Am J Cancer Res 2022;12(6):2686-2696
www.ajcr.us /ISSN:2156-6976/ajcr0141548

Original Article

Ubiquitin specific peptidase 38 promotes the progression of gastric cancer through upregulation of fatty acid synthase

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Received January 5, 2022; Accepted May 9, 2022; Epub June 15, 2022; Published June 30, 2022

Abstract: Gastric cancer (GC) is a malignant tumor with an adverse health effect worldwide, whereas the underlying mechanism of GC development remains controversial. Identification of biomarkers is critical for the treatment of GC. Increasing evidence demonstrates that protein modification plays a pivotal role in carcinogenesis. USP38 is a member of the ubiquitin-specific protease (USP) family, which promotes protein stability by deubiquitinating the target proteins. In this study, we focused on the effect of USP38 on the GC and explored its underlying mechanism. The Cancer Genome Atlas (TCGA) database was used to evaluate the expression of USP38. AGS and HGC27 cells were treated with siRNA targeting USP38 or plasmids overexpressing USP38 to disturb levels of USP38. Immumohistochemical staining was performed to detect the level of USP38 and FASN. RT-qPCR and Western blotting (WB) were used to analyze the expression of mRNA and protein respectively. CCK8 assay, colony formation, cell migration assay, and cell apoptosis and cell cycle were performed to assess cell proliferation and migration ability. A subcutaneous tumor mice model was carried to verify the effect of USP38 on the GC in vivo. In this research, we found that USP38 was overexpressed in GC tissues, and USP38 contributed to GC cell proliferation, migration and tumorigenesis. Cell cycle and apoptosis were also regulated by USP38. Mechanistically, USP38 interacted with FASN, which resulted in enhanced protein stability of FASN and increased triglyceride production. Furthermore, FASN was critical for GC cell growth, migration and tumor development triggered by USP38 overexpression because its inhibitor orilistat reversed phenotypes in USP38 overexpressed GC cells. Collectively, USP38 overexpression is critical for GC cell growth, migration and tumorigenesis. Targeting FASN with inhibitors could be used as a potential treatment for GC patients with highly expressed USP38.

Keywords: Fatty acid synthesis, FASN, gastric cancer, ubiquitination, USP38

Introduction

Gastric cancer (GC) is the fifth most common malignancy worldwide, with a high degree of lethality [1-3]. GC has a high relapse rate due to invasion and metastasis, both of which lead to poor prognosis in patients. However, the underlying mechanism leading to the invasion and metastasis of GC remains to be understood [4-6]. Therefore, it is critical to identify new biomarkers with higher specificity for GC patients, which will facilitate early diagnosis and improve overall survival.

USP38 is a member of the USP family with cysteine-type endopeptidase activity and thiol-dependent ubiquitin hydrolase activity [7]. There are several types of deubiquitinating enzymes; among which, the USP superfamily of ubiquitin-specific modification enzymes have the most diverse structures and are the most widely studied [8]. Current research shows that USP38 is involved in several diseases such as asthma, severe malaria, emphysema, and cancer [9]. However, the role of USP38 in GC is not clear. With tumor progression, the cancerous cells undergo comprehensive metabolic reprogram-

ming. Usually, most tumor cells undergo metabolic transformation via anabolic pathways. Therefore, abnormal fatty acid synthesis may be a critical change in the development of tumor features [10, 11]. Fatty acid synthase (FASN) is an essential protein that promotes the production of long-chain fatty acids. In mammals, normal cells obtain lipids through exogenous ingestion and express FASN at low levels [12, 13]. However, in cancer, FASN is upregulated to meet the increasing lipid demand for high cell proliferation. In GC tissues, FASN is highly expressed and is related to a poor prognosis in patients [14-16]. However, its potential mechanism of action remains unclear. Therefore, in this study, we investigated the role of USP38 and FASN in the development of GC.

Methods

Clinical samples and cells

A total of 18 cancer and 12 normal tissues were enrolled between March and September 2019 in the Beijing Friendship Hospital. The study was approved by the Ethics Committee of Beijing Friendship Hospital (2018-P2-015-02). Each patient had signed the written informed consent. This study was conducted following the principles of the Declaration of Helsinki. AGS and HGC27 cells was purchased from the ATCC. Both AGS and HGC27 cells were cultured in RPMI1640, which was supplied with 10% FBS.

Bioinformatic analysis

The mRNA level of USP38 in GC and normal tissues were calculated from The Cancer Genome Atlas (TCGA) database. 408 GC samples and 211 normal samples were enrolled in the present analysis. For the analysis of USP38 expression at different stages and grades of GC, 408 tumor tissues were available for comparison.

Immunohistochemistry

GC samples were fixed with formalin. 4-μm paraffin-embedded tissue sections were subjected to immunohistochemistry staining using rabbit polyclonal antibody USP38 (17767-1-AP, proteintech) and FASN (66591-1-Ig, proteintech). DAKO Envision Detection Kit (DAKO, Glostrup, Denmark) was used for color development.

