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High expression of USP18 is associated with the growth of colorectal carcinoma

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Summary. Aim. To investigate whether USP18 can be used as a predictive marker for the diagnosis and development of colorectal cancer.

Methods. The Gene Expression Omnibus (GEO) Dataset and the Cancer Genome Atlas (TCGA) database were used to select differential proteins for the ubiquitin-specific peptidases (USPs). The extensive target prediction and network analysis methods were used to assess the association with the USP18 interacting proteins, as well as the statistical correlation between USP18 and the clinical pathology parameters. The effects of USP18 on the proliferation of colorectal cancer were examined using CCK8. The effects of USP18 on the migration of colorectal cancer were examined using wound healing assays. Immunohistochemistry (IHC) was performed on the tissue microarray.

Results. The results showed that the expression of USP18 was related to age (P=0.014). The positive rates of the USP18 protein in T1, T2, T3, and T4 were 0.00%, 22.92%, 78.38%, and 95.35%, respectively (P<0.00). The positive rates of the USP18 protein in I, II, III, and IV were 47.43%, 83.12%, 66.67%, and 100.00%, respectively (P<0.00). The Western blot assay showed that the expression of USP18 in colorectal cancer tissues was significantly higher than that in matched paracancerous tissues (P<0.05). The CCK8 experiments suggested that USP18 promoted the migration of CRC cells. Wound healing assays suggested that USP18 promoted the proliferation of CRC cells.

Conclusion. This study showed that USP18 can promote the proliferation of colorectal cancer cells and might be a potential biomarker for the diagnosis of CRC.

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Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in the world (Siegel et al., 2020). If patients can be diagnosed early, 90% of patient deaths can be prevented (Smith et al., 2001). Currently, although the etiology, pathogenesis, and epidemiology of CRC are being conducted effectively, the molecular mechanism is still not understood. Although classical chemistry has achieved some success in the clinical treatment of cancer, the side effects are still large. Hence, alternative targets are still required to treat cancer in the early stages of development.

Studies have shown that most colon cancers originate from polyps. Therefore, early detection of polyps can prevent CRC. It can be seen that it is very important to identify CRC at an early stage. Although recent studies have shown that some molecules can be used as biomarkers for the early detection of CRC or the progression of CRC, they still cannot be used to diagnose a percentage of CRC (Lin et al., 2016). Therefore, there is an urgent need to conduct research in molecular and genetic terms to find more reliable and novel biomarkers for the early detection of colorectal cancer.

Ubiquitin-specific peptidase (USP) 18 is called interferon (IFN)-stimulated gene 15 (ISG15) isopeptidase and is a negative regulator of type I and type III IFN signaling (Ritchie et al., 2004). It has been reported that USP18 plays an important role in tumorigenesis. USP18 plays a tumor-promoting role in different types of cancer. Interference with the expression of USP18 can reduce the growth of acute promyelocytic leukemia cells and induce apoptosis, also the knockdown of USP18 in T98G glioblastoma cells can enhance the apoptosis of IFN cells. The growth rate of breast cancer tumors in USP18 knockout mice was

significantly lower than that in control mice, and knocking down USP18 in the MCF7 cell line *in vitro* resulted in an increase in apoptosis (Burkart et al., 2013). Similarly, overexpression of USP18 can enhance the proliferation of renal cancer cells (Shahidul Makki et al., 2013). USP18 is highly expressed in lung cancer tissues of patients (Guo et al., 2012). Low expression of USP18 can significantly prolong survival (Tan and Xia, 2014). In hepatitis C virus (HCV) therapy, USP18 regulates the anti-HCV type I IFN response as a therapeutic target (Randall et al., 2006). In summary, USP18 can be an important target for oncogenes and other diseases.

In the study of colorectal cancer, the expression of USP18 in SW480 and DLD1 and its biological function have been studied (Huang et al., 2020). In this study, the expression of USP18 is found in the colorectum by using a tissue microarray and the Oncomine database, and related investigations are then conducted using LoVo cells.

