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UBR5 promotes cell proliferation and inhibits apoptosis in colon cancer by destablizing P21

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UBR5 is recently recognized as a key player in a large number of prevalent cancers. In this study, we sought to explore the connection of UBR5 expression with cell proliferation, apoptosis, as well as the regulation mechanism in colon cancer cell line. SiUBR5 or oeUBR5 were separately applied to interfere the expression of UBR5. Western blot, DNA gel electrophoresis and qPCR were performed to detect the expression of UBR5 at mRNA and protein level. Then MTT and flow cytometry were used to explore the proliferation and apoptosis in a colon cancer cell line in vitro. Finally, we explored the interaction and correlation of UBR5 and P21 in the colon cancer regulation. We found that UBR5 was highly expressed in colon cancer not only at mRNA level but also at protein level. Moreover, UBR5 can promote the growth of colon cancer cells, and inhibit apoptosis. The mechanism exploration proved that UBR5 can degrade P21 via ubiquitination. All these findings suggest that UBR5 may be involved in progression of colon cancer and could be a new therapeutic target for this disease.

1. Introduction

Colon cancer is a common and major public health issue, the incidence of which continues to rise, leading to significant morbidity, mortality, and social and economic impact (Erenay et al. 2014; Yang et al. 2017). A large number of studies have shown that dietary habits, inflammation and other factors, such as genetic mutations can lead to the occurrence of colon cancer, but its inherent pathogenesis is not very clear (Hsu and Dotan 2014; Salazar 2012; Shearer et al. 2015; Yokota et al. 2014).

As an E3 ubiquitin ligase, UBR5 has unique structural features, and has been implicated in regulation of the ubiquitination of proteins involved in translation, DNA damage response, metabolism, transcription, and apoptosis (Matsuura et al. 2016; Muñozescobar et al. 2015; Subbaiah et al. 2015; Zhang et al. 2014). Hence, UBR5 is essentially involved in diverse types of cancers. The study of Matsuura et al. (2016) has shown that UBR5 downregulates proapoptotic MOAP-1 and can confer cisplatin resistance in ovarian cancer, thus, UBR5 may be an attractive therapeutic target for ovarian cancer treatment (Yang et al. 2016). Studies have reported UBR5 could promote the growth of gastric cancer cells recently. The investigation of Yang et al. (2016) has shown that UBR5 was increased in gastric cancer tissues compared with normal gastric tissues, and UBR5 knockdown inhibited proliferation and colony formation of gastric cancer cells. Thus, UBR5 plays an essential role in gastric cancer and may be a potential diagnosis and treatment target (Subbaiah et al. 2015). For UBR5 regulation mechanism investigation, it was found that UBR5 promotes tumor development mainly by mediating ubiquitin degradation of tumor suppressor genes, such as TIP60 and GKN1 (Subbaiah et al. 2015; Yin et al. 2016). But the function of UBR5 in colon cancer is far from well known.

In the present study, we applied western blot, DNA gel electrophoresis, qPCR to detect and compare the expression of UBR5 in tumor cells and normal tissues, and found that UBR5 was highly expressed in colon cancer. And then, we investigated the role of UBR5 in colon cancer cell proliferation and apoptosis. The results confirmed that UBR5 promotes cell viability and inhibits cell apoptosis in colon cancer. The furtherly mechanism exploration proved that UBR5 can promote P21 degradation via ubiquitination, thus playing a key role in promoting the cancer. Our study provides a new vision for the clinical diagnosis of colon cancer.

2. Investigations and results

2.1. UBR5 higher expressed in colon cancer

The western blot and DNA gel electrophoresis were performed to detect the expression of UBR5. The results are summarized in Fig. 1A, B. The DNA gel electrophoresis and western bolt results showed that UBR5 was high-expressed in the 5 couples of colon cancer cell samples, while low-expressed in the adjacent tissues. Moreover, a large number of clinical samples were used for the qPCR detection of UBR5 mRNA expression level. The results presented in Fig. 1C shows the UBR5 high-expressed in mRNA level. Next, we detected UBR5 expression level of colon cancer cell lines HCT116, HCT8 HT29, RKO, LOVO, SW480 and SW620, with 293T cell line as the comparing group as shown in Fig. 1D. The conclusion is the same than that described in Fig. 1A, B, C.