RT-qPCR

A TRIzol kit was used to extract the total RNA of GC cells. Reverse transcription was carried out by a cDNA synthesis kit (Takara Biotechnology, Co., Ltd., Dalian, China). A 2*SYRB Green qPCR master kit was used for RT-qPCR (Servicebio. Wuhan, China). The primers synthesized by Tianyi Huiyuan (Beijing, China), and the sequence of primers was showed as follows: USP38-upstream, 5'-TATGAGCTGTCCGTCGGTG-3'; USP38-downstream, 5'-CTGGAAATGGCCT-ATCGTTCG-3'; FASN-upstream, 5'-AACTCCTTG-GCGGAAGAG-3'; FASN-downstream, 5'-TAGGA-CCCCGTGGAATGTCA-3'; GAPDH-upstream, 5'-GACTCATGACCACAGTCCATGC-3'; GAPDH-downstream, 5'-AGAGGCAGGGATGATGTTCTG-3'.

Western blotting (WB) and immunoprecipitation (IP) assay

For western blotting assay, the protein of HGC27 or AGS cells were collected by RIPA buffer. After detected by BCA kit, a total of 30 ug proteins was analyzed by SDS-PAGE and visualized by a protein imaging system (ODYSSEY, LI-GOR). For IP experiment, the proteins were immunoprecipitated out using A-Sepharose and Flag antibodies. Then the protein of different groups (input, IgG and Flag) was also analyzed by SDS-PAGE and visualized by a protein imaging system (ODYSSEY, LI-GOR). Primary antibodies against USP38 (17767-1-AP), Flag (80010-1-RR) and FASN (66591-1-Ig) were purchased from Proteintech. Antibody against β-actin (A5316) was purchased from Sigma-Aldrich.

CCK8 assay

CCK8 assay was used to detect the AGS and HGC27 cell growth rate. Briefly, a total of 2000 AGS or HGC27 cells with USP38 knockdown or overexpression were planted into a 96-well plate, added 10 μl CCK8 solution, incubated at 37°C for 2 h. Then the absorbance at 570 nm was detected at day 1, 2, 3 and 4. When to evaluate the growth of HGC27 and AGS under FASN inhibitor treatment, a final concentration of FASN inhibitor was used at 10 μg/ml. Each measurement was performed in triplicate.

Colony formation

AGS and HGC27 cells with USP38 overexpressing or knockdown and its negative control seeded in a six-well plate at equal number. Fourteen days later, the cell colony was stained, photographed and counted.

Cell migration assay analysis

A total of 1×10^4 cells were suspended in 200 μl medium with FBS free. Then the cell suspension was planted into the upper chamber. While the lower chamber was contained with 500 μl completed medium. After 24 hours culture, the migrated cells were fixed, and stained by crystal violet.

Cell apoptosis and cell cycle

We used the Annexin V-FITC/PI apoptosis kit and PI staining cell cycle kit (YEASEN, Shanghai, China) to assess apoptosis and cell cycle, respectively. Cells fixed in 75% cold ethanol for 24 h were used for cell cycle analysis. Flow cytometry (Beckman) was used for measurement of apoptosis and cell cycle.

Establishment of a mouse subcutaneous tumor model

Lentivirus was used to establish a stable USP38 overexpressing cell line. BALB/c mice, 6 weeks old, were purchased from the Charles River Co. Ltd. (Beijing, China). PBS (0.2 ml) containing 1×10^7 HGC27 cells was subcutaneously injected into the left posterior flank area. The mice were sacrificed after 28 days. The tumor size was detected twice a week. Tumor volume was calculated with the following formula: $V=1/2\times a\times b^2$ (a, length; b, width). All procedures were performed according to the guidelines of the Institutional Animal Care and Ethics Committee of the Beijing Friendship Hospital.

Statistical analysis

GraphPad Prism 8.0 was used for statistical analyses. Data were expressed as mean ± SD. The student's *t*-test was used to calculate the difference between two groups, while differences among more than two groups was analyzed by one-way ANOVA followed by Tukey's post hoc test. The differences were considered statistically significant with a *P*-value <0.05.

Results

USP38 was upregulated in GC tissues

We firstly evaluated the expression of USP38 in GC tissues. Using TCGA database, we found that USP38 transcripts were greatly upregulated in GC tissues (**Figure 1A**). Further analysis showed that USP38 highly expressed in the tissues with higher stage and grade (**Figure 1B** and **1C**). To validate the results, we collected GC and normal samples and subjected them to RT-qPCR and immunohistochemistry (IHC) assay. Similarly, USP38 was higher expressed in GC samples than adjacent normal and non-adjacent normal samples (**Figure 1D** and **1E**). In addition, IHC showed that USP38 was overexpressed in GC tissues (**Figure 1F** and **Table 1**). Our data indicate that USP38 is upregulated in human GC.

USP38 promoted GC cell growth

To explore the effect of the abnormal expression of USP38 on GC cells, siUSP38#1 or siUSP38#2 were used to knock down USP38. The results indicated that USP38 was significantly reduced in GC cells transfected with siUSP38#1 and siUSP38#2 in protein level (**Figure 2A**). Plasmids were used to overexpress USP38 in two cell lines (**Figure 2B**). CCK8 assays demonstrated that the number of GC cells incubated with siUSP38#1 or siUSP38#2 were also significantly reduced than siRNA group (siCtrl) (**Figure 2C**). In the USP38 overexpression group, the survival of GC cells increased compared to control group (**Figure 2D**). Furthermore, downregulation of USP38 impaired the growth of GC cells (**Figure 2E** and **2F**), while USP38 overexpression promoted GC cells growth (**Figure 2G** and **2H**). Collectively, USP38 overexpression contributes to the growth of GC cells.