Materials and methods

Data processing

The GSE110224 dataset was downloaded from the GEO datasets (http://www.ncbi.nlm.nih.gov/geo/) (Barrett et al., 2013), and the expression profiling arrays were generated using GPL570 (HG-U133_Plus_2) Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). The Oncomine database (https://www.oncomine.org/) and the TCGA database (http://ualcan.path.uab.edu/index.html) were used to identify the differentially expressed USP18 in colon cancer tissue and tissue adjacent to the carcinoma. UACLAN (http://ualcan.path.uab.edu/index.html) enrichment of the gene ontology analysis was performed on the USP18 interacting protein.

Patients and clinical specimens of colorectal cancer

Clinical specimens and matched adjacent non-cancerous tissues of colorectal cancer (20 cases of tumors) were collected from patients diagnosed with primary colorectal cancer from the First Hospital of Jiaxing City. Tissue microarray (TMA) specimens were purchased from Alenabio (191 adenocarcinoma cases, one-ring cell carcinoma, 12 normal tissue cases, and four adjacent normal tissue cases). A total of 160 cases of cancer were screened based on actual conditions. The human research program was approved by the ethics committee of the Jiaxing First Hospital.

Cell lines and cell culture

SW620, RKO, and LoVo cells were obtained from the Cell Bank of the Chinese Academy of Sciences. The DLD1 cells were obtained from the American Type Culture Collection. The SW620 cells were cultured in Dulbecco's modified Eagle medium (HyClone, Logan, UT, USA). The LoVo and DLD1 cells were cultured in the Roswell Park Memorial Institute (RPMI) 1640 medium (HyClone, Logan, UT, USA). The RKO cells were cultured in the MEM medium (GIBCO, No. 41500034 with the addition of $NaHCO_3$ 1.5 g/L and sodium pyruvate 0.11 g/L). All of the cells were supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Immunohistochemistry

The tissue slices were placed at 60°C for 1 h, defatted using xylene, and then dried with ethanol. The slices were thawed in a citrate buffer for 15 min and washed with phosphate buffered saline (PBS) after cooling for 1 h. The slices were incubated in 3% H_2O_2 for 20 min. After washing with PBS, a blocking buffer containing normal goat serum was used for blocking at 37°C for 15 min, and it was then incubated with USP18 antibody at 4°C. After washing with the PBS, the sections were incubated with biotinylated secondary antibodies at 37°C for 40 min. The slices were washed with PBS and then incubated with streptavidin-horseradish peroxidase at 37°C for 40 min. The positive reaction was observed using a 3, 3 N-diamino benzidine trihydrochloro (DAB)-peroxidase substrate and stained with blood xylin for 30 s. An Olympus TH4-200 microscope was used (Tokyo, Japan).

Western blot assay

The LoVo, DLD1, SW620, and RKO cells were extracted using Radio Immunoprecipitation Assay (RIPA) lysate. The protein concentration was determined using a (bicinchonininc acid) BCA kit. The protein was separated using 10% SDS-PAGE and transferred to a PVDF membrane. A 5% skim milk sealing film was used at room temperature for 1 h, and USP18 and GAPDH were kept at 4°C overnight and washed with TBST and anti-rabbit IgG. The HRP-linked antibody was incubated at room temperature for 1.5 h, and the change in the band was detected using the electrochemiluminescence method.

Realtime PCR

The total RNA was extracted according to the manufacturer's instructions. The cDNA was synthesized with 0.5 μg RNA. Quantitative real-time PCR was used to detect the USP18 and GAPDH gene levels in the LoVo cells.

USP18-Forward: 3'- GGC TCC TGA GGC AAA TCT GT-5'; USP18-Reverse: 3'- CAA CCA GGC CAT GAG GGT AG-5'; GAPDH-Forward: 3'- TCG GAG TCA ACG GAT TTG GT-5'; GAPDH-Reverse: 3'- TTC CCG TTC TCA GCC TTG AC-5'.