Samples of different stages of colon cancer samples were used to detect the UBR5 expression level by qPCR as shown in Fig. 1E. The results in Fig. 1F show the Kaplan-Meier survival analysis data in 100 colon cancer patients and present UBR5 high-expression and low survival rate.

2.2. Knockdown of UBR5 inhibits cell proliferation and induces apoptosis in colon cancer

Then HT29 and SW480 cells were transfected with siUBR5 to downregulate the expression of UBR5. As shown in Fig. 2A, B, the UBR5 expression in HT29 and SW480 cells was successfully inhibited in both mRNA and protein levels. Then, we tested the cell viability of HT29 and SW480 cells after UBR5 knockdown. The results shown in Fig. 2C, D tell that UBR5 knockdown decreased the cell proliferation in both HT29 and SW480 cells. In addition, the AnncexinV/PI results certified UBR5 knockdown induced cell apoptosis in both HT29 and SW480 cells (Fig. 2E, F). Western blot

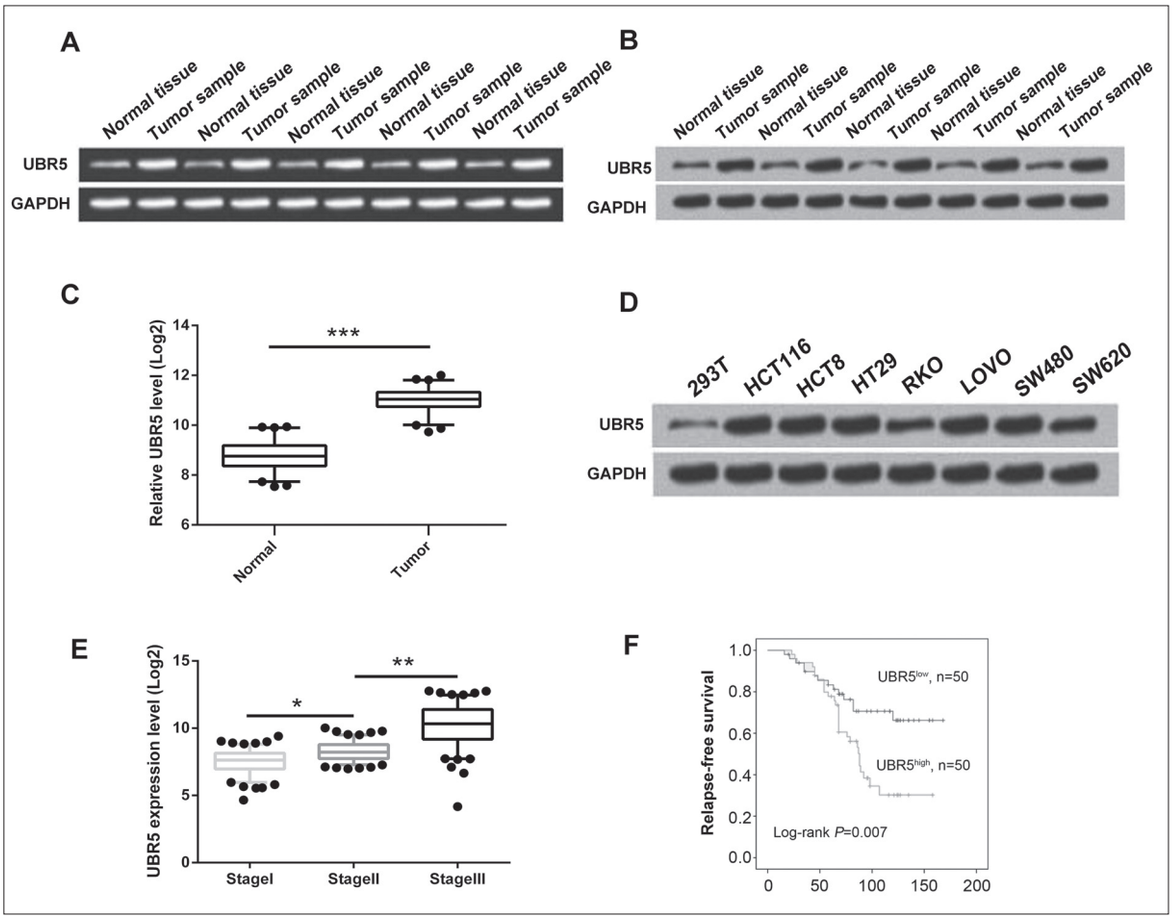

Fig. 1: Expression of UBR5 in colon cancer cells. a. DNA gel electrophoresis of UBR5 mRNA. b. Western blot results of UBR5 in colon cancer cells. c. UBR5 mRNA level statistics of colon cancer samples by qPCR assay. d. Western blot results of UBR5 expression in colon cancer cells lines HCT116, HCT8, HT29, RKO, LOVO, SW480, SW620, and 293T. e. UBR5 expression of different colon cancer stages by qPCR. f. Kaplan-Meier survival analysis in100 colon cancer patients.

assay was applied to detect the active caspase 3 expression and the results tell that UBR5 knockdown upregulate the expression of active caspase 3 (Fig. 2G).

2.3. Upregulation of UBR5 induces cell proliferation and decreases apoptosis in colon cancer

Then HT29 and SW480 cells were transfected with oeUBR5 to upregulate the expression of UBR5. As shown in Fig. 3A, the UBR5 expression in HT29 and SW480 cells was successfully promoted by western blot results. Then, we tested the cell viability of HT29 and SW480 cells. The results shown in Fig. 3B, C tell that UBR5 up-regulation increased cell viability in both HT29 and SW480 cells. Next, western blot assay was applied to detect the active caspase 3 expression and the results tell that UBR5 upregulation inhibits the expression of active caspase 3 (Fig. 3 D). Finally, the UBR5 was separately inhibited or high expressed in HT29 cells, and then injected into nude mice, detecting the tumor size after 4 weeks. We found that knockdown of UBR5 inhibits the tumor weight, while UBR5 high-expression increases tumor weight (Fig. 3E).

