

USP28 promotes aerobic glycolysis of colorectal cancer by increasing stability of FOXC1

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Aerobic glycolysis is essential for cancer cell metabolism and growth. Deubiquitinase, USP28 (ubiquitin specific peptidase 28), could maintain stability of proteins involved in tumor progression. This study was performed to investigate the role of USP28 in aerobic glycolysis of colorectal cancer. Our data showed that USP28 mRNA and protein expressions were enhanced in colorectal cancer tissues and cells. Functional assays demonstrated that overexpression of USP28 promoted cell proliferation and aerobic glycolysis of colorectal cancer, while USP28 inhibition could reverse these effects. Protein expression of Forkhead Box C1 (FOXC1) was increased by USP28 over-expression, whereas knockdown of USP28 aggravated cycloheximide (CHX; protein synthesis inhibitor) stimulated decrease of FOXC1. Moreover, proteasome inhibitor, MG132, could rescue USP28 silence-induced degradation of FOXC1. Overexpression of FOXC1 counteracted the suppressive effects of USP28 interference on colorectal cancer cell viability and aerobic glycolysis. In conclusion, USP28 enhanced cell viability and aerobic glycolysis of colorectal cancer by stabilizing FOXC1, suggesting that USP28-FOXC1 might be a novel therapeutic avenue for colorectal cancer.

Keywords: USP28, FOXC1, aerobic glycolysis, stability, colorectal cancer

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Abbreviations: CHX, cycloheximide; FBP1, fructose-1,6-bisphosphatase 1; FOXC1, Forkhead Box C1; qRT-PCR, quantitative reverse transcriptase PCR; USP28, ubiquitin specific peptidase 28

INTRODUCTION

Colorectal cancer is one of the most common cancers in the world (Yang, 2019). Although 5-year survival rate of colorectal cancer patients is improved via surgical resection, radiotherapy and systemic chemotherapy at an early stage, high recurrence rate caused by distant metastasis still threatens a large proportion of advanced stage patients (Prenen *et al.*, 2013). Recently, efforts have been made to elucidate the genetic and molecular characteristics of colorectal cancer for better prognosis and response to targeted therapies (Fleshman & Smallwood, 2015). Understanding the mechanisms involved in cell proliferation and metastasis of colorectal cancer might also improve treatment of the patients.

Tumor cells undergo abnormal glycolysis, accompanied by increased glucose uptake and lactic acid production, that is known as aerobic glycolysis or Warburg effect (Lu, 2019). Aerobic glycolysis plays an essential role

in tumor cell proliferation with the synthesis of nucleic acids, amino acids and fats, and the production of lactic acid in microenvironment that could enhance tumor metastasis and treatment resistance (Doherty & Cleveland, 2013). Activation of aerobic glycolysis has been shown to be associated with colorectal cancer metastasis and poor prognosis of the patients (Satoh *et al.*, 2017). Therefore, inactivation of glycolysis might facilitate for suppression of colorectal cancer metastasis (Hu *et al.*, 2020).

Ubiquitin specific peptidase 28 (USP28), member of USPs, contains a ubiquitin-recognition site and corresponding catalytic mechanism (Wang *et al.*, 2018). With the ability to regulate physiological homeostasis of ubiquitination process, tune DNA-damage response and maintain cell cycle, targeting USP28 has shown clinical significance in cancer therapy (Wang *et al.*, 2018). For example, USP28 could increase the characteristics of breast cancer stem cells by deubiquitinating lysine-specific demethylase 1 (Wu *et al.*, 2013). Inhibition of USP28 promotes degradation of lysine-specific demethylase 1 to repress gastric cancer cell metastasis (Chen *et al.*, 2018). In colorectal cancer, USP28 could regulate intestinal homeostasis and promote the progression of intestinal diseases and tumor progression (Diefenbacher *et al.*, 2014; Diefenbacher *et al.*, 2015). Inhibitor of USP28 could reduce cancer-related proteins, followed by suppressing colorectal cancer progression (Wang *et al.*, 2020). However, the mechanism of USP28 in colorectal cancer aerobic glycolysis has not been reported yet.

This study was performed to explore the role and molecular mechanism of USP28 in aerobic glycolysis of colorectal cancer. Results showed that USP28 could promote aerobic glycolysis of colorectal cancer and promote the cell growth through enhanced stability of Forkhead Box C1 (FOXC1), thus providing a novel therapeutic avenue for colorectal cancer.