Cell counting Kit-8

The 100 µl cell suspensions were prepared in a 96-well plate. The culture plate was pre-cultured in the incubator for 24 h (at 37°C and 5% CO_2). A total of 10 ul of different concentrations of substances to be tested were added to the culture plate. The culture plate was placed in the incubator for a suitable period of time. A total of 10 µl of the CCK8 solution (Beyotime Biotechnology) was added to each well. The culture plate was incubated in the incubator for 2 h. The absorbance at 450 nm was determined using an enzyme labeling instrument.

Wound healing assays

The cells were cultured in six-well plates until 90% confluence. The cells were damaged using a 200 µL sterile pipet head, and the suspension cells were washed. The scratches were photographed with a Leica microscope at 200x magnification from three fields of view.

Statistical analysis

The data were calculated and presented using GraphPad Prism 8.0 (GraphPad Software, CA, USA). The values were expressed as the means ± standard deviations (±SDs). In the cell line experiments, a Student's t-test was used to compare the properties between transfected cancer cells. A $P<0.05$ was determined to be statistically significant.

Results

Identification of the USP18 significantly upregulated in the GEO datasets, the Oncomine database, and the TMA

The USP family proteins were selected as the heatmap using the GEO database (GSE110224) (Fig. 1A), and the 10 most meaningful USP proteins (USP expression level) were used in the scatter maps (USP18, USP14, USP1, USP7, USP2, USP38, USP34, USP24, USP13, and USP30). Among them, USP18 was the most different protein (Fig. 1B). The difference in USP18 expression between normal tissues and colorectal cancer was verified using the Oncomine database. It was found that USP18 expression increased significantly in CRC (Fig. 1C). Next, tissue microarrays were bought and stained using immunohistochemistry. It was found that USP18 was highly expressed in the colon cancer tissues and had low expression in normal tissues (Fig. 1D). An analysis of the relationship between the USP18 expression and established clinicopathological parameters revealed no correlation at the gender level, histological type, grade, N stage, or M stage. The USP18 level correlated positively with age (years) (P=0.014), T stage (P=0.000), and TNM stage (UICC released. Version 8.) (P=0.000) (Table 1). Multivariate models were evaluated for the adjusted risk of USP18 in relation to CRC, including age (P=0.027, 1.115–5.936) and T stage (P=0.000, 0.048–0.202) (Table 2).

Table 1. Correlation between USP18 expression and clinicopathological characteristics.

Characteristics	N	USP18		χ^2	P
		Low	High		
Gender					
Male	110	37	73	0.001	1.000
Female	56	19	37		
Ages (years)					
<60	85	21	64	6.353	0.014
≥60	81	35	46		
Histological type					
Adenocarcinoma	155	50	105	2.282	0.185
other	11	6	5		
Grade					
High	38	11	27	0.790	0.674
Moderate	99	36	63		
Low	29	9	20		
T stage					
T1	1	1	0	63.440	0.000
T2	48	37	11		
T3	74	16	58		
T4	43	2	41		
N stage					
N0	151	53	98	1.583	0.453
N1	14	3	11		
N2	1	0	1		
M stage					
M0	161	56	105	2.625	0.169
M1	5	0	5		
TNM stage					
I	78	41	37	24.698	0.000
II	77	13	64		
III	6	2	4		
IV	5	0	5		

Table 2. Multivariate analyses for USP18.

	B	Std. Error	Wald	df	Sig.	Exp(B)	95% Confidence Interval for Exp(B)
Age	0.945	0.427	4.908	1	0.027	2.573	1.115-5.936
T stage	-2.320	0.369	39.634	1	0.000	0.098	0.048-0.202
TNM Stage	-0.163	0.912	0.032	1	0.858	0.849	0.142-5.071