2.4. UBR5 interact s with P21, regulating the P21 ubiquitination

In this part of study, yeast two-hybrid was applied to find the protein that interacted with UBR5. We finally confirmed that p21 is the protein that interacts with UBR5 as shown in Fig. 4 A. Then, we promoted the expression of flag-UBR5 and Myc-P21, validating the interaction by CoIP (Fig. 4B). Next, we tested the expression of recombinant protein GST-P21and Flag-UBR5, validating the interaction by Pulldown (Fig. 4C). Moreover, we detected the endogenous P21 protein levels at different time points (0/4/8/12/24h) after the UBR5 was inhibited in HT29 cells (Fig. 4D). Similarly, we detected the P21 protein levels at different time points (0/4/8/12/24h) after the UBR5 was high-expressed in HT29 cells (Fig. 4E). And then we used a ubiquitin antibody to testP21 ubiquitin modification level after inputting of MG-132 in silence or overexpression of UBR5.

2.5. UBR5 regulates cell proliferation and cell cycle by activating P21 ubiquitin degradation

In the following research, we explored the connection of UBR5 and P21 in the colon cancer progress. Firstly, we detected the expression of P21and found it down-expressed in colon cancer cells (Fig. 5A). Then, we investigated the Pearson's correlation and compared the expression of UBR5 and P21. The results shown in Fig. 5B tell that UBR5 and P21are negatively correlated. Next, Ki-67 FACS was used to detect the cell proliferation. The results of Fig. 5C confirm that overexpression of UBR5 promotes the cell proliferation, while the cell proliferation was inhibited after the interference of P21. Finally, we tested the effect of UBR5 and P21 on cell cycle. The results in Fig. 5D proved that the overexpression of UBR5 makes the colon cancer cells enter the cell cycle, while the overexpression of P21 makes them stay in G0.

3. Discussion

UBR5 is a gene with multiple functions, including E3 ligase activity based on a conserved cysteine residue at the C-terminus (Bolt et al.

