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Original Article

Porphyromonas gingivalis induced UCHL3 to promote colon cancer progression

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Abstract: *Porphyromonas gingivalis* (*P. gingivalis*), a Gram-negative oral anaerobe, was demonstrated to facilitate colonization and progression in colonic tumor, while the underlying mechanism still remains to be clarified. Here, we identified the proteome profile changed by *P. gingivalis* infection in HCT116 cells through label-free quantitative proteomics, and found that deubiquitinase UCHL3 was a key protein that response for *P. gingivalis* infection. By CCK8, colony formation, wound healing assays, and in vivo subcutaneous tumor mouse moudle, we proved that *P. gingivalis* could promote the proliferation and migration of colon cancer, while the process was inhibited by UCHL3 knock down. Through IP-MS, we identified GNG12 as the UCHL3 interacting protein. The protein level of GNG12 was significantly reduced when knock out UCHL3. Thus we propose that GNG12 is a substrate protein of UCHL3. Furthermore, we demonstrated that overexpression of GNG12 could restore the tumor inhibition effect caused by UCHL3 knock down, and UCHL3-GNG12 axis promote colon cancer progression via the NF-κB signal pathway. Collectively, this study unveiled that *P. gingivalis* infection up-regulated UCHL3 and stabilized its substrate protein GNG12 to activate the NF-κB signal pathway to promote colon cancer progression. Our study indicate that UCHL3 is a potential biomarker and therapeutic target for colon cancer which infected with *P. gingivalis*.

Keywords: *Porphyromonas gingivalis,* colon cancer, UCHL3, GNG12, NF-κB signal pathway

Introduction

Colon cancer is the third most common malignant tumor worldwide, with a high mortality rate [1-3]. During the development of colon cancer, microbiota composition is an important risk factor [4-7], imbalance of gut microbiota usually leads to chronic inflammatory in colon which is a major risk factor of carcinogenesis [8]. Except gut microbiota, some oral microbes have been found to be enriched in colon cancer [8-10] and participated in the occurrence and development of colon tumors [11-15]. *P. gingivalis*, a well-known oral microbe, is recently reported to be associated with colon cancer [16, 17]. Infection of *P. gingivalis* could stimulate the NLRP3 inflammasome [18] and activate MAPK/ERK, JNK, and NF-κB signaling in colon cancer [19]. However, the protein characteristics and pathogenesis that distinguish *P. gingivalis* infected colon cancer from other colon cancers have not yet been fully elucidated.

This study unveiled the proteome characteristics of *P. gingivalis* infected colon cancer cells using label-free quantitative proteomics technology. We found 335 proteins were significantly changed after *P. gingivalis* infection. Of the most up-regulated proteins, UCHL3, a kind of deubiquitinase, was confirmed to play important roles in the progression of colon cancer. Furthermore, we identified GNG12 as the substrate protein of UCHL3, and explored the function of the UCHL3-GNG12 signal axis in colon cancer progression. Our results disclosed the protein landscape of *P. gingivalis* promoting colon cancer progression, and we proposed that UCHL3 is a new potential biomarker and therapeutic target for *P. gingivalis* positive colon cancer.

Results

Proteome profile of P. gingivalis infected colon cancer cells

To reveal the proteome characteristics of *P. gingivalis* infected colon cancer, we treated HCT116 cells with *P. gingivalis* at a multiplicity of infection (MOI) of 100, PBS treatment was used as control group. The *P. gingivalis* were sampled when they grew to an OD_{600} of about 0.4 (Figure S1A). After infecting cells with *P. gingivalis*, the infection was confirmed to be successful through quantitative real time PCR (qPCR) and PCR detection (Figure S1B and S1C). After 48 hours treatment, the cells were collected and lysed for protein extraction. The extracted proteins were then subjected to label-free quantitative mass spectrometry analysis (**Figure 1A**). We identified a total of 6713 proteins, most of them are common between the two groups (**Figure 1B**). 227 proteins were found to be downregulated and 108 proteins were up-regulated after *P. gingivalis* infection (**Figure 1C**, **1D**; Tables S1 and S2). The principal component analysis (PCA) showed high degree of similarity in the samples from the biological replicates (**Figure 1E**). KEGG pathway analysis of the regulated proteins demonstrated that *P. gingivalis* infection altered many biological pathways of host cells, such as ubiquitin mediated proteolysis, mitophagy, pathways of neurodegeneration, carbon metabolism, and biosynthesis of amino acids (**Figure 1F**). Of these regulated proteins, UCHL3, CMC1 and PIK3R4 were up-regulated by more than 10-fold in response to *P. gingivalis* treatment (**Figure 1C**). We investigated the mRNA expression level of the 3 most up-regulated proteins in colon cancer using The Cancer Genome Atlas (TCGA) database, and found that UCHL3 was significantly positive related to colon cancer (**Figure 1G-I**). These results indicated that UCHL3 may play important roles in *P. gingivalis* infected colon cancer.

P. gingivalis infection promotes UCHL3 expression in colon cancer

Previous researches have put forward that UCHL3 could promote colon cancer progression [20], but the correlation between *P. gingivalis* and UCHL3 is unknown. To confirm whether *P. gingivalis* infection actually promotes UCHL3 expression in colon cancer. We detected the expression levels of UCHL3 in HCT116 cells and colon cancer tissues. Western blot and qPCR results showed that UCHL3 was significantly increased in *P. gingivalis* treated HCT116 cells or NCM460 (human normal colon epithelial) cells at a time and dose gradient dependent manner (**Figure 2A**, **2B**; Figure S2A, S2B). To check whether UCHL3 expression level was positive correlated with *P. gingivalis* infection, we first identified the of *P. gingivalis* infection in colon tissue through PCR and nucleic acid electrophoresis (Figure S1D), then we choose several colon cancer tissues and precancerous lesions to extract proteins for western blot analysis. The results showed that UCHL3 was significantly accumulated in *P. gingivalis* infected tissues (**Figure 2C**), and the mRNA level of UCHL3 in those samples were also up regulated (**Figure 2D**). Besides, the immunohistochemistry (IHC) assay was used to detect the expression level of UCHL3 in colon tumors. The results also showed that *P. gingivalis* infection led to higher level of UCHL3 in colon cancers compared to uninfected samples (**Figure 2E**). These results suggest that UCHL3 is positive correlated with *P. gingivalis* infection in colon cancer.