USP38 regulated GC cell apoptosis, cell cycle and migration

As shown in **Figure 3A**, when USP38 was silenced in the cells, the percentage of apoptotic cells increased, especially for HGC27 cells. By contrast, USP38 overexpression suppressed apoptosis (**Figure 3B**). In addition, USP38 overexpression reduced the G0/G1 phase in AGS and HGC27 cell cycle (**Figure 3C** and **3D**). Then, compared with the siCtrl group, cell migration ability of AGS and HGC27 was de-

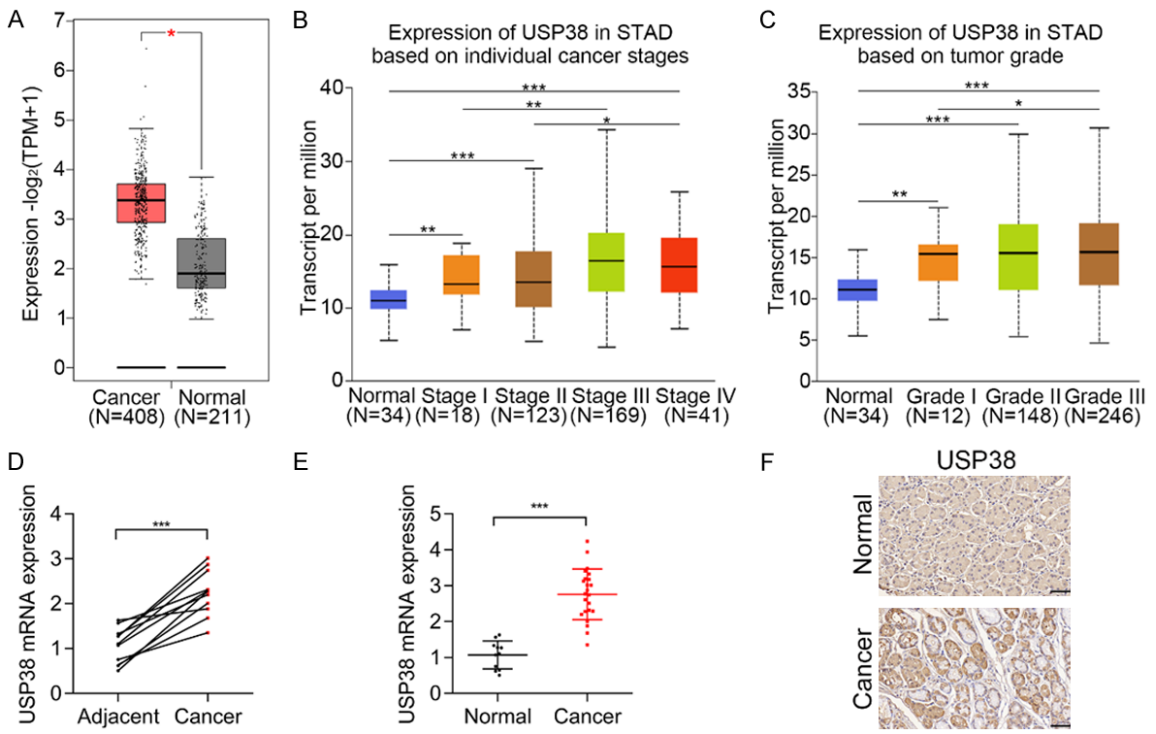

Figure 1. Upregulation of USP38 in GC specimens. A. mRNA level of USP38 was analyzed in GC and normal tissues based on TCGA database. B. Relative expression level of USP38 in different clinical stages. C. Relative expression level of USP38 at different clinical grades. D. The mRNA level of USP38 in GC and adjacent normal tissues. E. The mRNA level of USP38 in GC and normal tissues. F. IHC staining results of USP38 in GC and normal tissues. *P<0.05; **P<0.01; ***P<0.001.

Table 1. The protein abundance of USP38 and FASN in gastric cancer and normal tissues by IHC

Genes	Cancer	Normal	χ^2	*p* Value
USP38				
High expression	14	3	8.17	<0.01
Low expression	4	9		
FASN				
High expression	13	4	4.43	<0.05
Low expression	5	8		
Total	18	12		

creased in siUSP38#1 and siUSP38#2 groups (**Figure 3E**). Conversely, USP38 overexpression promoted AGS and HGC27 cell migration (**Figure 3F**). Thus, USP38 regulates GC cell apoptosis, cell cycle progression, and migration.