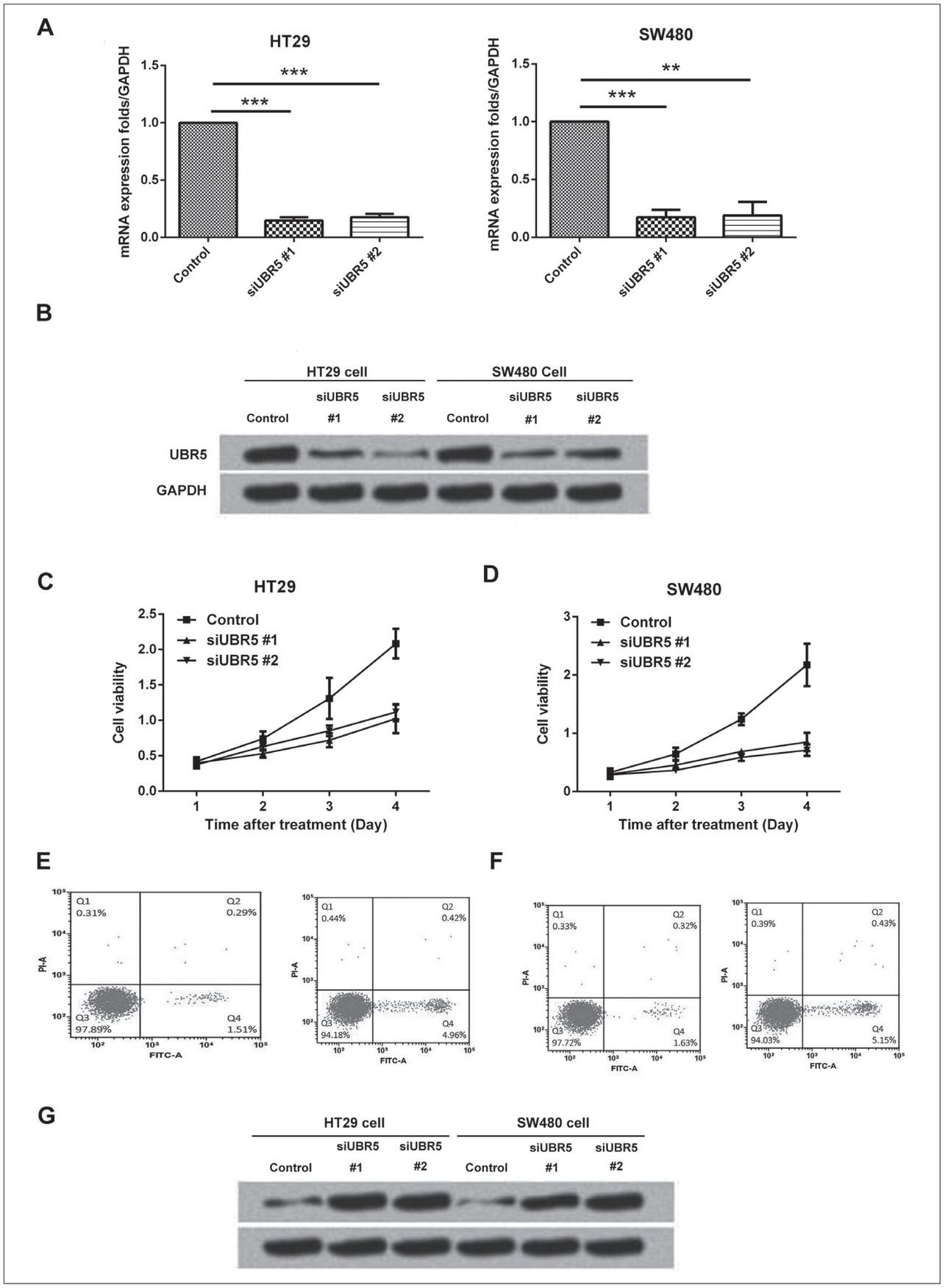

Fig. 2: Effects of UBR5 knockdown on cell proliferation and cell apoptosis. A, UBR5 expression was successfully inhibited in HT29 and SW480 cells in mRNA level. B, UBR5 expression was successfully inhibited in HT29 and SW480 cells in protein level. C.UBR5 knockdown inhibits HT29 cell proliferation by MTT. D. UBR5 knockdown inhibits SW480 cell proliferation by MTT. E. UBR5 knockdown promotes HT29 cell apoptosis. F. UBR5 knockdown promotes SW480 cell apoptosis. G. UBR5 knockdown promotes active caspase 3 expression.

