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Understanding The Regulatory Role of USP32 and SHMT2 in The Progression of Gastric Cancer

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Abstract

Objective: Gastric cancer is the fifth most common neoplasm and the fourth reason for mortality globally. Incidence rates are highly variable and dependent on risk factors, epidemiologic and carcinogenesis patterns. Previous studies reported that *Helicobacter pylori* (*H. pylori*) infection is one the strongest known risk factor for gastric cancer. USP32 is a deubiquitinating enzyme identified as a potential factor associated with tumor progression and a key player in cancer development. On the other hand, SHMT2 is involved in serine-glycine metabolism to support cancer cell proliferation. Both USP32 and SHMT2 are reported to be upregulated in many cancer types, including gastric cancer, but its complete mechanism is not fully explored yet. The present study explored possible mechanism of action of USP32 and SHMT2 in the progression of gastric cancer.

Materials and Methods: In this experimental study, Capsaicin (0.3 g/kg/day) and *H. pylori* infection combination was used to successfully initiate gastric cancer conditions in mice. It was followed by 40 and 70 days of treatment to establish initial and advanced conditions of gastric cancer.

Results: Histopathology confirmed formation of signet ring cell and initiation of cellular proliferation in the initial gastric cancer. More proliferative cells were also observed. In addition, tissue hardening was confirmed in the advanced stage of gastric cancer. USP32 and SHMT2 showed progressive upregulated expression, as gastric cancer progress. Immunohistologically, it showed signals in abnormal cells and high-intensity signals in the advanced stage of cancer. In USP32 silenced tissue, expression of *SHMT2* was completely blocked and reverted cancer development as evident with less abnormal cell in initial gastric cancer. Reduction of SHMT2 level to one-fourth was observed in the advanced gastric cancer stages of USP32 silenced tissue.

Conclusion: USP32 had a direct role in regulating SHMT2 expression, which attracted therapeutic target for future treatment.

Keywords: Cancer, Gastric Cancer, *H. pylori*, SHMT2, USP32

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Introduction

Gastric cancer is a condition of malignant tumour originated from gastric mucosa layer. Each year, more than 1 million people are newly developing gastric cancer, as the 3rd most leading form of cancer that results in death (1, 2). Another important fact behind the gastric cancer is that 70% of cases are reported from developing countries whereby China alone is reported with more than 50% of cases (3). Although there is some advancement happening in the treatment procedure, prognosis of gastric cancer in the early stage is still poor that makes the overall 5 years survival rate less than 20-30% (4). In the present scenario, it is essential to identify a new prognostic marker to identify gastric cancer in its early stage and to design new therapies, to improve 5 year survival rate (5).

Ubiquitin-specific protease 32 (USP32) is recognized as a new member of the ubiquitin-specific proteases subfamily. It alters protein stability and localization, thereby regulating their activity in different pathological stages of many human diseases, like cancer (6-8). So far, it has been reported that USP32 was over-expressed in lung and breast cancers, enhancing cellular proliferation and tissue metastasis (9). Recent knock-down studies with USP32 represented significant reduction of gastric cancer cell proliferation and cellular migrations both *in vitro* and *in vivo* (10). However, very little is known about the regulating effect of USP32 on other key proteins to promote gastric cancer.

Serine hydroxymethyltransferase-2 (SHMT2) is a key enzyme presented within the mitochondria. It is involved in the conversation of serine to glycine (11, 12). SHMT2 was highly expressed in many cancer cells like brain, colorectal,

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bladder and kidney, which enhanced (7) their proliferative ability (13, 14). SHMT2 enhanced serine catabolism and thereby supported cancer growth by increasing nucleotide synthesis (12), mitochondrial translation (15), redox balance (16), methylation of DNA and histone protein (12) and by suppression of retrotransposon activation (17). Higher expression of SHMT2 was recently reported in gastric, colon and esophageal cancers (18). Therefore, this needs to extensively studied in different stages of gastric cancer along with the other key linked protein. The present investigation explored relationship of USP32 with SHMT2 protein in progression of gastric cancer.

