACC and ACLY based on the H-Score. R represents the Pearson correlation coefficient.

Figure 5

USP22 promotes HCC cell tumorigenesis. a, Representative IHC staining of USP22 in HCC and paired normal tissues. Scale bars, 50 μ m. b, H-Score analysis of USP22 expression in HCC tissue microarrays. ** $p < 0.01$, paired two-sample Wilcoxon test. c, Kaplan–Meier curves of the survival analysis of USP22-positive patients based on the data of HCC tissue microarrays. d, Clone formation assay in MHCC-97H-shUSP22-1/2 cells. * $p < 0.05$, ANOVA test. e, EdU staining assay in MHCC-97H-shUSP22-1/2 cells. ** $p < 0.01$, ANOVA test. f and g, MHCC-97H-shUSP22-1 or Bel-7402-USP22 cells with Control cells were injected into the right flank of nude mice. Tumor volumes were measured every 3 days. Tumor images (f), growth curves and weight (g) were obtained at day 21 after dissection. * $p < 0.05$, ** $p < 0.01$, unpaired two-tailed Student's t test. h, Western blotting of USP22, ACC and ACLY in tumors derived from MHCC-97H-shUSP22-1 and shControl cells. i, Heatmap analysis of significantly changed metabolites in MHCC-97H-shUSP22-1 cells-derived tumors compared to shControl cells-derived tumors based on nontargeted metabolomic analysis. $p < 0.05$, unpaired two-tailed Student's t test.

Figure 6

USP22 participates in HCC tumorigenesis through ACC and ACLY regulated by PPAR γ . a and b, Western blotting of USP22, PPAR γ , ACC, ACLY and Actin in MHCC-97H cells transduced with USP22 shRNA or in combination with PPAR γ (a), and in MHCC-97L cells transduced with USP22 or in combination with PPAR γ shRNA (b). c and d, qRT-PCR analysis of ACC and ACLY in MHCC-97H cells transduced with USP22 shRNA or in combination with PPAR γ (c), and in MHCC-97L cells transduced with USP22 or in

combination with PPAR γ shRNA (d). * $p < 0.05$, ** $p < 0.01$, ANOVA test. e and f, The percentages of various isotopomers of palmitate (FA 16:0) after trace to [U-13C]glucose in MHCC-97H-related stable cells (shControl, shUSP22-1 and shUSP22-1+PPAR γ) (e) and MHCC-97L-related stable cells (Control, USP22, USP22+shACC and USP22+shPPAR γ) (f). * $p < 0.05$, ** $p < 0.01$, ANOVA test. g, h, i and j, MHCC-97H-related stable cells (shControl, shUSP22-1 and shUSP22-1-PPAR γ) and MHCC-97L-related stable cells (Control, USP22, USP22+shACC, USP22+shACLY and USP22+shPPAR γ) were injected into the right flanks of null mice. Tumor volumes were measured every 3 days. Tumor images (G and H), growth curves and weight (I and J) were obtained at day 21 after dissection. * $p < 0.05$, ** $p < 0.01$, ANOVA test. k, Representative IHC staining of USP22, PPAR γ , ACC and ACLY in xenograft tissues described in (H). Scale bars, 50 μ m

Figure 7

The USP22-PPAR γ /ACC/ACLY axis controlled HCC prognosis. a, Representative IHC staining of USP22 and PPAR γ in HCC tissue microarrays. Scale bars, 50 μ m. b, Correlation analysis between USP22 and PPAR γ based on the H-Score. R represents the Pearson correlation coefficient. c and d, Kaplan-Meier

curves of the survival analysis of PPAR γ -positive patients (C) and USP22 and PPAR γ -copositive patients (D) based on HCC tissue microarray data. e, Kaplan–Meier curves of the survival analysis of USP22, ACLY and ACC copositive patients based on the data of the TCGA HCC database. f, Diagram of the proposed mechanism.

Supplementary Files

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