P. gingivalis facilitates the proliferation and migration of colon cancer cells via UCHL3

We then try to demonstrate the function of UCHL3 on the tumorigenesis of *P. gingivalis* infected colon cancer. We constructed UCHL3 knock-down HCT116 cell line, and checked the knock down efficiency by western blot (**Figure 3A**). We treated cells with *P. gingivalis* under aerobic conditions, and took the treated cells or cell culture supernatant for anaerobic cultivation of *P. gingivalis* to calculate the colony forming unit (CFU). Initially, the control group had 19840 CFU/ml, the UCHL3 knockdown group had 18560 CFU/ml. After 24 hours, the CFU values in the supernatant and cells of the control group and UCHL3 knockdown group were both 0. This indicates that UCHL3 knockdown will not affect *P. gingivalis* growth (Figure S1E). By CKK8 assays, we confirmed that the relative cell viability was obviously increased after *P. gingivalis* treatment, while UCHL3 knock down significantly blocked the process (**Figure 3B**). The colony formation assay results suggested that *P. gingivalis* promoted colony numbers but was significantly reduced when knock down UCHL3 (**Figure 3C**). The EDU assay

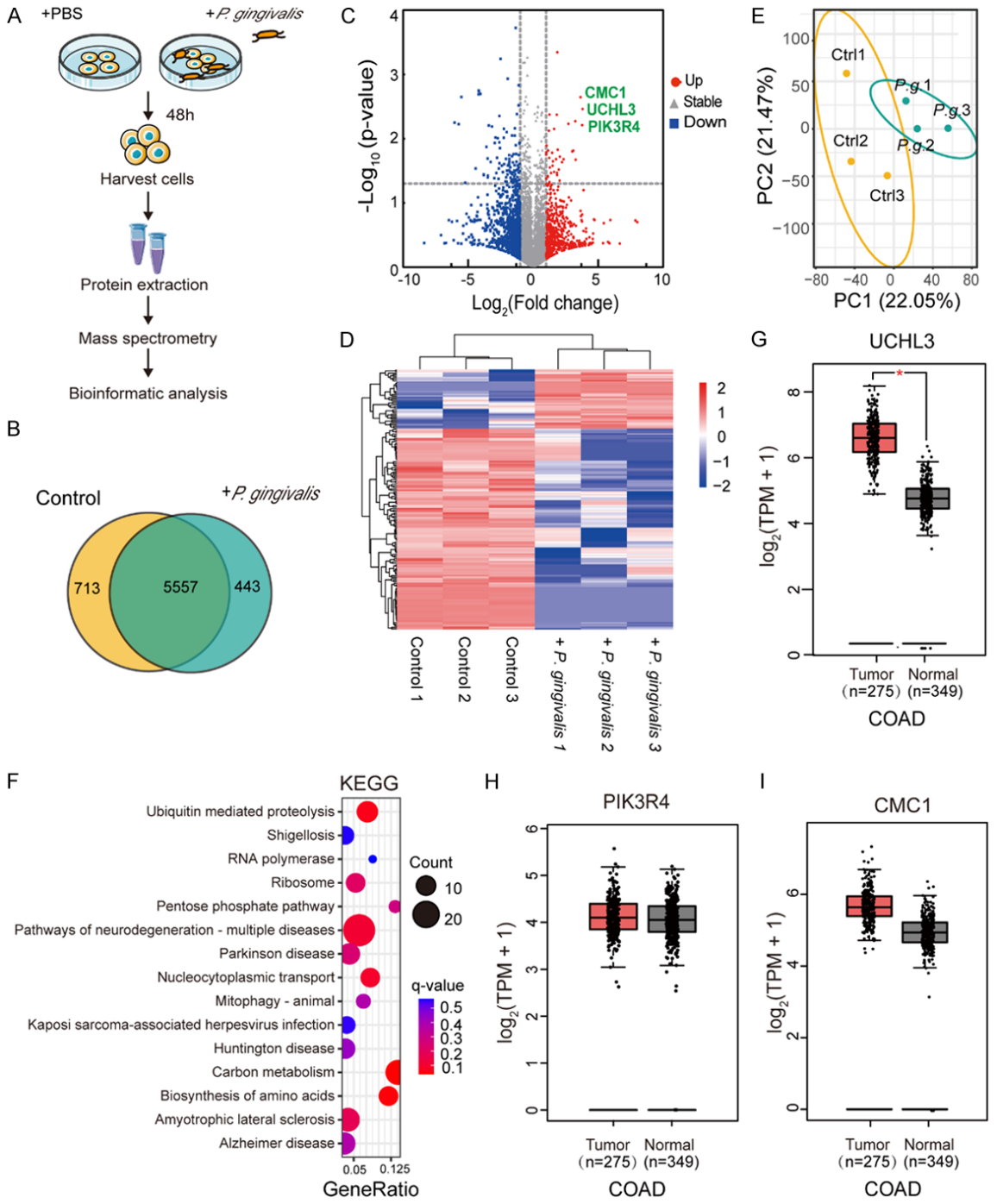


Figure 1. Proteomics study on *P. gingivalis* infected colon cancer cells. (A) Workflow for sample preparation and mass spectometry analysis. HCT116 cells were treated with *P. gingivalis* (MOI=100) for 48 h. Proteins were extracted and trypsin digested, then used for mass spectrometry analysis. (B) Venn diagram shows the overlap between the proteins in control group and *P. gingivalis* infected group. (C) The volcano plots show the differential expressed proteins. Blue dots showed the decreased proteins with *P. gingivalis* treatment and the red ones displayed the increased proteins. (D) Heatmap shows the quantification of differentially expressed proteins. Results from three biological replicates. (E) PCA analysis between the control group and *P. gingivalis* treated one. (F) KEGG analysis showed the pathway enrichment of differentially expressed proteins in colon cancer affected by *P. gingivalis* infection. (G-I) The mRNA exprssion level of UCHL3, PIK3R4 and CMC1 in colon cancer. TCGA database were used and analyzed using GEPIA (http://gepia.cancer-pku.cn/detail.php) online software, the red box represents the colonic tumor tissues and the gray box represents normal tissues.

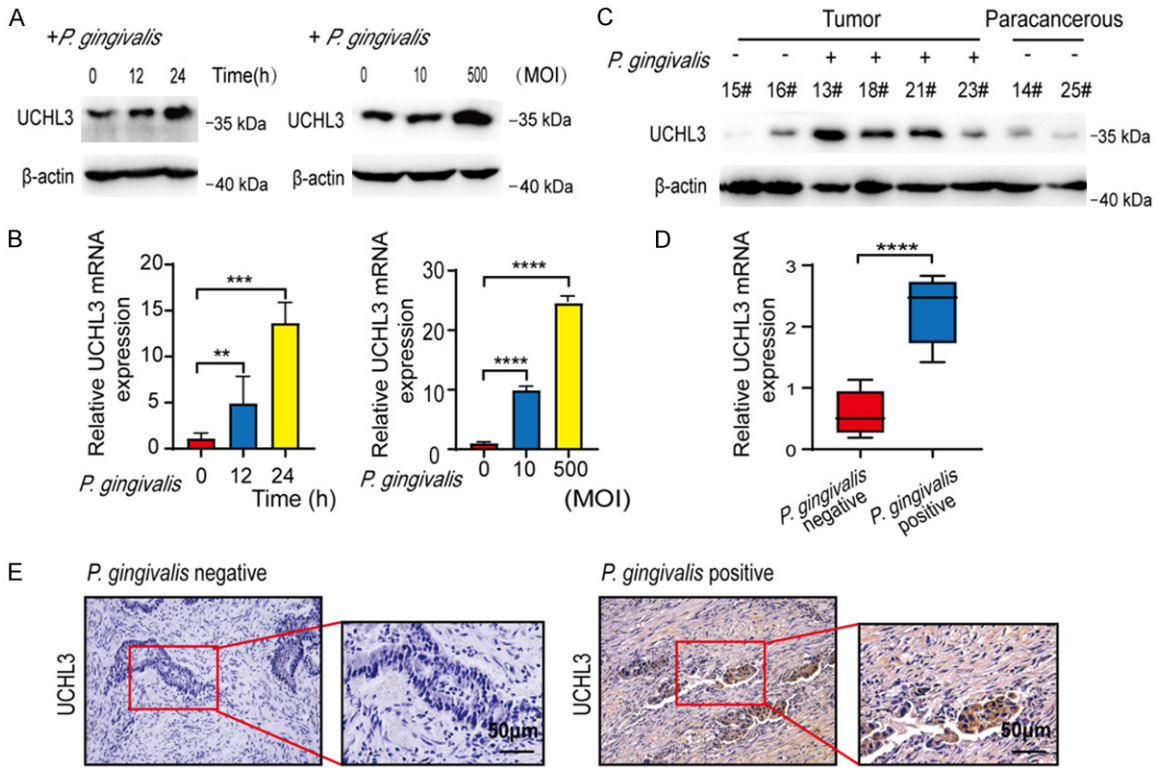