Materials and Methods

The experimental study was ethically approved by General Hospital of Ningxia Medical University, Ningxia, China (EXC/20180819).

Mice model and experimental design

Two-months-old male mice of strain C57-BL/6J-219 with an average weight of 20-22 g were purchased from Vital River Laboratory (Ningxia, China). The mice were free from Helicobacter spp., Salmonella spp and Citrobacter rodentium. They were maintained in laboratory environment with ambient temperature of 24ºC, humidity of 55-60% and light/dark cycle for every 12 hours interval. The mice were fed with a regular diet containing sterilized commercial pellets obtained from Beijing HFK Bioscience (China) and provided with sterilized water ad libitum. All of the experimental procedures, followed here, were performed after getting the institutional ethical committee approval and they were in accordance with the recent guidelines. The mice were randomly divided into three groups: negative control (NC) group, initial stage gastric cancer group and advanced stage gastric cancer group (n=10 for each group). After one week of the acclimation period, the mice were administrated capsaicin (0.3 g/kg/day) in dietary food for two weeks before *H. pylori* infection and the capsaicin administration was continued throughout the experiment, as described earlier (19). From the 3rd week of capsaicin injection, each mouse was administered a suspension of the *H. pylori* SS1 strain containing 10^8 CFUs/ml by gavage, three times per week. Additionally, for effective *H. pylori* colonization, pantoprazole (25 mg/kg) was administrated three times per week by gavage method to lower gastric acidity. The experimental procedure was continued: one group of animals was euthanized at the end of 40 weeks and the other groups were euthanized at 70 weeks. For control purpose, the mice were left untreated and euthanized at the end of 70 weeks. After euthanizing the mice, the stomach was excised out and the gastric tumour was microscopically dissected for further experiments.

Histological procedure

The dissected gastric cancer tissue with size of 3-5 mm was carefully transferred to 10% formalin solution and kept for 48 hours at room temperature. The tissues was washed with tap water and subjected to dehydration step using an increased concentration of ethanol (70-100%). The dehydrated tissue was transferred to xylene for the tissue clearing step and it was finally embedded with wax. Using microtome, 6 µm thin sectioning of the block containing tissues was done and the ribbon was placed on the glass slides. The glass slides containing the sections were dewaxed, dehydrated and stained with haematoxylin and eosin. The slides were finally mounted using DPX mounting solution (Sigma Aldrich, Germany) and examined under EVOS® FL Cell Imaging System (Thermo Fisher Scientific, USA).

Immunohistochemistry

The wax-embedded tissue was set up in the microtome and allowed for 5 µm uniform sectioning. After dewaxing and rehydration, the sections were subjected to antigen retrieval step using heat-induced method in which the slides were boiled for 10 minutes in 10 mM sodium citrate buffer (pH=6.0). The slides were pre-incubated with 5% bovine serum albumin (BSA, Thermo Fisher Scientific, USA) in 1X TBST for 1 hour prior to primary antibody incubation for background minimization. The primary antibodies anti-USP32 (ab251903, 1:800; Abcam, USA), anti-SHMT2 (12762, 1:1000; Cell Signaling Technology, USA) and anti-β actin (sc-47778, 1:1000; Santa Cruz, USA) were diluted in 5% BSA solution and overlaid with tissue samples. They were then incubated in 4ºC for 4 hours. The slides were washed with 1X TBST for three times to remove non-specific binding of antibody. Next, they were further incubated with HRP conjugated secondary antibody (ab97051, 1:1000; Abcam, USA) for 2 hours in room temperature. After washing, the slides were completely drain out and incubated with the HRP (Bio Rad, India) substrate solution of DAB (3,3´-Diaminobenzidine; Bio Rad, India) for 15 minutes in dark environment at 37ºC. After washing, the slides were counter stained with haematoxylin for 5 minutes and the sections were mounted using DPX solution. The slides were examined under EVOS® FL Cell Imaging System (Thermo Fisher Scientific, USA).