USP38 expression positively correlated with the expression of FASN

To explore the molecules downstream of USP38, we performed mass spectrum in USP38 overexpressing cells after IP with Flag or IgG. We found that FASN was a potential target for USP38 (**Figure 4A**). Next, we conducted WB and IP assay and found that USP38 could interact with FASN (**Figure 4B** and **4C**). The RT-qPCR and WB results suggested that USP38 promoted the expression of FASN protein, not mRNA expression (**Figure 4D** and **4E**), which indicated that USP38 interacted with FASN and promoted the protein stability of FASN through the thiol-dependent ubiquitin hydrolase activity. To explore the correlation between USP38 and FASN in GC patients, we performed IHC staining in the tissues, which were also used to analyze the expression of USP38. The IHC results indicated that FASN was overexpressed in GC tissues (**Figure 4F** and **Table 1**). In summary, we preliminarily confirmed that USP38 expression positively correlated with the FASN expression in GC cells and tissues.

FASN mediated fatty acid synthesis promoted the proliferation and migration of GC cells

FASN is a key enzyme in regulating the synthesis fatty acid, which usually formed triglyceride in cells and tissues. To understand the mechanism of USP38 involved in fatty acid synthesis, we first studied the production of triglyceride in

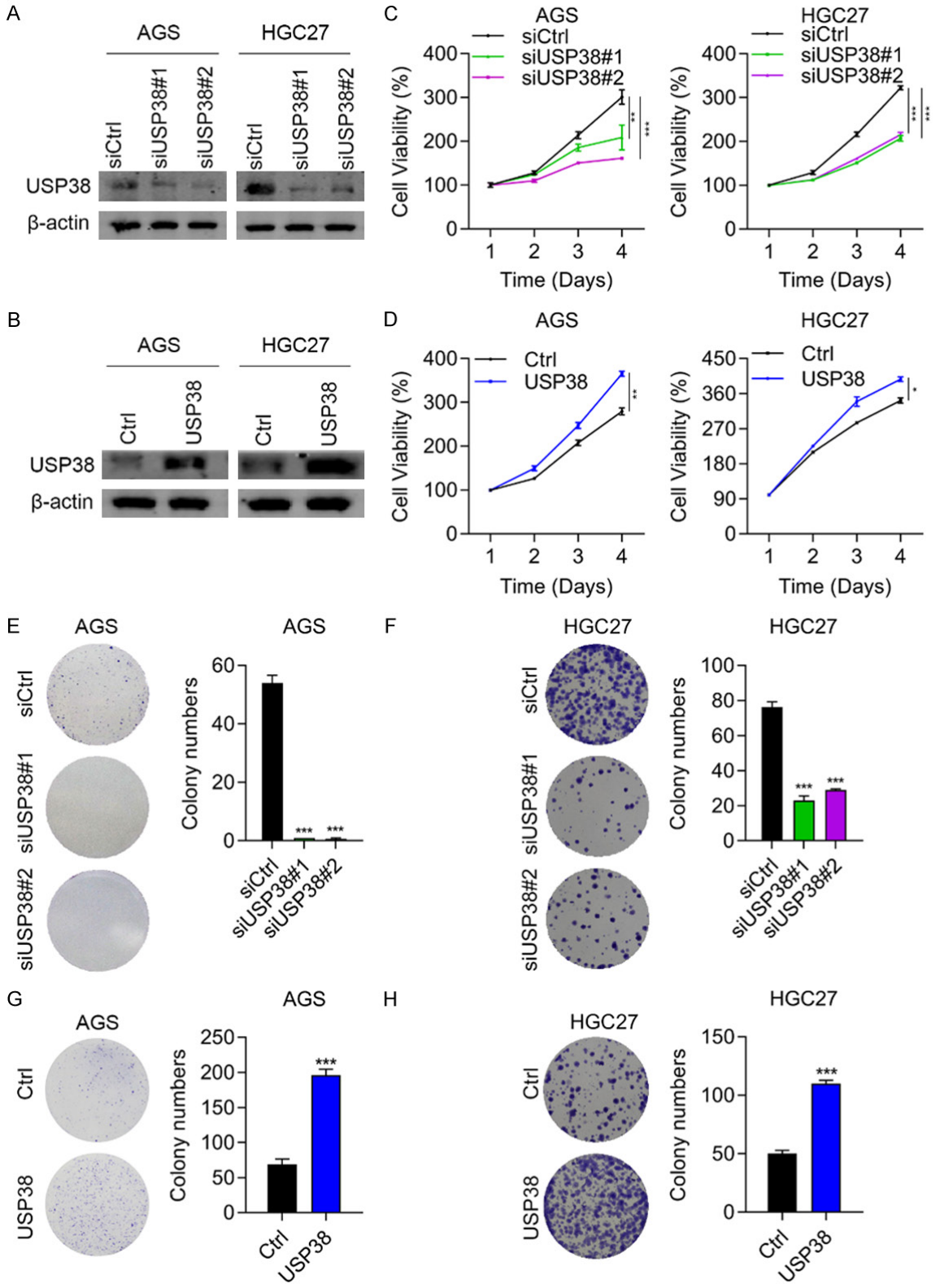

Figure 2. Effects of USP38 silencing and overexpression on the growth of GC cells. A. The USP38 protein level of GC cells after silencing of USP38. B. The USP38 protein level of GC cells after overexpression of USP38. C. Cell viability of GC cells after silencing of USP38. D. Cell viability of GC cells after overexpression of USP38. E, F. Cell colony formation of GC cells after silencing of USP38. G, H. Cell colony formation of GC cells after overexpression of USP38. *P<0.05; **P<0.01; ***P<0.001.