Figure 2. UCHL3 expression level is up regulated in *P. gingivalis* infected colon cancer (A, B). HCT116 cells were treated with *P. gingivalis* at dose of 0, 10, 500 MOI for 24 h, or treated with *P. gingivalis* at 500 MOI for time gradients of 0, 12, 24 hours, and then cells were collected for western blot (A) or qPCR assays (B). (C) Colon cancer or paracancerous tissues were checked whether have been infected with *P. gingivalis* firstly. Then tissue samples were minced and proteins were extracted for Western blot analysis. (D) Relative UCHL3 mRNA expression level was detected by qPCR in colon cancer tissues with or without *P. gingivalis* infection. (E) Colon cancer tissues were embedded in paraffin and used to detect the UCHL3 level through immunohistochemistry. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

results also showed more EDU incorporation in *P. gingivalis* treatment group than the control group, while knock down UCHL3 significantly inhibited this effect (**Figure 3D**). To check whether UCHL3 affect the migration of *P. gingivalis* infected colon cancer, we conducted wound healing experiments. The results showed that *P. gingivalis* infection significantly decreased the wound area, while knock down UCHL3 suppressed the process (**Figure 3E**). These foundings indicate that UCHL3 is necessary for *P. gingivalis* to promote colon cancer proliferation and migration.

Uchl3 is necessary for P. gingivalis to promote tumor progression in mice

To verify the function of UCHL3 on *P. gingivalis* infected colon cancer, we constructed *Uchl3* knock down MC38 cell line, and then validated the knock down efficiency of Uchl3 silence by qPCR (**Figure 4A**). For subcutaneous tumor formation experiment, 5×10^6 MC38 cells were injected into each BALB/c nude mice. After 5 days, 10^6 cfu *P. gingivalis* were injected in the grown tumor, and an equivalent amount of PBS was utilized for control. The mouse was sacrificed and the tumors were harvested after two weeks (**Figure 4B**). The *P. gingivalis* infection in control and Uchl3 knock down subcutaneous tumors were confirmed by qPCR (Figure S1F). Our results showed that the tumor size and weight were significantly increased after *P. gingivalis* infection, but not in the Uchl3 knock down group (**Figure 4C-E**). The consequences above indicate that *P. gingivalis* could promote colon cancer progression, and Uchl3 plays an important role in this process.