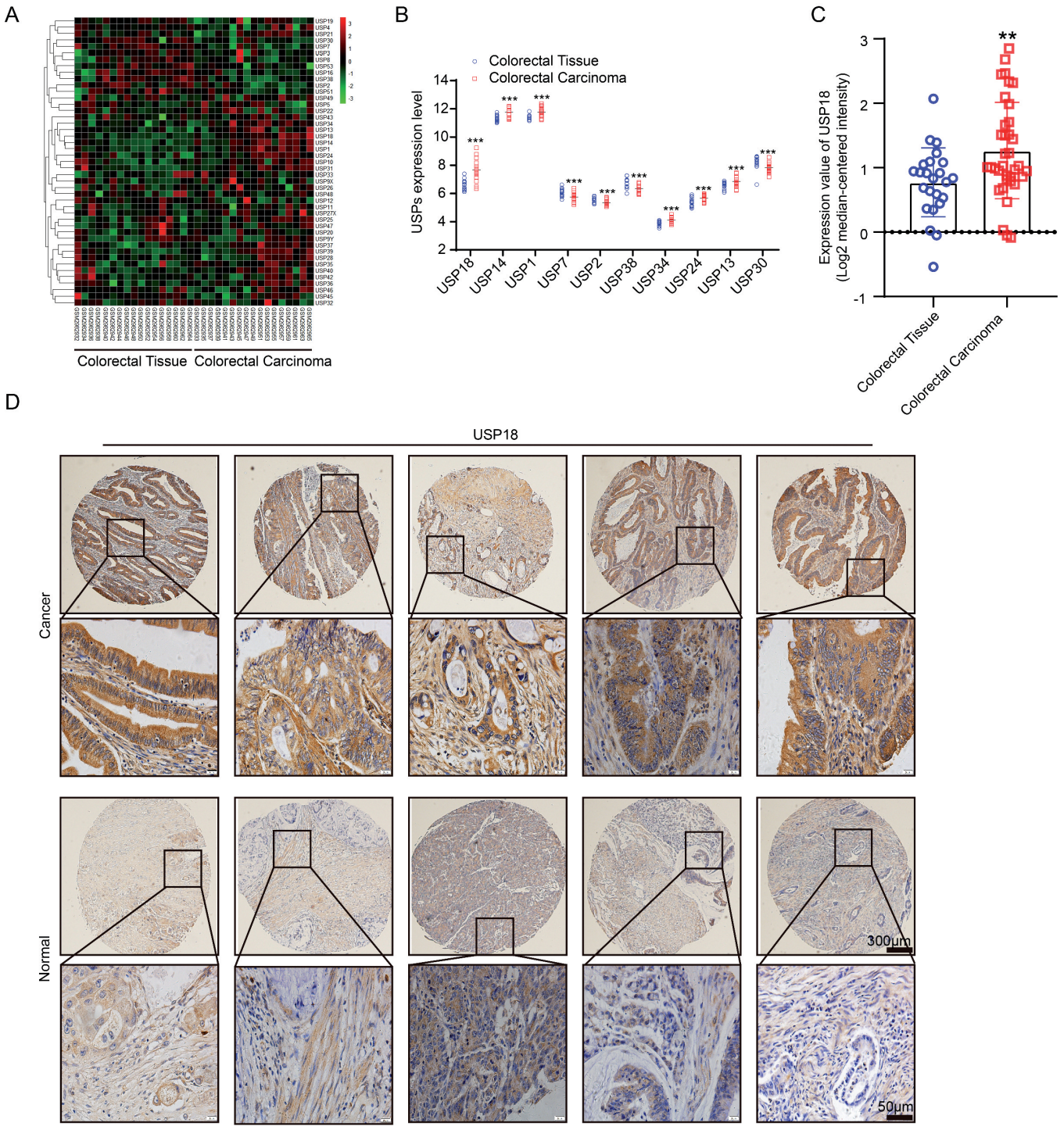

Fig. 1. Identification that USP18 is significantly upregulated in the GEO datasets, the Oncomine database, and in CRC tissues. **A.** The USP family proteins in the form of a heatmap. **B.** The 10 most meaningful proteins in the USP family were selected and represented using a scatter plot. **C.** The expression of USP18 in colorectal cancer and paracancerous tissues was observed in the Oncomine database, and the results are represented using a scatter plot. **D.** Immunohistochemical staining of the USP18 in adjacent non-tumor tissue and the CRC specimens.