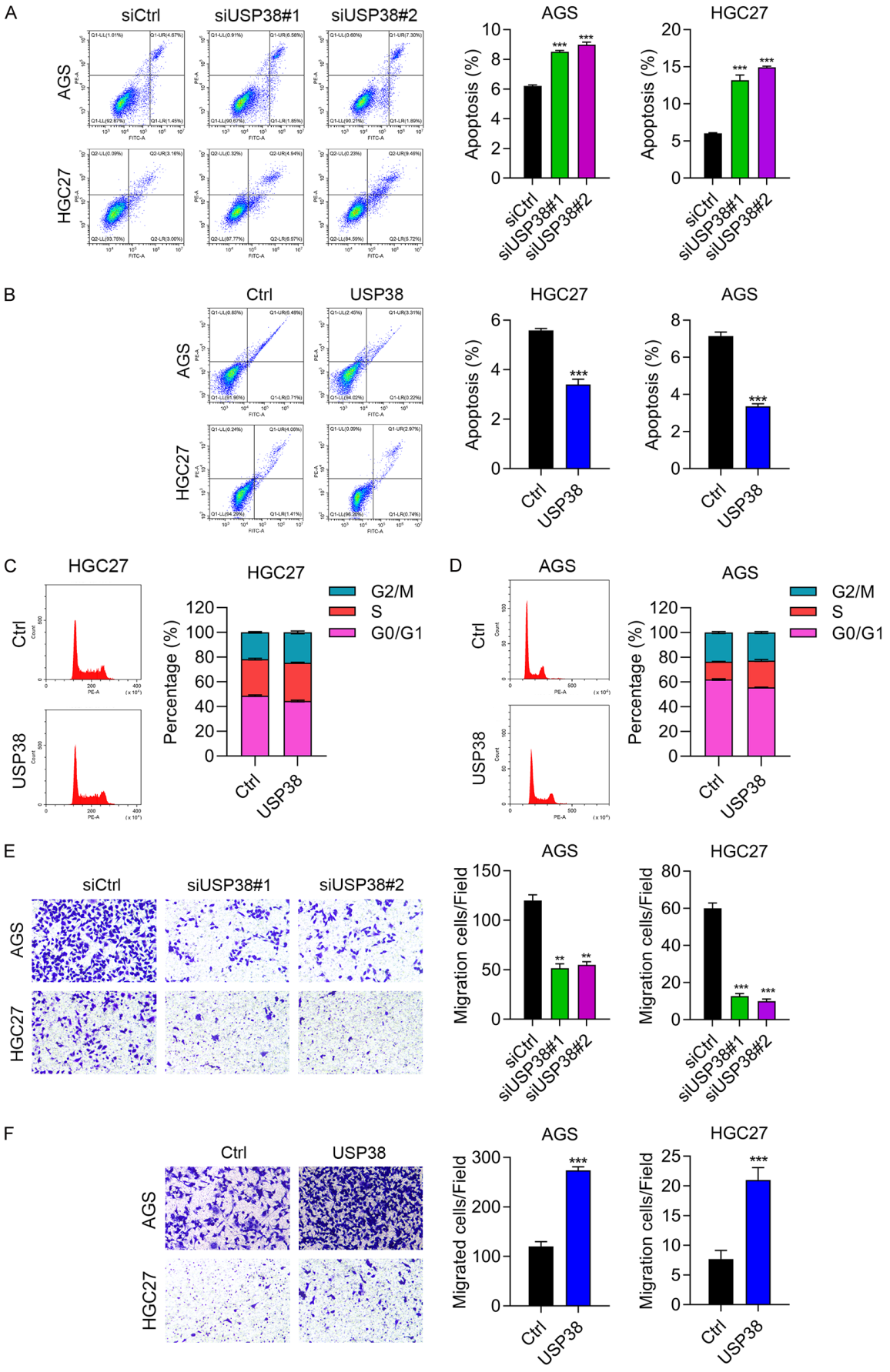
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Figure 3. USP38 regulates apoptosis, cell cycle, and cell migration. (A) Knockdown of USP38 induces apoptosis of GC cells. (B) USP38 overexpression inhibits the apoptosis of in GC cells. (C, D) USP38 overexpression promotes cell cycle progression in HGC27 (C) and AGS (D) cells. (E) Knockdown of USP38 inhibits GC cells migration. (F) USP38 overexpression promotes GC cells migration. ***P<0.001.