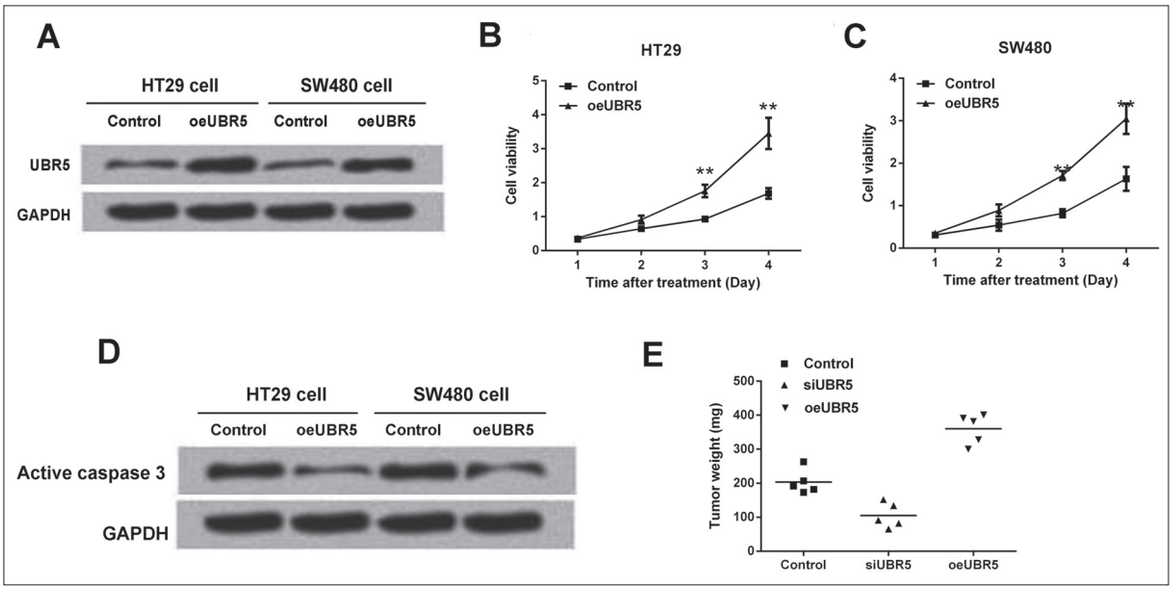

Fig. 3: Effects of of UBR5 up-regulation on cell proliferation and cell apoptosis. A, UBR5 expression was successfully up-regulated in HT29 and SW480 cells by western blot results. B.UBR5 up-regulation promotes cell viability in HT29 by MTT. C .UBR5 up-regulation promotes cell viability in SW480.D. Expression of active caspase 3 was tested by western blot assay after UBR5 inhibition or promotion. E. Tumor weight was detected after UBR5 inhibition or promotion.