UCHL3 interacts with and stabilizes GNG12

Protein ubiquitination and deubiquitination are important post-translational regulatory processes that affect protein intracellular localiza-

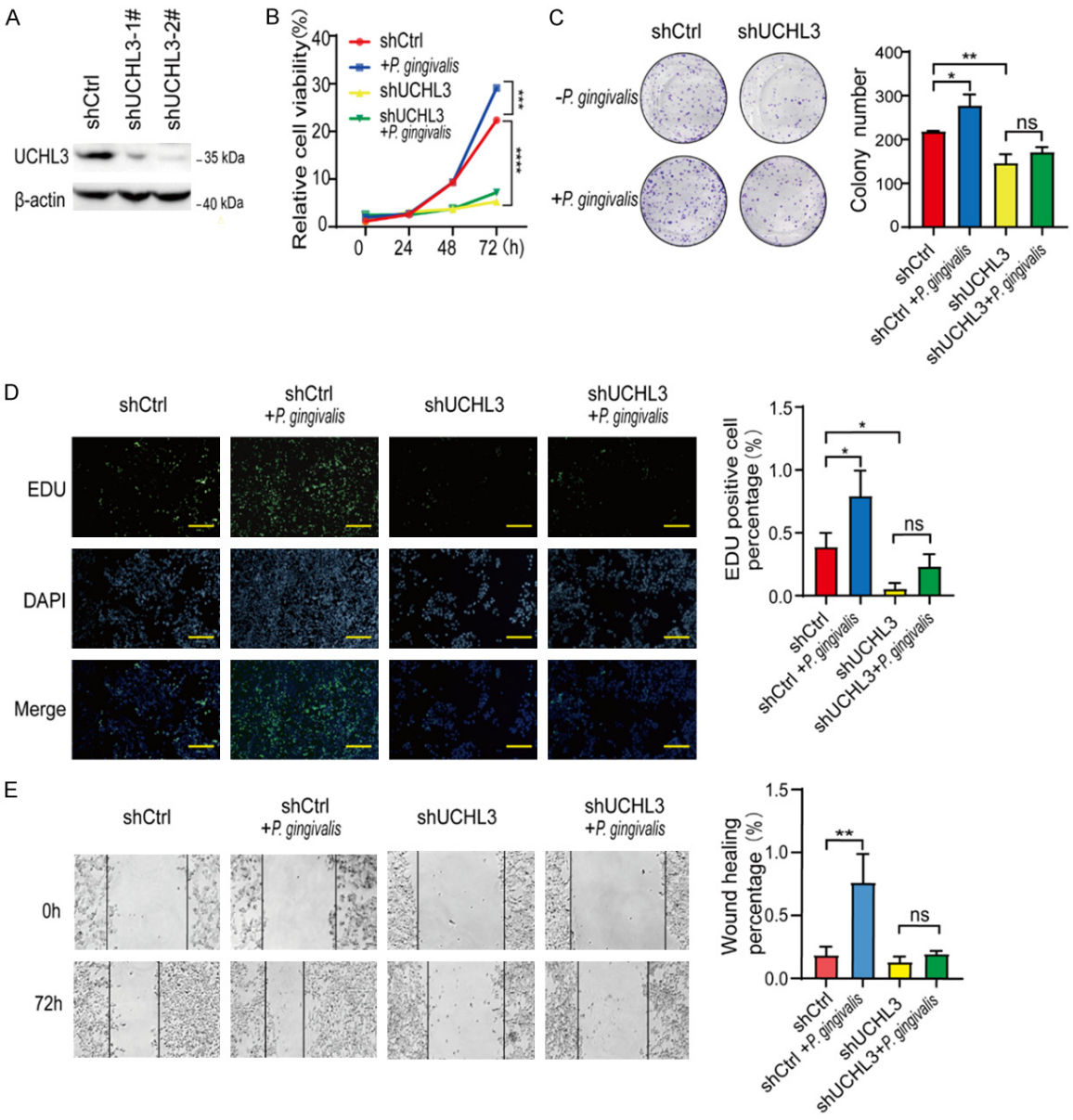


Figure 3. Knock down UCHL3 block the effect of *P. gingivalis* infection which promotes the proliferation and migration of colon cancer cells. (A) UCHL3 was knocked down by shRNA lentivirus in HCT116 cell line, the cells were selected by 1 μg/ml puromycin for two weeks and then checked the knock down efficiency by western blot. (B-E) shCtrl and shUCHL3 HCT116 cells treated with or without *P. gingivalis* infection were used for cell viability assays (B), colony formation assays (C), EDU assays (D), and wound healing assays (E). The *P. gingivalis* was used at MOI 100. The CKK-8 assay was performed to quantify cell viability. EDU incorporation was detected with fluorescent microscopy. The green dots indicate EDU positive cells and the blue ones represent nuclei (scale bar: 200 μm). All the graphs were drawn by Graph Prism and analyzed with one-way ANOVA. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

tion, interactions, and enzymatic activities, thereby influencing many biological processes such as cancer progress [21]. UCHL3 is a deubiquitination enzyme, which removes the polyubiquitin chains from substrate protein and prevent it's degradation [22]. To find the substrate protein of UCHL3 which affected colon cancer progression, we expressed 3× FLAG tagged UCHL3 in HCT116 cells, and purified the interacting proteins by FLAG beads, and identified them by mass spectrometry (**Figure 5A**). We selected some candidate proteins for further