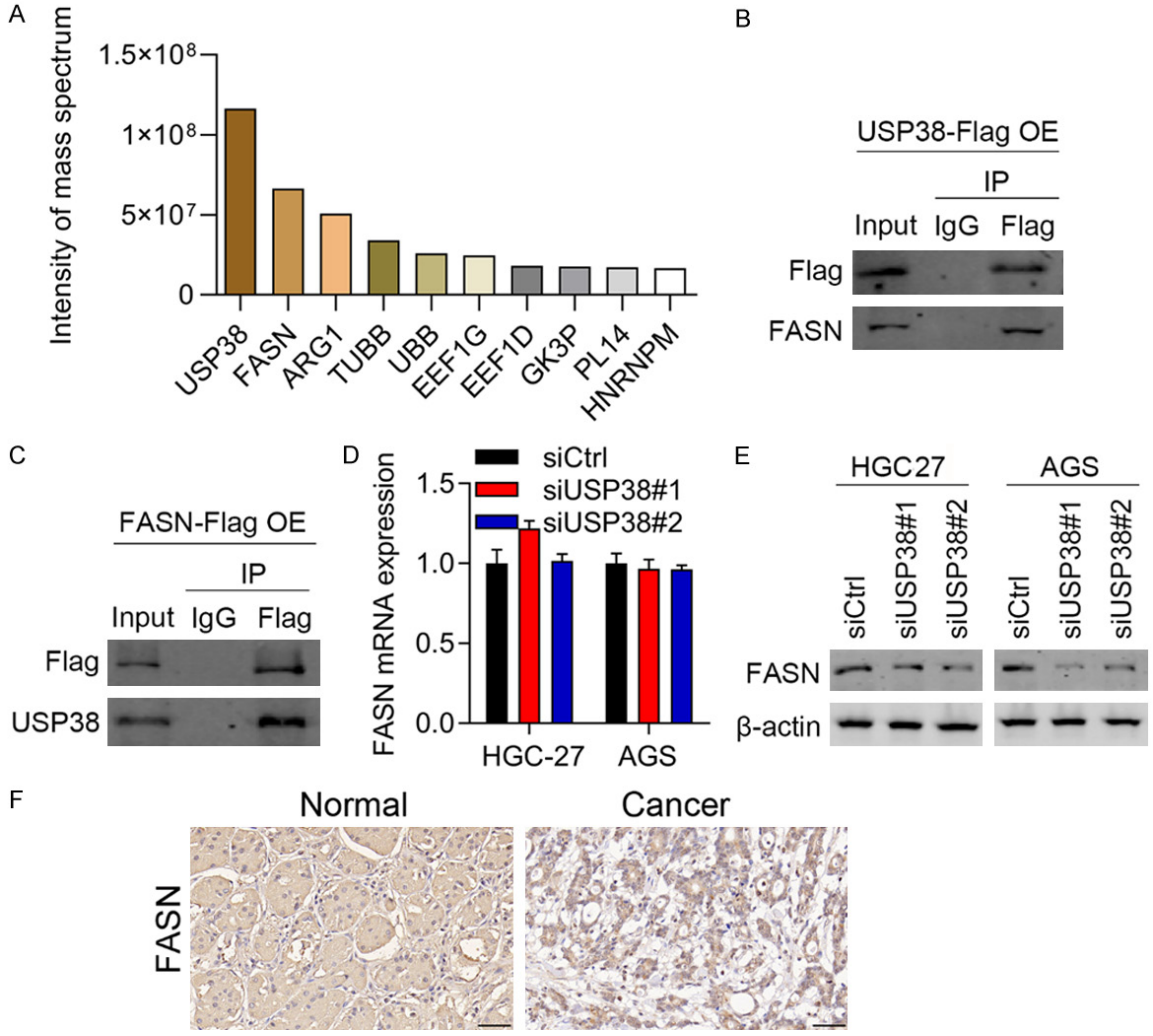

Figure 4. USP38 promotes expression of FASN in GC cells and tissues. (A) Mass spectrum analysis of proteins that potentially interacted with USP38. (B, C) IP assays were performed with Flag and IgG in USP38-Flag (B) and FASN-Flag (C) overexpressing cells to examine the interaction between USP38 and FASN. (D) RT-qPCR analysis of FASN in negative control and USP38-knockdown AGS and HGC27 cells. (E) USP38 knockdown inhibits FASN protein expression in HGC27 and AGS cells. (F) IHC results for the expression of FASN in GC and normal tissues.

GC cells after knocking down USP38. Compared with siCtrl group, lipid synthesis was significantly decreased in AGS and HGC27 cells (**Figure 5A**). To determine the role of FASN and FASN-mediated fatty acid synthesis on GC cell growth and migration, we treated Ctrl and USP38 overexpressing cells with or without FASN specific inhibitor, orlistat. Overexpression of USP38 enhanced the inhibitory effect of orlistat on the proliferation of GC cells (**Figure 5B** and **5C**). Moreover, orlistat treatment had a higher inhibitory effect on triglyceride synthesis in USP38-overexpressing cells (**Figure 5D** and **5E**). The same result was obtained in the clone-formation and migration results (**Figure 5F** and **5G**). Therefore, FASN mediated fatty acid production is critical for GC cell growth and migration.

USP38 promotes gastric tumorigenesis through FASN-mediated triglyceride synthesis

To verify the effect of USP38 in vivo, we generated nude mice with subcutaneous tumors.