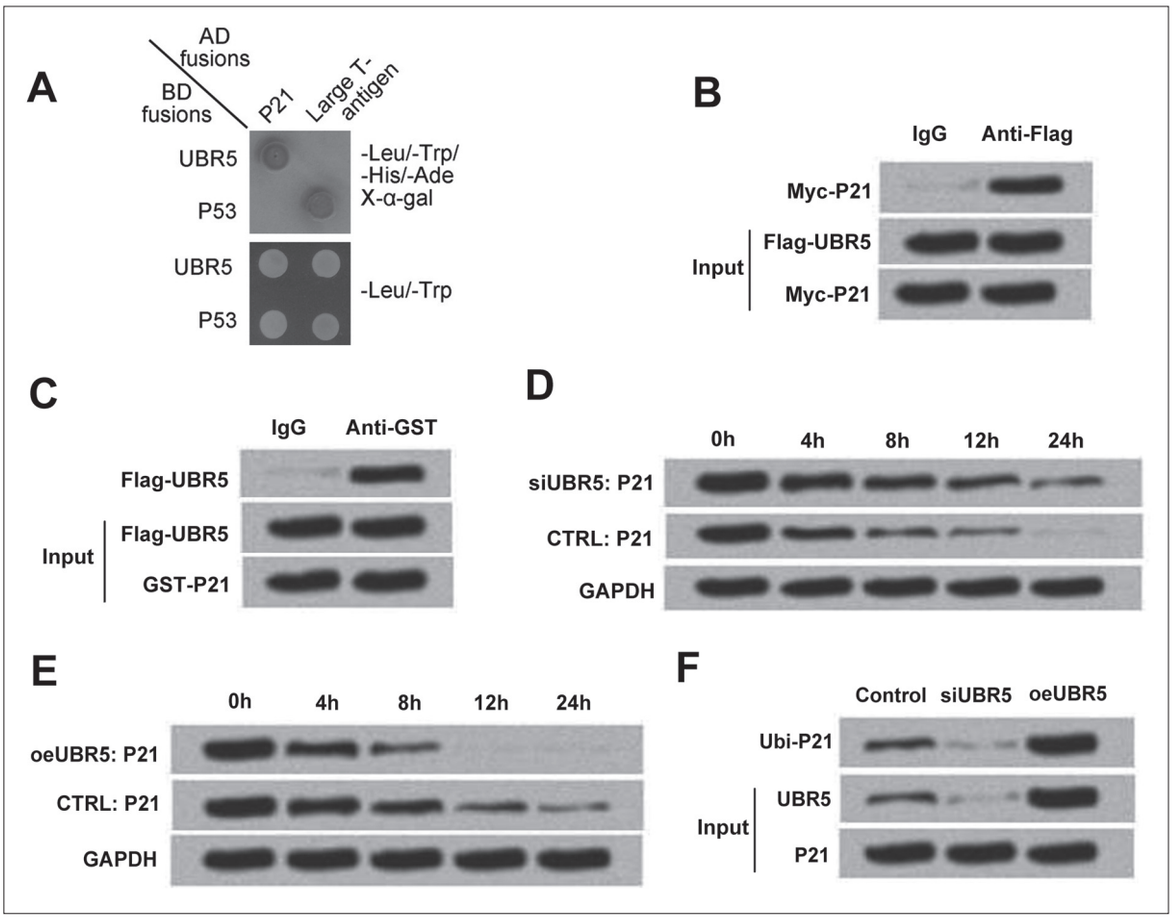

Fig. 4: Correlation of UBR5 and P21. A. p21 was confirmed as the protein interactive with UBR5 by yeast two-hybrid assay. B. CoIP validates the UBR5 and P21 interaction. C. GST Pulldown validates the UBR5 and P21 interaction. D. p21 expression was detected by western blot after knockdown of UBR5. E. p21 expression was detected by western blot after highexpression of UBR5. F. Ubiquitin antibody was used to detect the function of UBR5 on P21 ubiquitin modification level.