siRNA against *USP32*

For *USP32* silencing purposes, commercially available siRNA against *USP32* 5´-GACCUGUGGACUCUCAUAUTT-3´ (si-USP32-homo-386) was purchased from Gene Pharma, China along with the NC 5′-UUCUCCGAACGUGUCACGUdTdT-3´ (si-NC) (10). The siRNA samples were dissolved in water to achieve final concentration of 0.6 µg/µl. It was next mixed with 0.5 µl of 50 nM Lipofectamine 2000 prior to injection to improve transfection efficiency. Injection of siRNA was performed through the tail vein exactly before

one month of mice scarification.

RNA isolation and quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from tissues using Trizol reagent (Invirogen, USA) as per the standard protocol. Synthesis of complementary DNA (cDNA) was performed using RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific, USA). For evaluating the relative transcript levels of different genes, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with the help of SYBR Green RT-PCR kit (Takara Shuzo Co. Ltd, Japan). The thermo-cycling was performed on the Quant Studio 5.0 Real-Time PCR system (Applied Biosystems, USA). Quantitative assessment of mRNA expression levels was made using the $2^{-\Delta\Delta Ct}$ method. *GAPDH* was used as an endogenous expression controls. Sequences of the qRT-PCR primers used were:

USP32-

F: 5′-GGCTGCTCGTGATATGCTGTTC-3′
R: 5′-GTTTCTGGGCTGACACCTTGC-3′

SHMT2-

F: 5′-AGTCTATGCCCTATAAGCTCAACCC-3′
R: 5′-GCCGGAAAAGTCGAGCAGT-3′

GADPH-
F: 5′-CATCTCTGCCCCCTCTGCTGA-3′
R: 5′-GGATGACCTTGCCCACAGCCT-3′.

$2^{-\Delta\Delta Ct}$ method was used for estimating the relative expression levels.

Western blotting

The dissected gastric cancer tissue samples were crushed with 2X samples buffer using a pre-cooled mortar and pestle. The cell lysate was heated in a boiling water bath for 10 minutes and later cooled. The concentration of protein samples were determined using Bradford method. The equal quantity of protein samples (70 µg/well) were loaded in the 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and separated at 100V for 4 hours. The separated proteins in the gel were transferred to the PVDF membrane effectively using the semi-dry method. To minimize the background signals, the PVDF membrane was incubated with blocking solution containing 5% BSA solution in 1X TBST buffer for 2 hours at 37ºC. The membrane was then incubated with primary antibodies like, anti-USP32 (ab190184, 1:800; Abcam, USA), anti-SHMT2 (12762, 1:1000; Cell Signaling Technology) or anti-β actin (sc-47778, 1:1000; Santa Cruz, USA) for 4 hours at 4ºC. After terminating incubation, the membrane was washed with 1X PBS for three times, each time containing 10 minutes each. Finally, the blot was incubated with the secondary antibodies (ab205718, 1:5000 or ab205720, 1:15000; Abcam, USA) and further developed with Pierce™ DAB Substrate Kit (Thermo Fisher Scientific, USA). The band intensity developed in the membrane was documented using gel documentation system and analysed using ImageJ software.

Statistical analysis

For achieving statistical significance, the experiments were repeated for at least three or more times and the obtained results were denoted in mean ± SD. Student's t test and ANOVA using posthoc Tukey's t test were used to estimate significance of the statistical difference between two/among many data points. The obtained results were considered as statistically significant when the $P<0.005$.