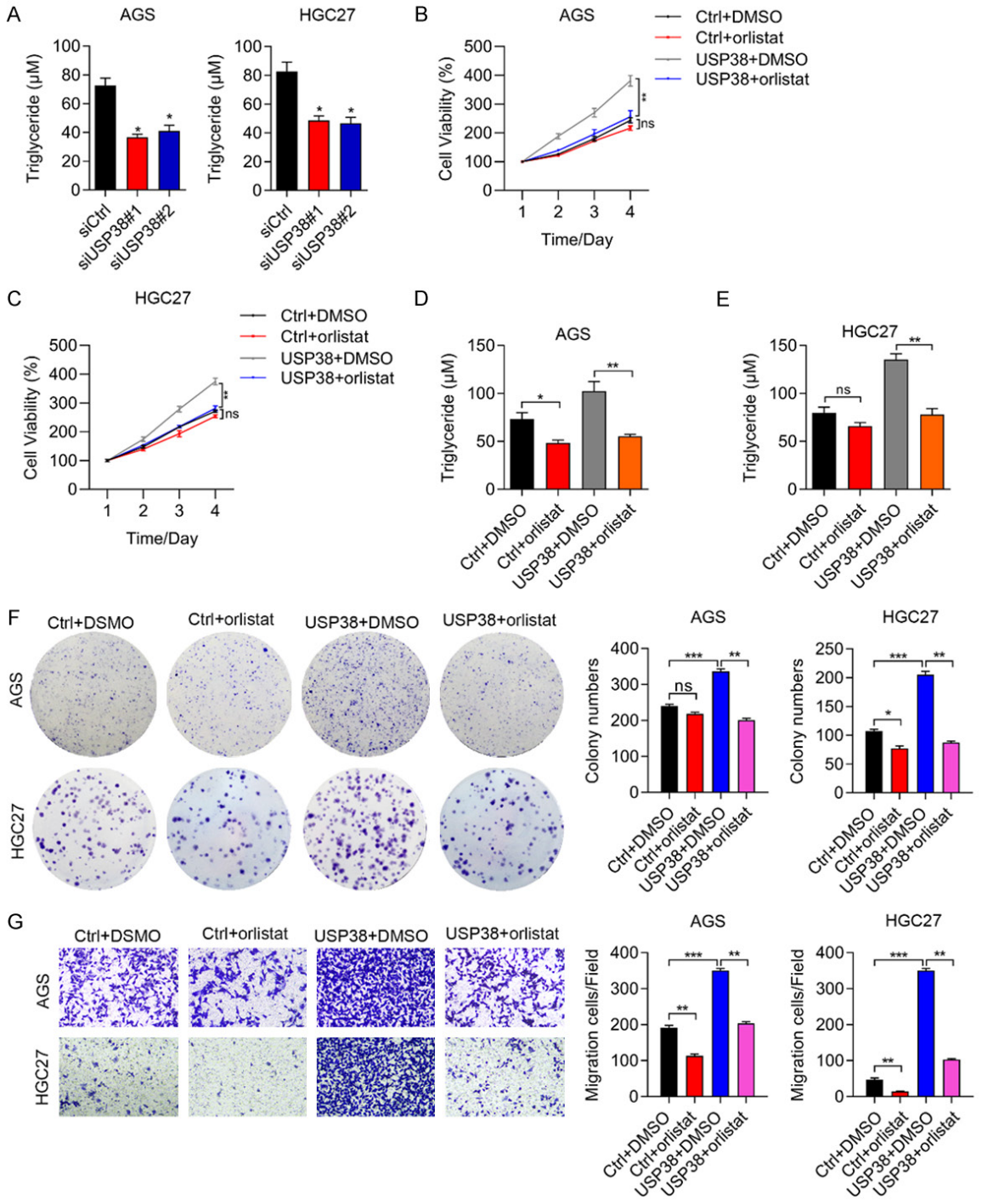

Figure 5. USP38 promotes GC progression through FASN mediated lipid synthesis. (A) Triglyceride concentration was detected in AGS and HGC27 cells after USP38 knockdown. (B, C) The proliferation was examined in Ctrl and USP38 overexpressing AGS (B) and HGC27 (C) cells treated with DMSO or 10 μg/ml orlistat. (D, E) The concentration of triglyceride was measured in Ctrl and USP38 overexpressing AGS (D) and HGC27 (E) cells treated with DMSO or 10 μg/ml orlistat. (F) Clone formation was examined in Ctrl and USP38 overexpressing GC cells treated with DMSO or 10 μg/ml orlistat. (G) Migration ability was examined by Transwell in Ctrl and USP38 overexpressing GC cells treated with DMSO or 10 μg/ml orlistat. ns, not significant. *P<0.05; **P<0.01; ***P<0.001.