Identification of the proteins interacting with USP18 and the GO-term pathway enrichment

Next, the expression of USP18 in colorectal cancer was screened out using the UALCAN website (http://ualcan.path.uab.edu/analysis.html), and once again, the high expression of USP18 in colorectal cancer was verified (P=2.41009999999431E-05) (Fig. 2A). In addition, the interacting proteins of USP18 were screened out. Five proteins with the highest score

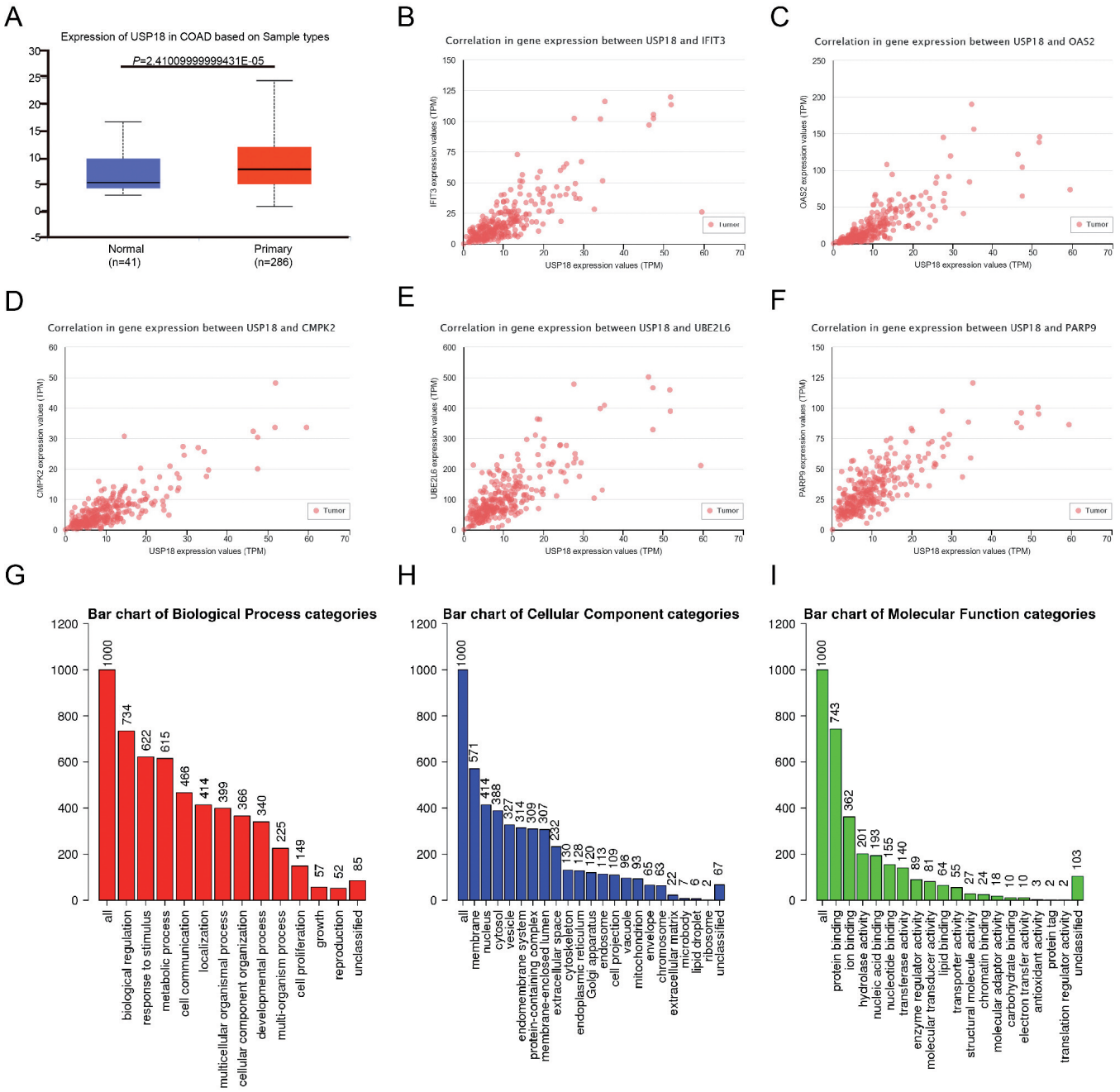

Fig. 2. Identification of the proteins that interact with USP18 and the GO-term pathway enrichment. **A.** The expression of USP18 in colorectal cancer and paracancerous tissues was observed using the TCGA database, and the results are represented as a scatter plot. **B-F.** The proteins that may interact with USP18 were screened using the UALCAN database, and the five proteins with the highest scores are shown in the chart. **G-I.** Functional enrichment-based clustering analysis for quantified succinylated proteins. According to the functional differences observed between the increased and decreased proteins, a GO-term association and enrichment analysis using the UALCAN program were performed. (**G.** Biological process analysis. **H.** Cellular component analysis. **I.** Molecular function analysis).