Results

Induction of initial and advanced stages of gastric cancer

In the control gastric tissue, the cells were normal without any abnormal variations and the cells were packed in regular interval (Fig.1A). In initial stage of gastric cancer, tissue showed histological variations like formation of signet ring cell along with initiation of more proliferative cells which disturb the normal arche structure of gastric tissue arrangement (Fig.1B). In the advanced stage of gastric cancer, there were complete change of normal gastric tissue structure with more proliferative cells which were ultimately resulted in more cellular density and tissue hardening (Fig.1C). In *USP32* silenced tissue, the histopathological observation in initial gastric cancer stage revealed no signet ring cell formation and reduced proliferative nature of cells were also seen (Fig.1D). Similarly, in *USP32* silenced tissue, the histopathological observation in advanced gastric cancer stage revealed no tissue hardening and highly reduced proliferative nature of cells (Fig.1E).

USP32 and SHMT2 showed elevated expression as gastric cancer progress

Following the confirmation of initial and advanced stages of gastric cancer, expression of USP32 and SHMT2 were analyzed upon the progression of gastric cancer, as shown in Figure 2. In the control gastric tissue, expression of USP32 was difficult to locate within the tissue layer (Fig.2A). However, in initial tumour tissue, expression of USP32 was substantially upregulated within the abnormal cells (Fig.2B). As the cancerous condition progresses, expression of USP32 showed strong signals throughout the tissue layer (Fig.2C). Similarly, SHMT2 expression levels were examined in the control gastric tissue (Fig.2D). SHMT2 showed progressive overexpression with respect to cancer stages (Fig.2D-F). Expression of SHMT2 was localized to abnormal cells in the initial stage of gastric cancer (Fig.2E). However, expression of SHMT2 was widespread (with high intensity signals) throughout the tissue in advanced stage of gastric cancer (Fig.2F).