MATERIALS AND METHODS

Tissue specimens and immunohistochemistry

Sixty-three colorectal patients were recruited from First People's Hospital of Yuhang District, Hangzhou. The tumor tissues and adjacent healthy tissues were acquired from patients with informed consent. Ethics approval was acquired from the Ethics Committee of First People's Hospital of Yuhang District, Hangzhou. For immunohistochemical analysis, paraffin-embedded colorectal tumor tissues were cut into 4 μ m sections. Sections were deparaffinized, dehydrated, blocked, and incubated with primary anti-USP28 antibody (1:100; Miltenyi Biotec, Belgium, Germany). The sections were

incubated with horseradish peroxidase-labeled secondary antibody (Abcam, Cambridge, UK) followed by staining with diaminobenzidine and counterstaining with hematoxylin. Slides were visualized under a light microscope (Olympus, Tokyo, Japan).

Cell culture, treatment and transfection

Different cell lines T84, LoVo, HCT116, HCT8, SW480 and NCM460 (ATCC, Manassas, VA, USA) were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) at 37°C. Full length of USP28 and FOXC1 were inserted into pcDNA3.1 (Invitrogen). USP28 shRNAs (shUSP28#1 or 2#) were acquired from GenePharma (Suzhou, China). For cell transfection, T84 or SW480 were transfected with shRNAs or vectors using Lipofectamine 2000 (Invitrogen). For cell treatment, two days after T84 or SW480 transfections, cells were incubated with 50 µg/mL CHX (Sigma Aldrich, St. Louis, MO, USA) for 0, 4, 8, 12 hours or 25 µM MG132 (Sigma Aldrich) for 12 hours before western blot analysis.

MTT and colony formation assays

T84 or SW480 transfections were seeded into plates at the density of 4×10^3 cells/well at indicated time (0, 24, 48, 72, 96, 120 hours). MTT reagent (20 µL; Sigma Aldrich) was added to the plates two hours before the measurement of absorbance at 490 nm by microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA). For colony formation assay, T84 or SW480 transfections were seeded into plates at the density of 1×10^2 cells/well, and then cultured for 12 days. Colonies in each well were measured under microscope (Olympus) following fixation in methanol and staining in 0.05% crystal violet.

Measurement of glucose uptake, lactate and ATP levels

For glucose uptake analysis, T84 or SW480 transfections were seeded into plates for 48 hours. The cultured medium was collected and the cells were harvested. Protein concentration in cells was determined by Pierce bicinchoninic acid protein assay kit (Thermo Scientific) and the glucose concentration was determined by glucose assay kit (Shanghai Rongsheng Biotech, Shanghai, China). The values of glucose uptake were normalized to the total protein number. For lactate and ATP levels determination, the cultured medium was conducted with lactate or ATP assay kits (Nanjing KeyGen Biotech, Nanjing, China). The values of lactate and ATP levels were also normalized to the total protein number.

Extracellular acidification rate (ECAR)

XF Glycolytic Rate Assay kit (Agilent Technologies, Santa Clara, USA) with Seahorse Bioscience XF Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, USA) was used to determine ECAR for the evaluation of glycolytic function. T84 or SW480 was seeded into XF24 plates for 24 hours. XF Base Media (5 mM HEPES, 10 mM glucose, 1 mM sodium pyruvate, and 2 mM L-glutamine) without phenol red was then used to detect the glycolytic rate under WAVE version 2.6.0 (Agilent Technologies). Oligomycin (1 µM) and 2-deoxyglucose (50 mM) were added as inhibitors into the plate.

Western blot

Total of 25 µg proteins were separated by SDS-PAGE, and then transferred to polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). Following block with 5% skim milk, membrane was probed with antibodies, including anti-USP28 (1:2000, Abcam, Cambridge, UK), anti-FOXC1 (1:2500, Abcam), anti-FBP1 (1:3000, Abcam), anti-β-actin (1:3500, Abcam) and anti-GAPDH (1:3500, Abcam). β-actin and GAPDH were used as the reference genes. Membrane was subsequently probed with horseradish peroxidase-labeled second antibody, and the bands were visualized using ECL Western blotting detection kits (Millipore, Billerica, MA, USA). The densitometry of the bands was quantified by ImageJ k 1.45 software.

Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA (1 µg), isolated from frozen tissues by TRIzol reagent (Thermo Scientific), was reverse transcribed into cDNA under conditions: 37°C 15 minutes, 85°C 5 seconds and 4°C 10 minutes. The cDNA was then conducted with qRT-PCR analysis with SYBR Premix Ex Taq kit (Takara, Otsu, Japan) under conditions: 95°C 5 minutes; 45 cycles of 95°C for 10 seconds, 60°C 10 seconds and 72°C for 10 seconds. The primer sequences were listed as below: USP28 (F: 5'-ATCITTCAGGCTGCCATTGCT-3' and R: 5'-CTAGCTGGAATGCGTCTCT-3') and β-actin (F: 5'-TGCCCATCTACGAGGGGTATG-3' and R: 5'-CTCCCTAATGTACGCACGATTTC-3').

Statistical analysis

Data were presented as mean ± SEM from at least three independent experiments. Statistical analysis was conducted with SPSS 22.0, and evaluated by Student's t-tests or one-way analysis of variance. $p < 0.05$ was considered as statistically significant difference.

RESULTS

Enhanced USP28 in colorectal cancer

To evaluate the expression level of USP28 in colorectal cancer tissue, qRT-PCR and western blot analysis were performed. Results showed that mRNA (Fig. 1A) and protein (Fig. 1B and 1C) expression of USP28 were enhanced in the tumor tissues. Similarly, up-regulation of USP28 was also validated in colorectal cancer cells compared to NCM460 (normal human colon mucosal epithelial cell line) (Fig. 1D), suggesting potential regulatory role of USP28 on colorectal cancer.

USP28 promoted colorectal cancer cell growth

T84 was transfected with pcDNA-USP28 (Fig. 2A) and SW480 was transfected with shUSP28#1 or 2# (Fig. 2B) to exploit the functional role of USP28 on colorectal cancer. shUSP28#1 showed lower expression of USP28 than shUSP28#2 and was used for the following functional assays. Data from MTT and colony formation assays indicated that overexpression of USP28 promoted cell viability (Fig. 2C) and proliferation (Fig. 2D) of T84, while knockdown of USP28 reversed the promotive effects on cell viability (Fig. 2C) and proliferation (Fig. 2D) in SW480. These results indicated that USP28 promoted colorectal cancer cell growth.

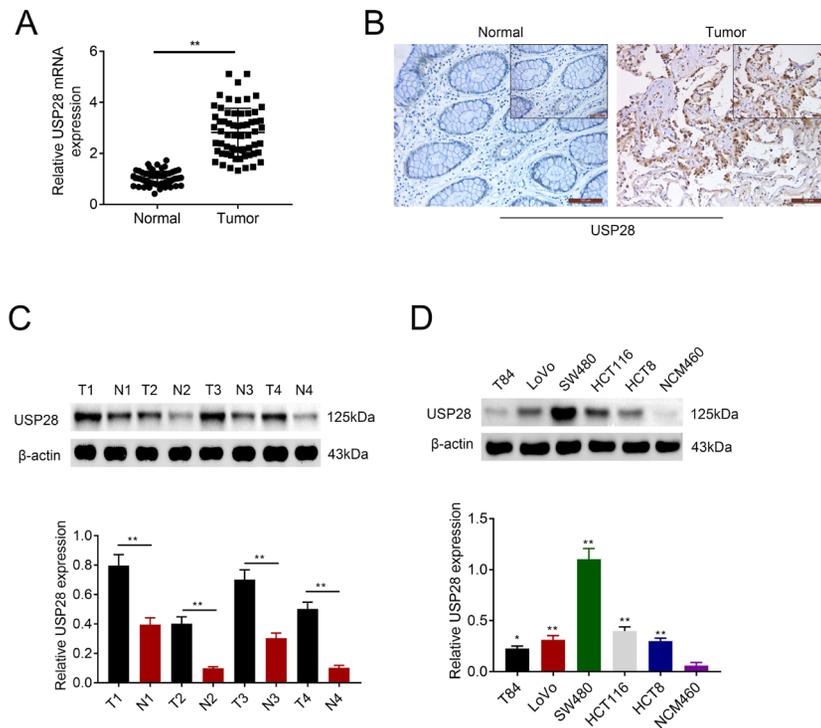


Figure 1. Enhanced USP28 in colorectal cancer

Increased mRNA expression of USP28 in colorectal cancer tissues compared to normal adjacent tissues indicated the upregulation of USP28 in tumor tissues. Immunohistochemical analysis of colorectal cancer tissues compared to normal adjacent tissues indicated the upregulation of USP28 in tumor tissues. Induced protein expression of USP28 in colorectal cancer tissues compared to normal adjacent tissues. Protein expression of USP28 was enhanced in colorectal cancer cells compared to NCM460 (normal human colon mucosal epithelial cell line). * $p < 0.05$; ** $p < 0.01$.