(Pearson-CC) (IFIT3, OAS2, CMPK2, UBE2L6, and PARP9) were selected, and these five proteins were listed with their correlation in the gene expression of USP18 (Fig. 2 B-F). A Go analysis was performed based on 1000 selected proteins that interact with USP18. The top five GO terms of related biological processes among those genes were biological regulation, response to stimulus, metabolic process, cell communication, and localization (Fig. 2G). There was a significant correlation in the membrane, nucleus, cytosol, vesicle, and endomembrane system in relation to the cellular components (Fig. 2H). In addition, the terms that related molecular functions involved in protein binding, ion binding, hydrolase activity, nucleic acid binding, and nucleotide binging (Fig. 2I) were significantly enriched.

USP18 promoted LoVo cell proliferation

To further verify whether USP18 was highly expressed and played a role in colorectal cancer, cancer tissues and paracancerous tissues were collected from 20 cancer patients. It was found that USP18 was highly expressed in cancer tissues (Fig. 3A,B) compared with paracancerous tissues, according to the Western blot assay. Four colon cancer cell lines (SW620, RKO, LoVo, and DLD1) were selected to detect the changes in the USP18 protein expression. The results showed that the expression of USP18 was the highest in the LoVo cell line and the lowest in the RKO cell line (Fig. 3C) according to mRNA levels. Then, the USP18 overexpression and interference plasmid was transfected