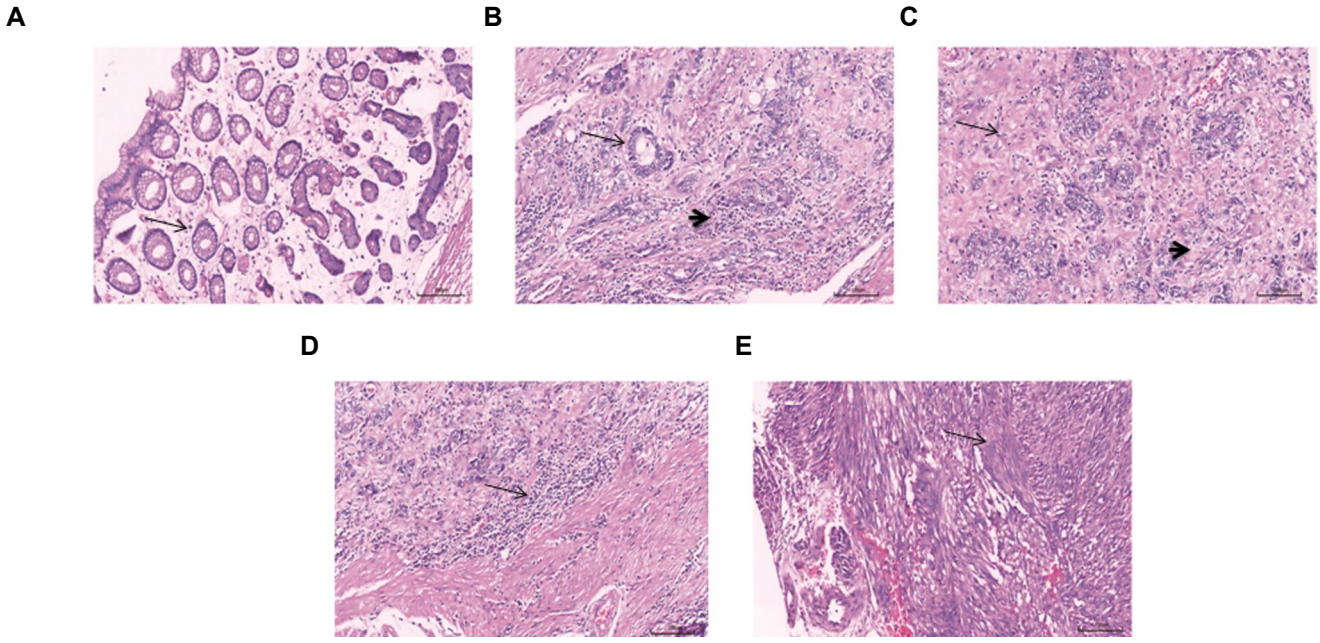

Fig.1: Histopathogy of gastric cancer progression. **A.** The control gastric tissue shows uniformly distributed cellular arrangement. **B.** Initial gastric cancer tissue showing signet ring cell (arrow) and proliferative cells (arrow head). **C.** Advanced stage of gastric cancer tissue with more proliferative cells (arrow) with tissue hardening (arrow head). **D.** *USP32* silenced the initial gastric cancer tissue without signet ring cell with less proliferative cells (arrow). **E.** *USP32* silenced advanced gastric cancer tissue without tissue hardening and with moderate proliferative cells (arrow) (scale bar: 50 µm).

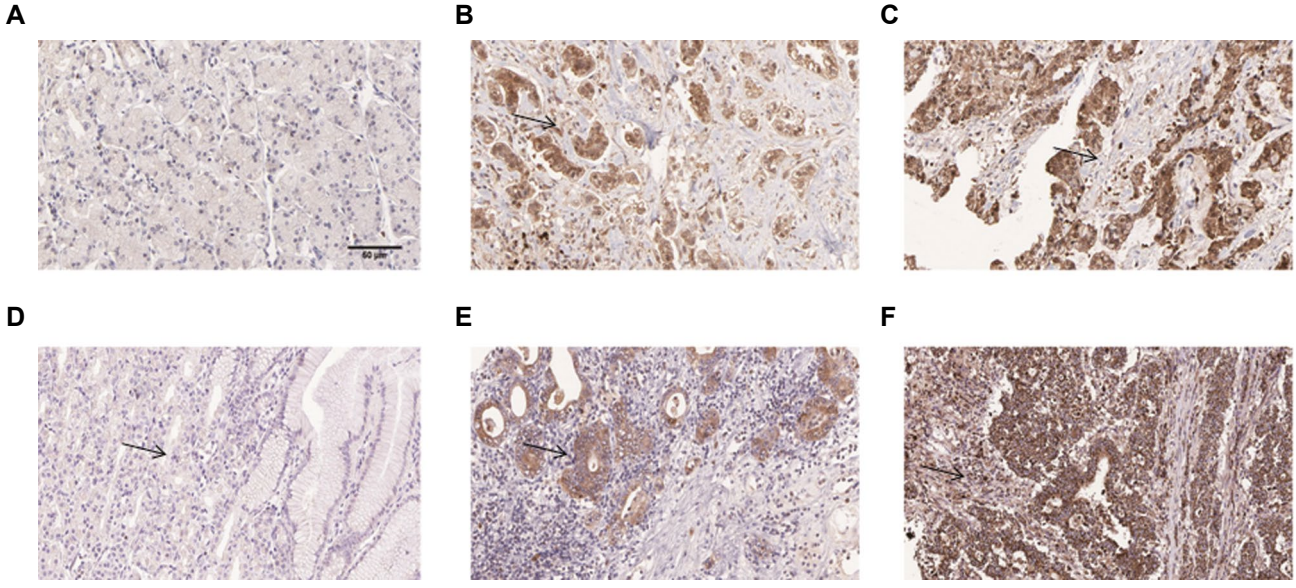

Fig.2: USP32 and SHMT2 expression analyses using immunohistochemistry. **A.** Control gastric tissue showing less signals for USP32. **B.** Progressive signal for USP32 observed in the initial gastric cancer tissue (arrow). **C.** Abundant and intense signal for USP32 observed in advanced stage gastric cancer (arrow). **D.** SHMT2 signal are very limited in the control gastric tissue (arrow). **E.** SHMT2 shows upregulated expression in initial gastric cancer tissue (arrow). **F.** Overexpression of SHMT2 was observed in the advanced stage of gastric cancer tissue (arrow) (scale bar: 50 µm).