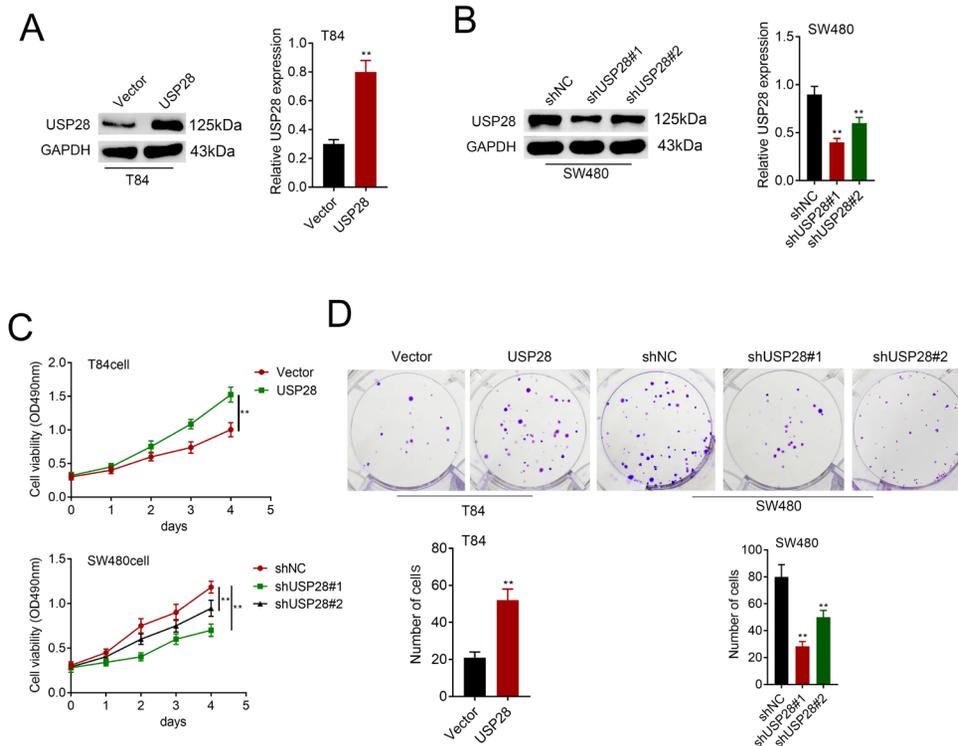


Figure 2. USP28 promoted colorectal cancer cell growth

Up-regulation of USP28 in T84 transfected with pcDNA-USP28. Down-regulation of USP28 in SW480 transfected with shUSP28 #1 or #2. Increased cell viability in T84 was transfected with pcDNA-USP28 and decreased cell viability in SW480 transfected with shUSP28#1 and #2. Increased cell proliferation in T84 was transfected with pcDNA-USP28 and decreased cell proliferation in SW480 transfected with shUSP28#1 and #2. ** $p < 0.01$.