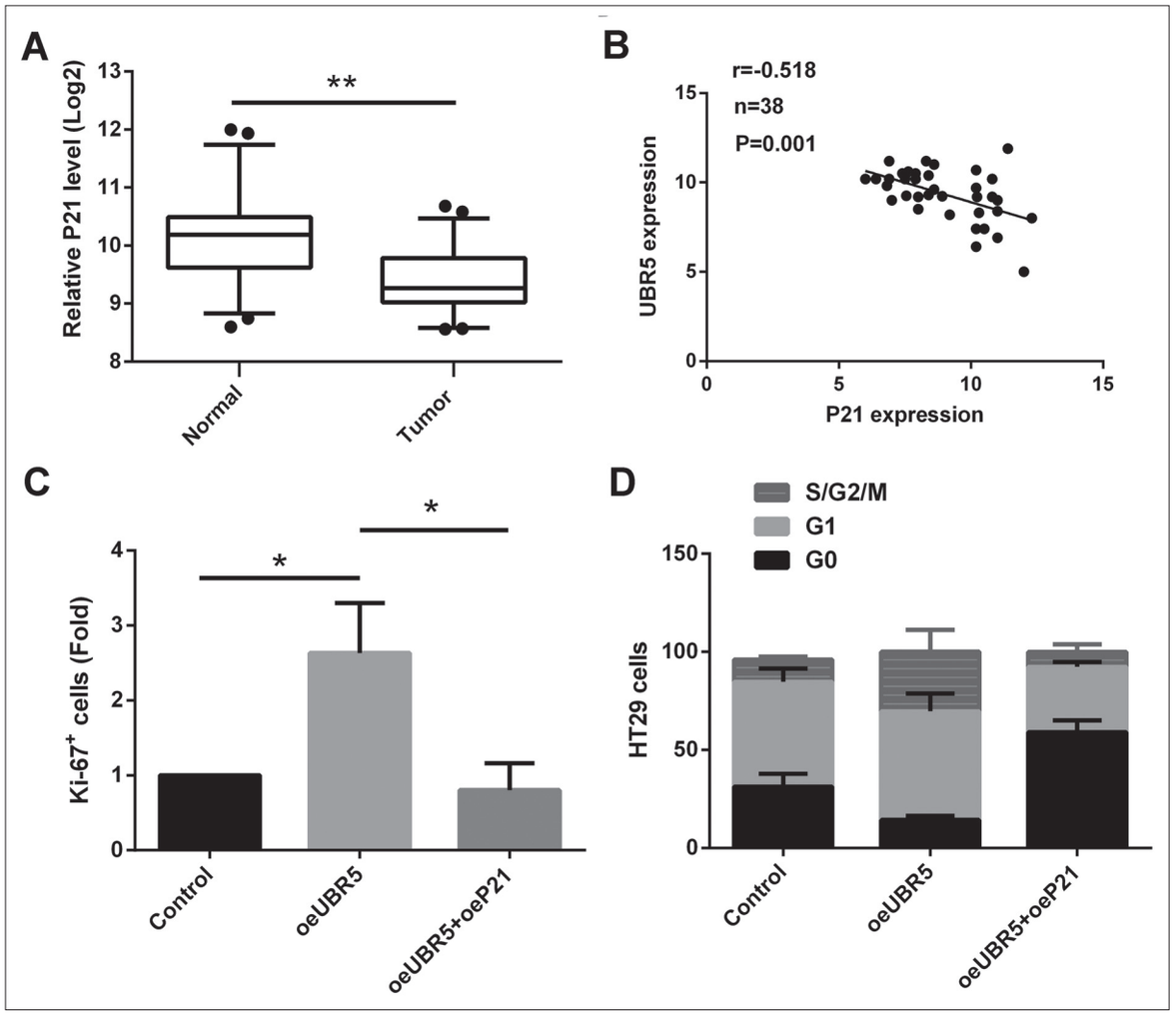

Fig. 5: Role of UBR5 and P21 in cell cycle. A. Detection of P21 expression in colon cancer cells. B. Correlation of UBR5 and P21 expression in colon cancer. C. Cell proliferation detection of colon cancer after overexpression of UBR 5 and P21. D. FACS was applied for the cell proliferation detection of colon cancer after overexpression of UBR 5 and P21.

2015). It is also conceived a key regulator of cell signaling relevant to broad areas of cancer biology, such as breast cancer, ovarian cancer (Bethard et al. 2011; Gudjonsson et al. 2012; Obchoei et al. 2016). However, no studies have specifically targeted the function of UBR5 in colon cancer progress.

In the present study, HT29 and SW480 were applied to investigate the role of UBR5 in the occurrence of colon cancer. Firstly, we found UBR5 high expressed in colon cancer cell line and tumor tissues. In addition, the survival analysis has proved that samples show lower survival rate when UBR5 is high expressed. Moreover, we found that UBR5 can promote colon cancer cell proliferation and inhibit apoptosis by upregulating or downregulating the expression of UBR5.