USP32 silencing diminishes the *SHMT2* expression in the both initial and advanced stages of gastric cancer

To understand role of USP32 in regulating *SHMT2* expression, gene silencing experiments were performed. siRNA against *USP32* was found to completely block expression of *USP32* in the control tissue (Fig.3A) and it substantially reduced *USP32* expression in the initial gastric cancer tissue (Fig.3B). Additionally, downregulated expression of *USP32* was observed in the advanced stage of gastric cancer following siRNA injection against *USP32* (Fig.3C). Alteration of *SHMT2* expression, following *USP32* silencing was also investigated and it was observed that expression of *SHMT2* was completely downregulated in the control and initial stages of gastric cancer (Fig.3D, E). *USP32* silencing in advanced stage gastric cancer, reduced expression of *SHMT2* to a moderate level (Fig.3F).

USP32 specifically alters *SHMT2* expression

Following immunohistochemistry, western blot and

qRT-PCR experiments were performed to observe expression levels of USP32 and SHMT2 in the initial and advanced stages of gastric cancer. As shown in Figure 4A and B, expression level of USP32 was increased progressively in the initial and advanced stages of gastric cancer compared to the control tissue. Similarly, expression of SHMT2 showed progressive increase in the initial and advanced stages of gastric cancer compared to the control tissue. Silencing *USP32*, reduced expression of SHMT2 in the both initial and advanced stages of gastric cancer compared to the control tissue. Similarly, *SHMT2* silencing drastically reduced expression of *USP47* (almost not able to detect) in the control tissue and initial stage gastric cancer tissue. In the advanced stage, expression of USP47 was reduced compared to the non-silenced tissue. As shown in Figure 4C, similar results, as obtained in western blotting, were obtained by qRT-PCR.