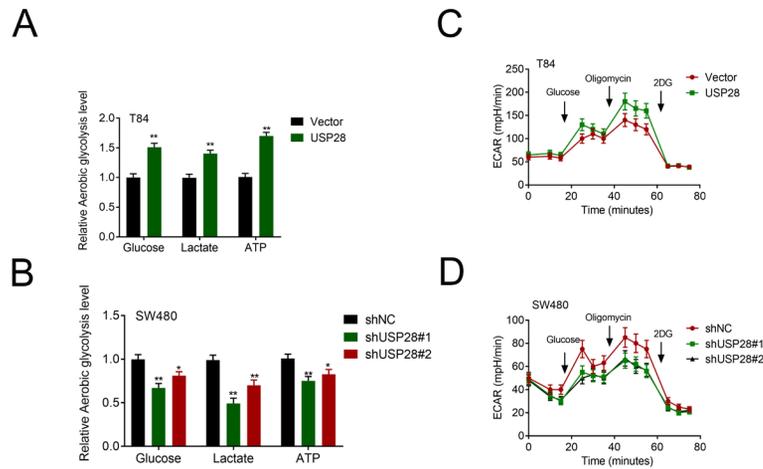


Figure 3. USP28 promoted aerobic glycolysis of colorectal cancer cell

Increased glucose uptake, lactate and ATP levels in T84 transfected with pcDNA-USP28. Decreased glucose uptake, lactate and ATP levels in SW480 transfected with shUSP28#1 and #2. Increased ECAR in T84 transfected with pcDNA-USP28. Decreased ECAR in SW480 transfected with shUSP28#1 and #2. ** $p < 0.01$.

USP28 promoted aerobic glycolysis of colorectal cancer cells

The role of USP28 on colorectal cancer cell metabolism was then evaluated to examine the involvement of USP28 in colorectal tumor development. Energy production was induced by USP28 overexpression with increased glucose uptake, lactate and ATP levels in T84 (Fig. 3A). However, knockdown of USP28 decreased glucose uptake, lactate and ATP levels in SW480 (Fig. 3B). Moreover, USP28 overexpression enhanced the ECAR (Fig. 3C), while knockdown of USP28 reduced the ECAR (Fig. 3D), indicating the promotive effect of USP28 on aerobic glycolysis of colorectal cancer cell.

USP28 promoted stability of FOXC1

To uncover mechanism involved in USP28-mediated colorectal cancer progression, protein expression of USP28 was determined in T84 and SW480. Results revealed that overexpression USP28 increased FOXC1 expression in T84 (Fig. 4A), while USP28 knockdown decreased the expression (Fig. 4B). Expression of FOXC1 was time-dependently decreased in SW480 transfected with shNC or shUSP28#1 post cycloheximide treatment (Fig. 4C). However, SW480 transfected with shUSP28#1 demonstrated lower expression of USP28 compared to that with shNC (Fig. 4C). Moreover, proteasome inhibitor MG132 attenuated USP28 interference-induced decrease of FOXC1 (Fig. 4D), suggesting that USP28 might pro-

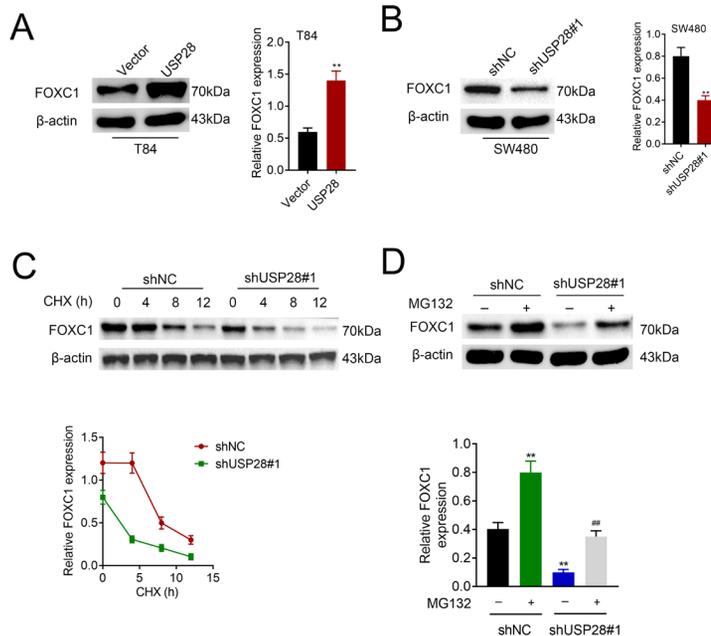


Figure 4. USP28 promoted stability of FOXC1

Increased FOXC1 expression in T84 was transfected with pcDNA-USP28. Decreased FOXC1 expression in SW480 transfected with shUSP28#1. Decreased FOXC1 expression in SW480 transfected with shNC or shUSP28#1 post cycloheximide treatment. Increased FOXC1 expression in SW480 transfected with shNC or shUSP28#1 post MG132 treatment. ** $p < 0.01$.