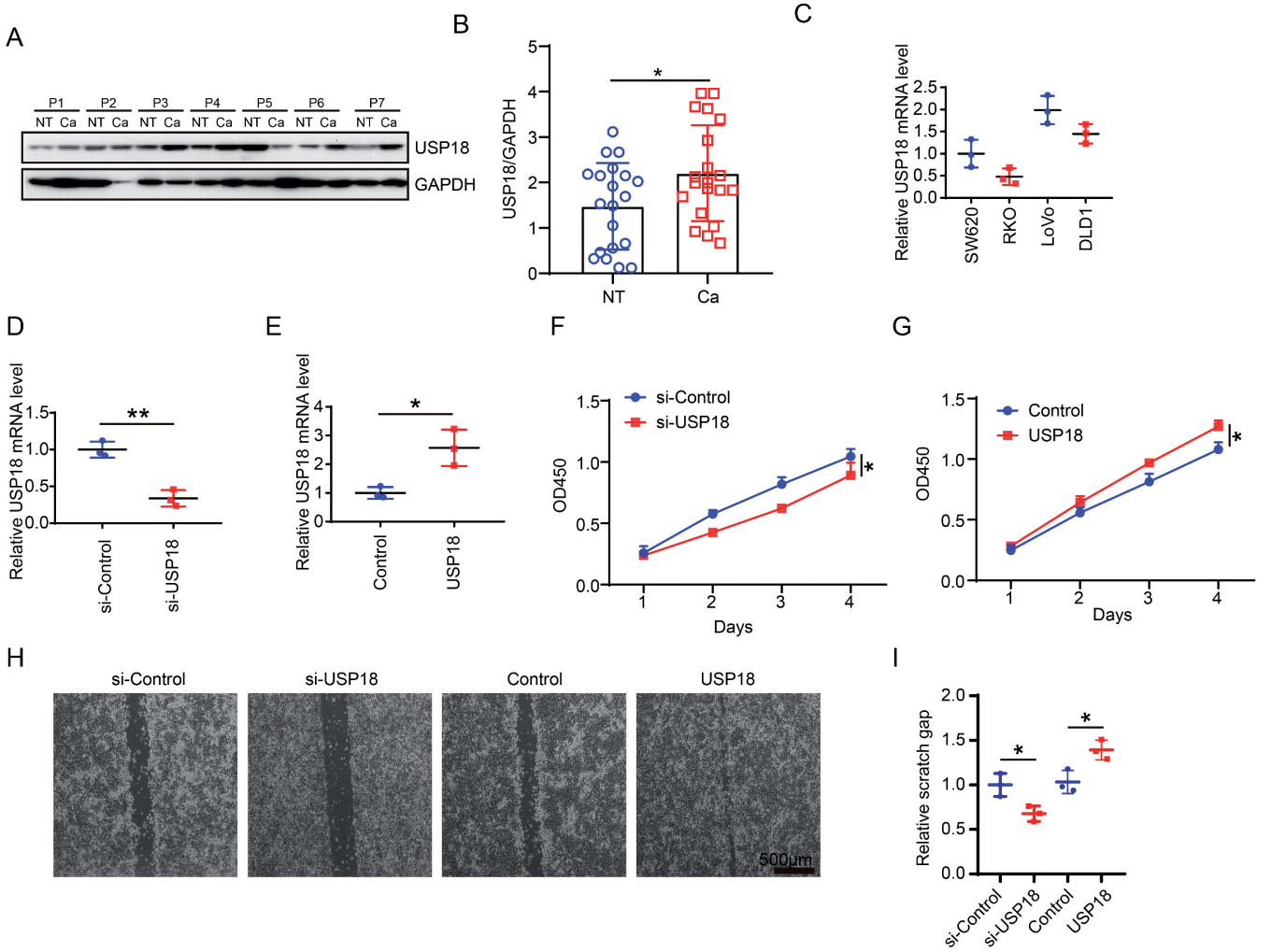

Fig. 3. USP18 promoted LoVo cell proliferation. **A, B.** The expression of USP18 in 20 pairs of matched adjacent non-tumor (NT) and cancer (Cai et al., 2017) tissues were detected using a Western blot assay. **C.** The USP18 expression levels in the CRC cells were measured using a Western blot assay. **D.** The mRNA expression of the USP18 transfected by si-USP18 or si-control was detected using qPCR. **E.** The mRNA expression of the USP18 or control transfected by USP18 was detected using qPCR. **F.** Cell proliferation was analyzed using CCK-8 in the LoVo cells that were transfected with the si-USP18 or si-control. **G.** Cell proliferation was analyzed using CCK-8 in the LoVo cells that were transfected with USP18 or the control. **H, I.** The cell migration was analyzed using a wound-healing assay.

into the LoVo cell line (Fig. 3D,E) according to the mRNA levels. By using the CCK8 experiments, it was found that USP18 was overexpressed and promoted the proliferation of the LoVo cells (Fig. 3F). Therefore, interference with USP18 inhibited the proliferation of LoVo (Fig. 3G). Next, we verified the effect of USP18 on cell migration by Wound healing assays. The results showed that overexpression of USP18 significantly promoted cell migration, and interference with USP18 significantly inhibited cell migration.