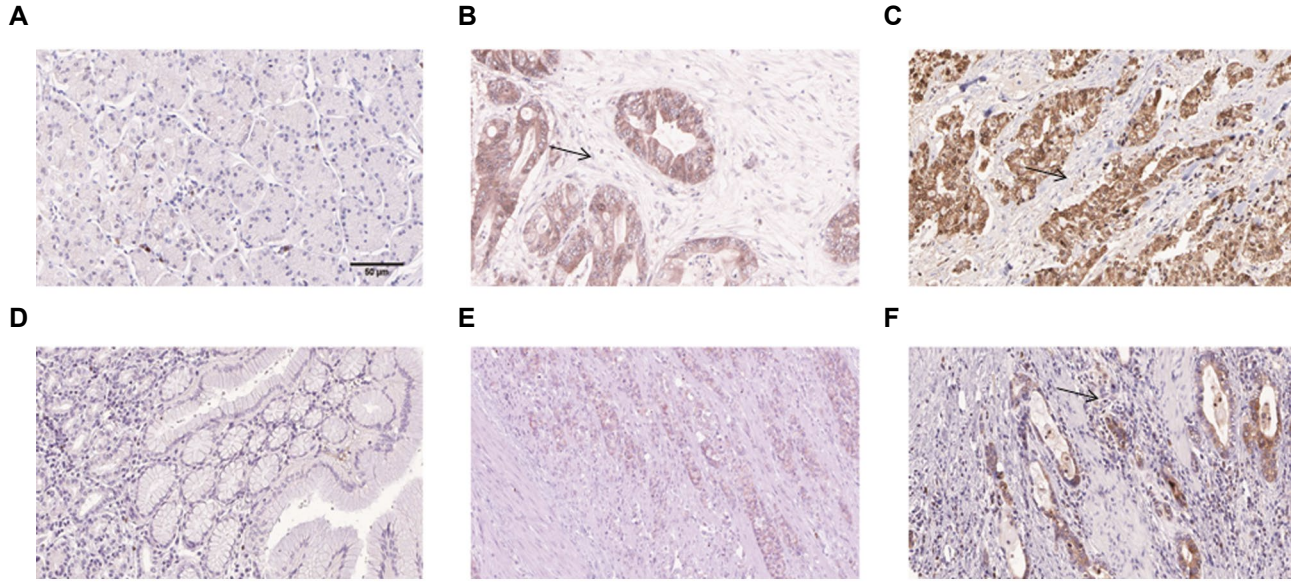

Fig.3: *USP32* silencing and *SHMT2* suppression. **A.** *USP32* silenced the gastric control tissue without *USP32* signal. **B.** *USP32* silenced in the initial gastric cancer tissue show limited expression of *USP32* (arrow). **C.** *USP32* silenced in the advanced stage gastric cancer tissue with moderate *USP32* expression (arrow). **D.** *USP32* silenced in the control gastric tissue without *SHMT2* expression. **E.** *USP32* silenced in the initial gastric cancer tissue show no *SHMT2* expression. **F.** *USP32* silenced in the advanced stage gastric cancer tissue with moderate *SHMT2* expression (arrow) (scale bar: 50 µm).

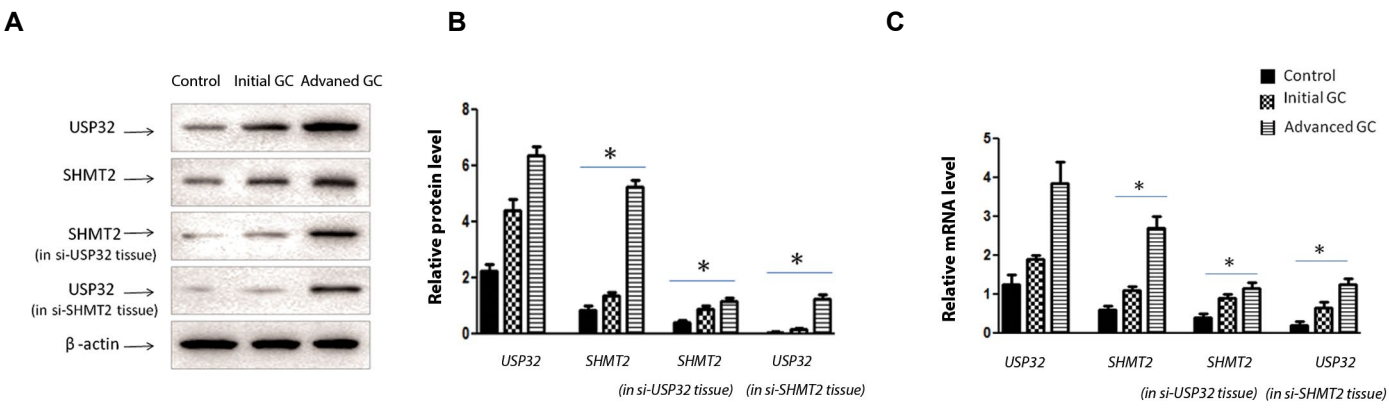

Fig.4: Expression analysis of USP47 and SHMT2. **A**, **B.** Protein expression level of USP32 was significantly and progressively increased in initial and advanced stages of gastric cancer than control tissue. Similarly, SHMT2 shows slight expression in the control tissue but its expression was significantly and progressively increased in the initial and advanced stages of gastric cancer. In *USP32* silenced tissue, expression of SHMT2 was drastically reduced in the control and initial gastric cancer tissues. However, in the advanced gastric cancer stage, the *USP32* silenced tissue showed significantly reduced expression of SHMT2 compared to the non-silenced tissue. Similarly, in the *SHMT2* silenced tissue, expression of USP47 was drastically reduced (almost not able to detect any band) in the control and initial stage gastric cancer tissue. However, in the advanced gastric cancer stage, *SHMT2* silenced tissue showed significantly reduced expression of USP47 compared to the non-silenced tissue. **C.** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis: mRNA expression level of *USP32* was significantly and progressively increased in the initial and advanced stages of gastric cancer than the control tissue. Similarly, *SHMT2* shows lower mRNA expression in the control tissue, but its expression was significantly and progressively increased in the initial and advanced stages of gastric cancer. In the *USP32* silenced tissue, mRNA expression of *SHMT2* was reduced in the control and initial gastric cancer tissues. However, in the advanced gastric cancer stage, *USP32* silenced tissue showed significantly reduced mRNA expression of *SHMT2* compared to non-silenced tissue. Similarly, in the *SHMT2* silenced tissue, mRNA expression of *USP47* was reduced in the control and initial stage gastric cancer tissue. However, in the advanced gastric cancer stage, the *SHMT2* silenced tissue showed significantly reduced mRNA expression of *USP47* compared to the non-silenced tissue. $2^{-\Delta\Delta Ct}$ method was used for estimating the relative expression levels. All of the experiments were performed thrice and (*) shows P<0.005.