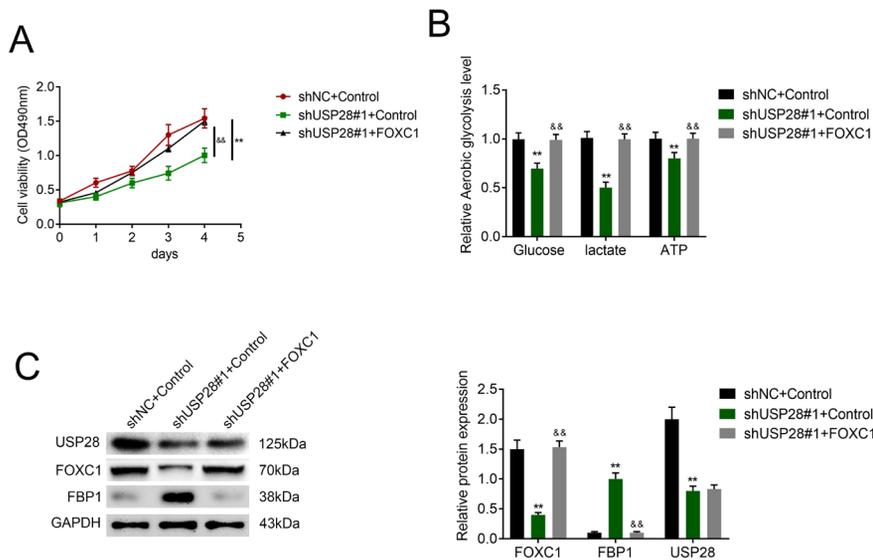


Figure 5. USP28 promoted growth and aerobic glycolysis of colorectal cancer cell through FOXC1

Decreased cell viability of SW480 induced by shUSP28#1 was reversed by over-expression of FOXC1. Overexpression FOXC1 attenuated shUSP28#1-induced decrease of glucose uptake, lactate and ATP levels. Knockdown of USP28 decreased FOXC1 to increase FBP1, and over-expression of FOXC1 counteracted with the promotive effect of USP28 silence on protein expression of FBP1. **,&& $p < 0.01$.

mote stability of FOXC1 through inhibition of proteasome-mediated degradation in colorectal cancer cells.

USP28 promoted growth and aerobic glycolysis of colorectal cancer cell through FOXC1

To determine role of USP28/FOXC1 axis on colorectal cancer, SW480 was co-transfected with shUSP28#1 or shUSP28#2 and pcDNA-FOXC1. Decreased cell viability induced by knockdown of USP28 was reversed by overexpression of FOXC1 in SW480 (Fig. 5A and Supplementary Fig. S1A at <https://ojs.ptbioch.edu.pl/index.php/abp>). FOXC1 Overexpression attenuated the decrease of glucose uptake, lactate and ATP levels by USP28 knockdown (Fig. 5B and Supplementary Fig. S1B at <https://ojs.ptbioch.edu.pl/index.php/abp>). Moreover, knockdown of USP28 could decrease FOXC1 to increase glycolytic enzyme, fructose-1,6-bisphosphatase 1 (FBP1), thus regulating the aerobic glycolysis (Fig. 5C). However, over-expression of FOXC1 counteracted the promotive effect of USP28 knockdown on protein expression of FBP1 (Fig. 5C). Therefore, USP28 could promote growth and aerobic glycolysis of colorectal cancer cell through up-regulation of FOXC1 and down-regulation of FBP1.

DISCUSSION

Cycles between ubiquitination and deubiquitination are essential for various biological processes, and the dysregulation of deubiquitination is involved in tumorigenesis (Sacco *et al.*, 2010). For example, USP13 functioned as tumor-suppressor through stabilization of PTEN (Zhang *et al.*, 2013). USP7 could stabilize Mdm2 to promote degradation of p53 (Sheng *et al.*, 2006). Recently, deubiquitination by USP37 has been reported to regulate aerobic glycolysis of tumors (Pan *et al.*, 2015). Considering the promotive effect of USP28 on colorectal (Diefenbacher *et al.*, 2014; Diefenbacher *et al.*, 2015), the role of USP28 on aerobic glycolysis of colorectal cancer was then evaluated in this study.