Discussion

Colorectal cancer (CRC), the third most common cancer in the world, is also the second leading cause of cancer death in the world (Jemal et al., 2011). However, if patients with colorectal cancer can be diagnosed and treated early in the development of the tumor, many patients can avoid death (Zauber et al., 2012). Therefore, new biomarker molecules are urgently needed to better predict the clinical outcomes of patients with colorectal cancer.

USP18 is located on chromosome 22q11.2 and is a ubiquitin-specific protease, but also an interferon (IFN)-stimulated gene 15 (ISG15) isopeptidase (Schwer et al., 2000; Malakhova et al., 2006). In recent years, USP18 has been gradually discovered and understood as an anti-tumor target. It is expressed in a variety of tumors and the reduction of its activity plays an effective inhibitory role in different tumors. For example, the expression of USP18 can up-regulate the level of EGFR protein, and then activate the Akt /Skp2 pathway, and finally promote the growth of breast cancer. USP18 can decoupling ISG15 from oncoproteins PML/RARα, Cyclin D1 and KRAS through its deubiquitination function, thereby improving the stability of these proteins and thereby increasing the formation and growth of lung cancer. Currently, the role of USP18 in tumors has been reported mainly in these types of tumors, such as hepatitis B virus-associated bladder cancer (Kim et al., 2014), hepatocellular carcinoma (Cai et al., 2017), melanoma (Hong et al., 2014), breast cancer (Burkart et al., 2013), and others. In addition, its role in colorectal cancer has not be reported on.

In this study, bioinformatics was used to find that USP18 was the most significant protein in colorectal cancer in the USP family. Subsequently, immunohistochemistry was used in tissue microarrays to find that USP18 was highly expressed in colorectal cancer tissues and lowly expressed in normal tissues. This result was consistent with Huang's research (Huang et al., 2020). This suggested that USP18 plays an important role in colorectal cancer. Combined with an analysis of the clinicopathological data, it was found that the expression of USP18 was closely related to age, T stage, and TNM stage, but not to gender, histological type, grade, N stage, of M stage of tumor patients. This suggested that an increase in USP18 in colorectal cancer may be related to the proliferation and metastasis of the colorectal cancer. However, the results are not exactly the same as what has been published. This may be due to the type of databases used (Huang et al., 2020). Twenty cases of fresh colorectal cancer tissues were then collected, and it was found that the protein levels of USP18 in the colorectal cancer tissues were significantly higher than those in the normal colorectal cancer tissues. The target proteins that USP18 may interact with were then identified using bioinformatics, and the five most likely proteins were listed. Finally, it was shown that the overexpression of USP18 can promote the proliferation of LoVo cells, and vice versa. Studies have shown that the loss of USP18 in the breast cancer cell line, MCF-7, can induce an increase in apoptosis induced by chemotherapy and interferon α (IFN- α) (Potu et al., 2010; Fang et al., 2018). The knockdown of USP18 can directly lead to a decrease in proliferation and an increase in apoptosis of APL cells in acute promyelocytic leukemia, which can be used as a clinical anti-tumor therapeutic target for the treatment of APL. These reports showed that USP18 is closely related to the proliferation and development of tumors. In terms of the mechanism, it has been reported that USP18 affects tumor development primarily by regulating the interferon signaling pathway, because IFNs can inhibit protein ISGylation and play an important role in the anti-tumor response. Other studies have shown that the deletion of the USP18 gene might inhibit tumor activity and regulate the tumor microenvironment by upregulating Cxcr3 ligands. USP18 can regulate the anti-tumor effect of $CD4^+$ T cells. Activated USP18 enzymes can directly affect the stability of Cyclin D1. The knockout of UBP43 can inhibit the expression of Cyclin D1 and promote apoptosis. In a follow-up study, the molecular mechanism of USP18 will be reexamined for promoting the invasion and proliferation of colorectal cancer and its clinical value in the treatment of colorectal cancer.

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Conflict of Interest. The authors declare that there are no conflicts of interest.

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