Discussion

Combination of capsaicin and *H. pylori* infections contribute to developing gastric inflammation and finally gastric cancer. The synergy of capsaicin and *H. pylori* combination is able to induce gastric tumorigenesis by inducing inflammatory-related proteins and cytokines (19). Treated mice for 40 weeks showed signet ring cells, which are the sign of tumour initiation (20). In the initial gastric stage, the cells started to proliferate more, which was evident in tumour initiation (21). Following 70 weeks of treatment procedure, more proliferative cells were observed throughout the tissue layer. Along with this, tissue hardness were observed. These were due to the changes in extracellular matrix protein, as a consequent of extensive changes in cellular morphology (22). *USP32* silencing experiment was fascinating, as it reduced level of proliferative cells in the initial and advanced stages of gastric cancer. From the result, it is proposed that *USP32* silencing can inhibit progression of gastric cancer. It may also have a possible control over cellular migration and invasion, although this needs to be investigated. Earlier, similar type of link has been found between USP32 and SMAD Family Member 2 (SMAD2), in the development and progression of gastric cancer (10).

USP32 showed a progressive upregulated expression in the initial and advanced stages of gastric cancer, and careful examination showed that in the initial stage gastric cancer, its expression was restricted within the abnormal cells. USP32 may promote key cancer-related proteins through deubiquitinating in abnormal cells, which may promote cell migration (23), regulate stemness or be involved in epithelial-mesenchymal transition (24). Signal intensity for USP32 was high in the advanced stage of gastric cancer which may represent it strongly regulated different gastric cancer-related proteins as the cancer progresses. Usually, SHMT2 is highly expressed in mitochondria, while it is also detected in cytoplasm and nucleus (25). After reprogramming normal cell to gastric cancer cell, elevated serine/glycine metabolism is essential for cellular proliferation (26). With this evidence, the progressive upregulated expression of SHMT2 was observed with more cytoplasmic and nuclear expression.

In *USP32* silenced initial gastric cancer, SHMT2 expression was completely diminished. This implied its control over USP32. The expression of SHMT2 was also reduced in the *USP32* silenced advanced stage of gastric cancer. It implied its tight regulation even in the advanced stage. SHMT2 overexpression frequently occurs in the advanced grade of glioma condition and it has a role in cellular proliferation and invasion (18). Therefore, suppression its activity by inhibiting USP32 can hinder the root of SHMT2 activation and it may be used as a future therapeutic target.

Conclusion

Our findings provided a new insight into expression and function of USP32 in gastric cancer. USP32 was found to regulate SHMT2 expression in initial and advanced stages of gastric cancer and vice versa. Targeting USP32 and SHMT2 might be the potential therapeutic strategy for gastric cancer.

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Authors' Contributions

J.L.; Conception and design of the manuscript. Y.B.; Data acquisition or data analysis and interpretation. B.D.; Performs experimental work and drafting the manuscript. L.W.; Final approval of the manuscript, statistical analysis and supervision. All authors read and approved the final manuscript.

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