The tumor volumes and tumor weight derived from HGC27 cells with stable USP38 overexpression were larger than control groups, while treatment with orlistat could decrease the pro-

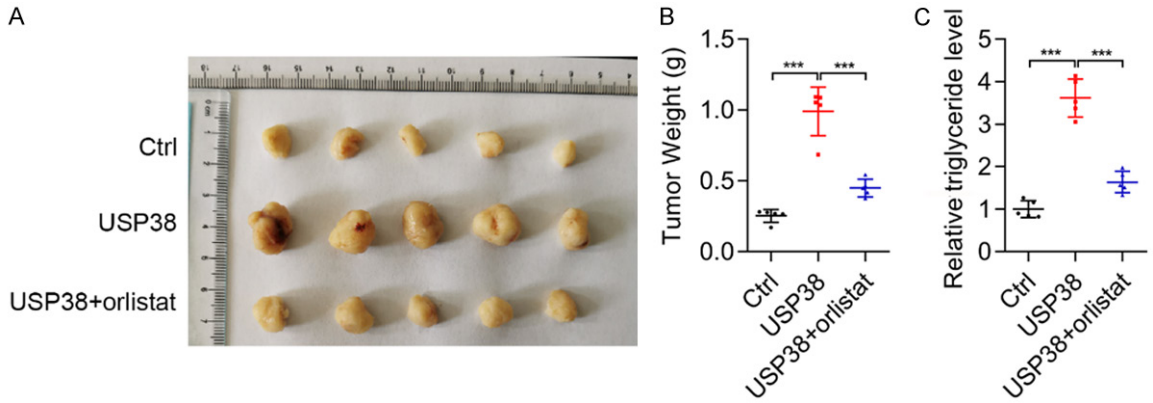

Figure 6. USP38 promotes GC cell growth dependent on enhancing lipid synthesis *in vivo*. (A) Xenografts images at 28 days after subcutaneous injection of HGC27 cells with control or USP38 overexpression treated with or without 240 μg/g of orlistat. (B) Tumor was weighted at day 28. (C) Triglyceride production was determined in the tumor tissues from (A). ***P<0.001.

moting effect induced by USP38 overexpression (**Figure 6A** and **6B**). For the triglyceride metabolic alterations, USP38 expression increased triglyceride production, and orlistat treatment significantly decreased the upregulated triglyceride production induced by USP38 (**Figure 6C**). Taken together, USP38 promotes tumorigenesis of GC cells by activating FASN.

Discussion

GC is one of the most common cancers worldwide, particularly in Asian countries [17]. Previous studies have shown that deubiquitinases is critical for tumorigenesis by regulating cancer stem cells [18]. Hence, it is essential to study the potential mechanisms of GC progression. Initially, USP38 has been reported to consider as a negative regulator of type I interferon [6]. Furthermore, the study showed that USP38 was involved in the pathogenesis of asthma [7]. Moreover, USP38 modifies the protein lysine-specific histone demethylase 1A by splicing its ubiquitin chain 17, thereby stabilizing key histones [8].

Previously, numerous studies have shown that ubiquitin-specific proteases exhibit diagnostic potential in GC patients. For example, USP22 was abnormal expressed in GC tissues, and higher expression of USP22 promoted GC cell malignancy [19]. Other oncogenic USPs in the progression of GC include USP39, USP32 and USP29 [20-22]. Recently, it has been demonstrated that USP38 regulates the histone modification status of regulatory genes in colorectal cancer stem cells [9]. Nevertheless, the role of USP38 in GC requires further study. In this study, USP38 was found to be a novel regulator of GC. USP38 was upregulated in GC tissues, similar with USP22, USP39, USP32 and USP29. In addition, USP38 overexpression promoted GC cells proliferation and migration, while its suppression suppressed GC cells growth and migration. In addition, USP38 suppressed apoptosis and impaired cell cycle progression in both AGS and HGC27 cells. Furthermore, we established a xenotransplantation model of USP38 overexpression in nude mice and found that USP38 could promote the tumor formation. Taken together, our findings suggest that USP38 is a component of GC progression and can be used as a predictor in GC patients.

FASN is a well-known oncogenic protein [23, 24]. Lipid metabolism and FASN provide new information for the treatment of human cancers [25-27]. FASN was reported to be upregulated in the tissues of patients with early stage of cancer. Additionally, the FASN upregulation in cancer cells implies the activation of fatty acid synthesis pathways [28, 29]. In this study, we found that FASN can interact with USP38 and is positively regulated by USP38 in GC cells. Additionally, orlistat, which is a FASN inhibitor that prevents the hydrolysis of triacylglycerols into free fatty acids and monoacylglycerides, had higher inhibitory effect on the proliferation and migration of GC cells with overexpression of USP38. In vivo, orlistat treatment could reverse the tumor promoting function of USP38. We also found that orlistat reduced tri-

glyceride production in GC cells and tumors with USP38 overexpression. Therefore, we concluded that USP38 promoted the triglyceride production, growth, migration and tumorigenesis of GC cells through upregulation of FASN.

The limitation of our study is that we did not study the correlation between the expression of USP38/FASN and the prognosis of patients with GC. This limitation needs to be addressed in the future by collecting a large amount of GC tissues.

Conclusions

Overall, our study showed that the upregulation of USP38 promoted cancer cell proliferation and migration. We further elucidated FASN as the downstream effector of USP38 in vitro and in vivo. This study will help us understand the pathological mechanism of GC and will provide a theoretical basis for further research on its early diagnosis and biological treatment.

Acknowledgements

This study was supported by grants from the Beijing Municipal Science and Technology Commission (No. D171100006517003), the Beijing Municipal Administration of Hospitals Incubating Program (No. PX2020001), and the Research Foundation of Beijing Friendship Hospital, Capital Medical University (No. Y2018-3).

Disclosure of conflicts of interest

None.

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