P21 is a kind of cell cycle cyclin-dependent kinase inhibitors (Hrgovic et al. 2016; Hu et al. 2016).The study of Hrgovic et al. (2016) demonstrated the role of TSA – a pan-HDACi in primary human lymphatic endothelial cells and found it has distinct anti-lymphangiogenic effects by activating cell cycle arrest via p21-dependent pathways (Mansilla et al. 2016). Moreover, as to endogenous P21, it is reported functional and necessary to preserve the genomic stability of unstressed cells (Liu et al. 2016). Additionally, the study of Liu et al. (2016) has indicated that ASTs induced G2/M cell cycle arrest and cellular apoptosis and the cell cycle arrest caused by ASTs was associated with increases of p21 expression levels (Wu et al. 2016). Knowledge of the regulation and function of p21 in cancer cells has opened up several areas of investigation and has led to novel therapeutic strategies (El-Deiry 2016; Wu et al. 2013). As a tumor suppressor gene, it has been studied extensively in basic cellular growth control, stem cell phenotypes and the physiology of differentiation (Armstrong et al. 2012; Majumder et al. 2016; Morrison et al. 2016; Thuraisingam et al. 2016).

However, the connection of UBR5 and P21 in colon cancer progress was not clearly reported. So in this study, we conducted a hybrid screening experiment and found that UBR5 can interact with P21. UBR5 is an E3 ubiquitin ligase, we has proved the UBR5 can regulate P21 ubiquitin modification. P21 is a cell cycle inhibitory protein; the further exploration in this study confirms the UBR5 control the colon cancer cell cycle through regulating the P21 ubiquitination, which ultimately affects the cell proliferation and apoptosis.

Taken together, all our findings demonstrated that UBR5 play an important role in the colon cancer, which provides perspectives for tumor treatment.

4. Experimental

4.1. Cell culture

Seven human colorectal cancer cell lines (HCT116, HCT8, HT29, RKO, LOVO, SW480, SW620) and 293T cell line were purchased from the American Type Culture Collection (ATCC) and cultured according to their instructions. All cell lines used in this study were authenticated through short tandem repeat profiling less than six months ago when this project was initiated, and the cells have not been in culture for more than two months (Wang et al. 2016).

4.2. Transfection and stable cell line construction

Lentiviral constructs expressing UBR5 shRNA (UBR5-shRNA-LV) were purchased from Genechem (Shanghai, China). UBR5-shRNA-LV was transfered into cells at a multiplicity of infection (MOI) of 60 using polybrene (10 μg/ml) and Enhanced Infection Solution (Genechem, China). Meanwhile, for the control group, a non-target negative control virus GFP-LV was transered into cells. As for the negative control group, the cells were transfected with lentiviral particles expressing UBR5 or GFP for UBR5 overexpression. Infected cells were selected with media containing 5 μg/ml puromycin (Meissner et al. 2013).

4.3. MTT assay

The cell proliferative capacities were determined using a 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyl-2Htetrazolium bromide (MTT) colorimetric assay according to standard methods described before.

4.4. Apoptosis assay

Apoptosis analysis was performed to identify and quantify the apoptotic cells by an Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The cells were cultured in 6 well-plate and treated cells were washed twice with cold PBS and then resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

4.5. qRT-PCR

Total RNA was isolated from transfected cells by using TRIzol reagent (Invitrogen) and treated with DNaseI (Promega). Reverse transcription was performed by using the MultiscribeRTkit (Applied Biosystems) and random hexamers or oligo(dT). The reverse transcription conditions were 10 min at 25 °C, 30 min at 48 °C, and a final step of 5 min at 95 °C.

4.6. Western Blot

The protein used for western blotting was extracted using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4 °C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the Polyvinylidene Difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

4.7. Evaluation of tumorigenicity

Tumorigenicity was determined 4 weeks later after the subcutaneously injecting 5×10^4 shCtrl or shUBR5 or oeUBR5 HT29 cells into the flanks of 6-week-old female BALB/c-nu mice (Animal Center of Chongqing Medical University, China) (Meissner et al. 2013).

4.8. Statistics

The results of multiple experiments are presented as the mean±SD. Statistical analyses were performed using SPSS 19.0 statistical software. The P-values were calculated using a one-way analysis of variance (ANOVA). A P-value of <0.05 was considered to indicate a statistically significant result.

Conflict of interests: There is no conflict of interests.

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