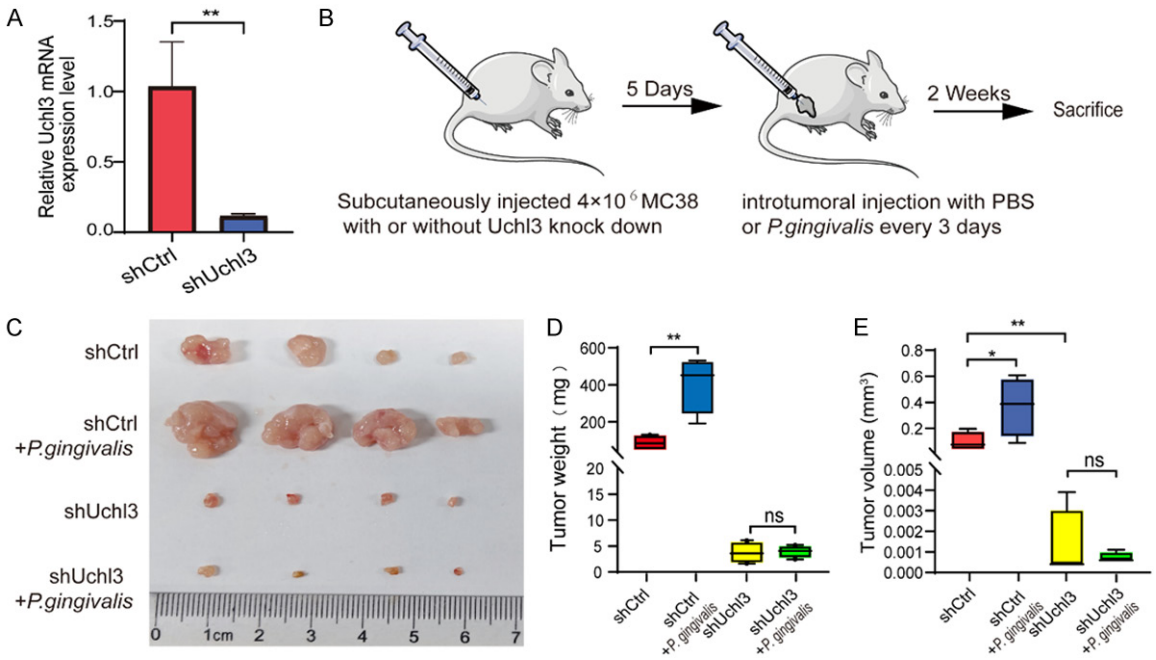


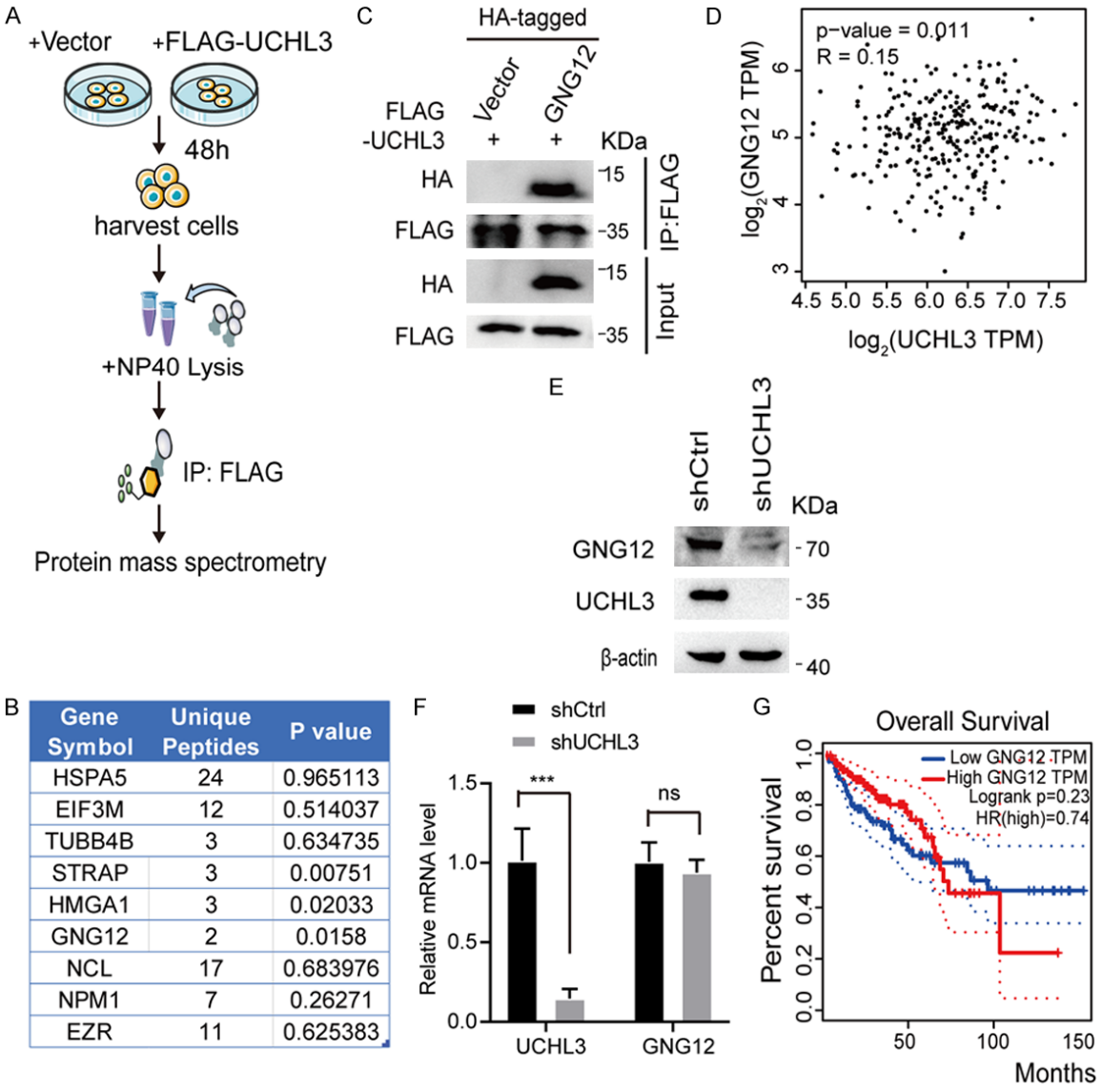
Figure 4. *P. gingivalis* promotes tumorigenesis via Uchl3 in mice. (A) Uchl3 was knocked down by pLKO.1 shUchl3 lentivirus in fection in MC38 cells. The knock down efficiency was detected by qPCR. (B) Schematic diagram displayed the experimental procedure for subcutaneous tumor treatment. BALB/c nude mice were used for the mouse model experiment. (C-E) Control and Uchl3 knock down MC38 cells were injected for subcutaneous formation. After 5 days growth, *P. gingivalis* was injected into the grown tumor every 3 days. The mouse were sacrificed and tumors were collected (C). Tumor weight (D) and tumor volume (E) were calculated with the forluma: $V=L\times W^2/2$. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