Previous study has shown that USP28 was enhanced in colorectal cancer tissues, and USP28 could form a positive feedback loop with oncoprotein, c-MYC, to promote colorectal cancer progression (Diefenbacher *et al.*, 2014). Our results also showed that up-regulation of USP28 in colorectal cancer led to cell proliferation. Since cancer cells in aerobic glycolysis increased lactate production during the growth (Soga, 2013), the aerobic glycolysis in colorectal cancer was promoted by USP28 with increased glucose uptake, lactate and ATP production. As is well known, c-MYC is a transcriptional factor to regulate metabolic enzymes, such as hexokinase 2, phosphofructokinase and lactate dehydrogenase A, involved in aerobic glycolysis and tumor progression (He *et al.*, 2015). Inhibition of c-MYC could suppress aerobic glycolysis in colorectal cancer (Xu *et al.*, 2015). USP28 could interact with ubiquitin ligase, F-box and WD repeat domain containing 7 and further stabilize c-MYC (Popov *et al.*, 2007; Chakravorty *et al.*, 2020). Silencing USP28 destabilized c-MYC in esophageal cancer cells (Weili *et al.*, 2019). Therefore, the downstream target of USP28 involved in colorectal cancer progression needs to be further investigated.

FBP1, key glycolytic enzyme in aerobic glycolysis, was a target gene of FOXC1 to enhance aerobic glycolysis in colorectal cancer (Li *et al.*, 2019). Moreover, FOXC1 could bind to integrin $\alpha 7$ or fibroblast growth factor receptor 4 to promote colorectal cancer metastasis (Liu *et al.*, 2018), and stabilization of FOXC1 could enhance the metastasis of colorectal cancer (Zhang *et al.*, 2020). Therefore, we suggested that USP28 might promote aerobic glycolysis in colorectal cancer by stabilizing FOXC1. Knockdown of USP28 decreased expression of FOXC1 in colorectal cancer cells post protein synthesis inhibitor (cycloheximide) treatment. Moreover, proteasome inhibitor MG132 attenuated USP28 interference-induced decrease of FOXC1. These results suggested that USP28 regulated stability of FOXC1 depending on inhibition of proteasome-mediated degradation in colorectal cancer cells. Functional assays further indicated that over-expression of FOXC1 counteracted the suppressive

effects of USP28 knockdown on cell viability and aerobic glycolysis of colorectal cancer. Moreover, in line with previous report that FOXC1 could down-regulate FBP1 in colorectal cancer cells (Li *et al.*, 2019), our results revealed that knockdown of USP28 decreased FOXC1 and increased FBP1, while over-expression of FOXC1 attenuated promotive effect of USP28 knockdown on protein expression of FBP1. Thus, USP28 could sustain growth and aerobic glycolysis of colorectal cancer cells through up-regulation of FOXC1 as well as down-regulation of FBP1. The present study for the first time showed that the deubiquitination of FOXC1 through USP28 could increase aerobic glycolysis in colorectal cancer. However, cycles of ubiquitination and deubiquitination have been shown to mediate activation of AKT (Yang *et al.*, 2013), which regulated aerobic glycolysis in tumors (Elstrom *et al.*, 2004), and phosphorylation of ser241 and ser272 of FOXC1 when p38 suppressed the ubiquitination and degradation of FOXC1 (Zhang *et al.*, 2020). Therefore, whether AKT or p38 pathways were involved in USP28/FOXC1/FBP1 axis-mediated colorectal cancer aerobic glycolysis would be further investigated in further research.

CONCLUSION

In summary, the present study illustrated that USP28, overexpressed in colorectal cancer tissues, could promote cell proliferation and enhance the aerobic glycolysis of colorectal cancer cells. Mechanistically, USP28 could block the degradation of FOXC1 to down-regulate FBP1, thereby promoting aerobic glycolysis. Our data demonstrated that USP28 and FOXC1 might be the potential therapeutic targets in colorectal cancer due to the biological significance of USP28/FOXC1/FBP1 axis.

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Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

Ethical approval was obtained from the Ethics Committee of First People's Hospital of Yuhang District, Hangzhou.

Statement of Informed Consent

Written informed consent was obtained from a legally authorized representative(s) of anonymized patient information to be published in this article.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Zhaohui Liu and Min Chen designed the study, supervised the data collection, Xiaoping Xu analyzed the data, interpreted the data, Lei Zhang, Yuan Pan and Dong Chen prepare the manuscript for publication and

reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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