western blot confirmation (**Figure 5B**), and revealed that GNG12 binds with UCHL3 directly (**Figure 5C**). We also found that the mRNA level of GNG12 is positive correlated with UCHL3 by Gene Expression Profiling Interactive Analysis (GEPIA) (**Figure 5D**). GNG12 is a particular G protein-coupled receptor, which was reported to participate in cancer immunity [23], but the degradation mechanism of GNG12 is unknown. To verify whether GNG12 is the substrate of deubiquitinase UCHL3, we knocked down UCHL3 in HCT116 cells. The western blot results showed that GNG12 was significantly reduced when UCHL3 was knocked down (**Figure 5E**), but the mRNA level of GNG12 is not affected by UCHL3 knockdown (**Figure 5F**). This supported that UCHL3 is necessary for the deubiquitination and stability of GNG12. From TCGA database, we can see high expression of GNG12 was relative to the poor overall survival of colonic neoplasms (**Figure 5G**). So we strongly suspected that UCHL3 interacted with and stabilized GNG12, thus promote colon cancer progression.

P. gingivalis promotes colonic neoplasm via UCHL3-GNG12 axis

We showed that *P. gingivalis* promotes colon cancer progression needs UCHL3, and GNG12 was the tumorigenesis-related substrate of deubiquitinase UCHL3. To clarified whether *P. gingivalis* promotes colon cancer via the UCHL3-GNG12 axis. We carried out colony formation, and EDU Cell Proliferation assays to detect whether GNG12 can rescue the inhibitory effect UCHL3 knock down in HCT116 cells. The results showed that cell proliferation was significantly inhibited when UCHL3 was knocked down, while over expression of GNG12 could partially rescue the inhibitory effect (**Figure 6A**, **6B**). The migration assays showed that knock down UCHL3 significantly inhibited cell migration ability of HCT116, but over express GNG12 could partially rescue the inhibitory effect (**Figure 6C**).

It was reported that GNG12 could activate NF-κB pathway then up-regulate PD-L1 to promote cancer progression [23-26]. So we



Gene Symbol	Unique Peptides	P value
HSPA5	24	0.965113
EIF3M	12	0.514037
TUBB4B	3	0.634735
STRAP	3	0.00751
HMGA1	3	0.02033
GNG12	2	0.0158
NCL	17	0.683976
NPM1	7	0.26271
EZR	11	0.625383

Figure 5. UCHL3 interacts with and stabilizes GNG12. (A) Workflow for UCHL3 interacting protein identification. (B) Protein list identified by FLAG-UCHL3 immunoprecipitation and mass spectrometry. (C) HCT116 cells were co-transfected with pCDNA3.1-FLAG-UCHL3 and pCDNA3.1-HA-GNG12, or pCDNA3.1 empty vector as control. Cells were collected for immunoprecipitation and western blot after 48 h transfection. (D) Correlation analysis of GNG12 and UCHL3 by GEPIA online software. (E, F) shScramble or shGNG12 HCT116 cells were growth and collected for western blot analysis (E) and qPCR (F). (G) The association between GNG12 and overall survival rate in the TCGA database.

hypothesized that that *P. gingivalis* induced UCHL3 may promote the colon cancer progression by GNG12 caused NF-κB signal activation. The qPCR results showed that knock down GNG12 significantly suppressed the mRNA level of PD-L1, but over express UCHL3 couldn't rescue the inhibitory effect (**Figure 6D**). The results indicate that GNG12 plays a role in NF-κB signal activation, and GNG12 exert its effect at the downstream of UCHL3. Taken together, these results suggested that *P. gingivalis* induced UCHL3 promotes colon cancer progression depend on GNG12 activated NF-κB signal pathway.

Discussion

Imbalance of microbial microbiota is one of the main causes of cancer [27]. Reorganization of bacterial floras in intestine influences the reconstruction of tumor microenvironment [28]. Oral microbiota is the second largest sym-

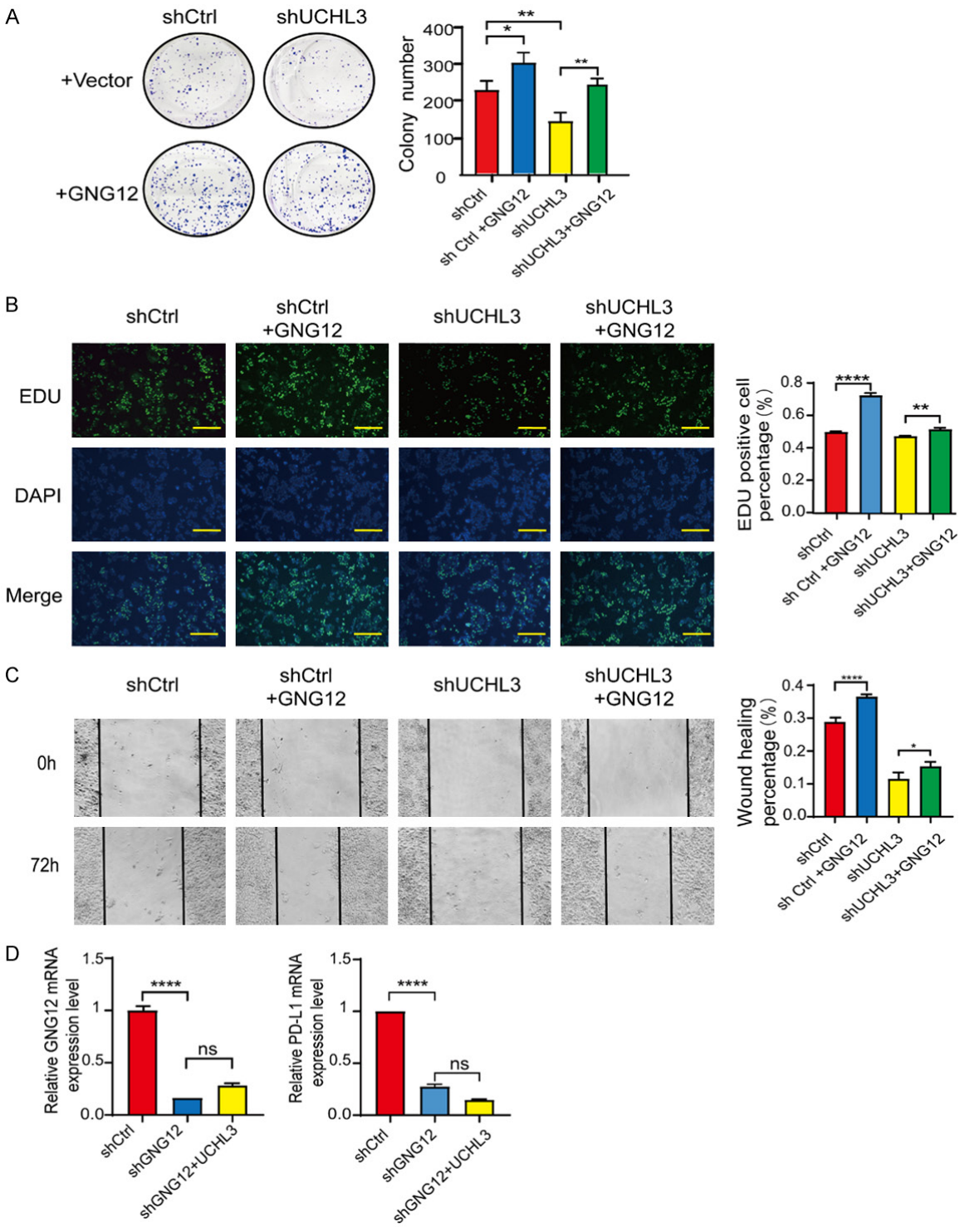


Figure 6. *P. gingivalis* promotes colonic neoplasm via UCHL3-GNG12 axis. (A) shCtrl and shUCHL3 HCT116 cell lines transfected with or without pCDNA3.1-GNG12 were used for colony formation assays. (B) EDU assay was applied to test the proliferation ability with GNG12 over expression (scale bar: 200 μm). (C) Wound healing rate was calculated to test the migration ability of HCT116 cells affected by UCHL3 knocked-down, and check the rescue effect of GNG12. (D) mRNA expression level of GNG12, PD-L1 in control cells, shGNG12 cells and shGNG12 cells with UCHL3 over expression. All the graphs were drawn by GraphPad Prism and analyzed with one-way ANOVA. P<0.05 was considered significant data. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

biotic flora in the human body, and are relative to many diseases including cancers [9, 29-33]. *P. gingivalis*, the main pathogenic bacteria of the oral cavity, was reported to be colonized in colon cancer tissues and promote the development of colon cancer [16, 18]. Additionally, *P. gingivalis* positive rates ranged from 2.6 to 5.3% in colon cancer patients and were 0 in healthy individuals [34]. Moreover, a few meta analysis revealed the level of serum antibody against *P. gingivalis* is closely related to the overcome of colon cancer [17]. Our study also validated that *P. gingivalis* could colonized in colon cancer tissues. Recently, it had been realized that *P. gingivalis* plays a crucial role in the progression of colon malignant tumors [35]. However, little is known that how *P. gingivalis* influences the process of colon tumor progression.

Proteins are the functional executives in the process of proliferation [36, 37], aging [38, 39] and apoptosis, while there is still a lack of research on the protein level changes caused by *P. gingivalis* in colon cancer. This study identified the proteome expression profiles in colon cancer cells affected by *P. gingivalis* treatment using label free quantitative mass spectrometry, and we found an important protein, UCHL3, which significantly high expressed and plays an important role in *P. gingivalis* infected colon cancer. UCHL3, one kind of deubiquitinases, belongs to the subfamily of ubiquitin C-terminal hydrolases and can remove ubiquitin from the specific proteins [40]. Recently, it had been disclosed that ubiquitylation modification is essential in the tumorigenesis of cancers [41]. UCHL3 was reported to promoted the oncogenesis and metastasis of cancers by regulating DNA repair [42], cell cycle [43, 44], tumor immunity [45] and epithelial to mesenchymal transition [46, 47]. Our study showed that knocking down UCHL3 obviously inhibits the progression of the colon cancer, reflecting the important role of ubiquitination in colon cancer infected with *P. gingivalis,* and UCHL3 might be a potential target for the clinic treatment of colon cancer.

We identified GNG12 as one of the UCHL3 interaction proteins, and we confirmed that UCHL3 is crucial for the stability of GNG12. GNG12 is a guanine nucleotide binding protein that participates in various cellular signal transduction pathways [23]. It has been revealed that GNG12 might be a predictor of cancers, such as osteosarcoma [48], glioma [49], pancreatic cancer [24] and so on. Furthermore, GNG12 was been demonstrated to influence tumor immune evasion [48], cell proliferation [50] and migration [51], which means GNG12 is a potential oncoprotein in tumorigenesis of colon cancer. In this study, knock down UCHL3 significantly inhibit the cell proliferation and migration ability of colon cancer cells, while over expression GNG12 can partially rescue the inhibitory effect. While, over expression UCHL3 can not rescue the inhibitory effect on NF-κB pathway by GNG12 knock down. Thus we supposed that *P. gingivalis* induced UCHL3 stabilizes GNG12 to activate NF-κB pathway in colon cancer. Finally, we propose that UCHL3 may be a valuable biomarker and drug target for *P. gingivalis* infectious colon cancer.

The study demonstrated UCHL3-GNG12 axis is important for *P. gingivalis* to activating NF-κB signal pathway and promote conlon cancer progression. But the transcription regulatory mechanisms by which *P. gingivalis* induced the expression of UCHL3 has not yet been revealed, and the ubiquitination sites of GNG12 and how UCHL3 deubiquitinates GNG12 needs further investigation.

Materials and methods

Patients

Colon tumors (n=22) and Precancerous lesions (n=6) were provided by Shanghai Tenth People's Hospital (Shanghai, China). The above samples were obtained from endoscope surgery without occurrence and survival data. All tissues were conserved at -80°C for the following experiments.

Cell culture and treatment

The colon cancer cell line HCT116 and MC38, and HEK293T cells were purchased from American Type Culture Collection (ATCC, America), and cultured in high glucose DMEM (Gibico, 11965118, America) with 10% FBS and 1% Penicillin-streptomycin at 37°C incubator with 5% CO_2. For bacteria treatment, HCT116 was cocultured with *P. gingivalis* at multiplicity of infection (MOI) 100 for 24 h.

Bacteria culture

P. gingivalis (33277) is obtained from ATCC. Brain Heart Infusion (BHI) broth is used to culture the *P. gingivalis* at the anaerobic environment of 37°C, 200 rpm. The full culture medium is composed of 18.5 g BHI (OXOID, CM1032B, America), 0.5 g yeast extract (OXOID, LP0021T, America), and 0.25 g L-cysteine (Merck, 168149, China) per 500 mL used after autoclaved. When the *P. gingivalis* grow to an OD of about 0.4, samples are taken. The bacterial culture solution is centrifuged at 4000 rpm, washed three times with PBS, and then resuspended with PBS. The bacterial concentration was calculated under a microscope, and frozen at -80°C for later use.

Western blot and antibodies

Cells were harvested and lysed with RIPA lysis (50 mM Tris, 150 mM NaCl, 1% TritonX-100, 0.01% SDS, 10 mM NaF, 5 mM EDTA) buffer containing 1 mM PMSF for 30 mins at 4°C. Adding the corresponding volume of loading buffer and boiled at 99°C for 10 mins. The proteins were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as the protocol described [52] and transferred to PVDF membranes. Then the membranes were blocked by 5% milk, and then incubated with primary antibody overnight at 4°C. After washed with PBS for 3 times, the secondary antibody was added and incubate for 1 h. Finally, the membranes were examined by enhanced chemiluminescence system. The following antibodies were used: UCHL3 polyclonal antibody (proteintech, 12384-1-AP, America), Anti-G gamma12 antibody (bioss, bs-13242R, China), β-actin monoclonal antibody (Sigma, A2228, America), Flag M2 monoclonal antibody (Sigma, F3165), HA-tag rabbit mAb (cell signaling technology, 3724, America). The secondary antibodies were purchased from Thermo Fisher: Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (A32731, America), Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (A32723).

DNA extraction and detection of P. gingivalis

The DNA of tumor tissues was extracted with Trizol (Vazyme, R401-01, China)-based method as previously described [53] with the purpose of obtaining RNA and DNA concurrently. 50 ng DNA is utilized for *P. gingivalis* detection by polymerase chain reaction assay. The detection primer sequences of *P. gingivalis* are shown in **Table 1**.

RNA extraction and qPCR

The total RNA of colon cancer cells was extracted with Trizol (Vazyme) as previously described [54]. Then 2 ng of RNA was reverse transcriptased with 1st Strand cDNA Synthesis SuperMix for qPCR kit (Yeasen, 11141ES60, China). The products were utilized in realtime-qPCR to detect the relative mRNA changes with SYBR Green Master Mix (No Rox) (Yeasen, 11201ES03). The primers' sequences are listed in **Table 1**.

Plasmid construction

The full sequence of UCHL3 was cloned from HCT116 cDNA with the primers as **Table 1** describes. The product was cleaved with restriction enzyme EcoR1 (Biolabs, R3101T, America) and Not1 (Biolab, R3189S), and polymerized with the cleaved vector of N-terminally tagged pCDH-3Flag by T4 ligase (Thermo Fisher, EL0016). The cDNA of colonic neoplasms tissues was used as the template for a clone of GNG12. Digesting the vector of N-terminally HA-tagged pcDNA3.1 and the fragment of GNG12 with enzyme EcoR1 (Biolabs, R3101T) and BamH1 (Biolabs, R3136M), then we set up ligation of the two parts.

For silencing target genes, pLKO.1 vector containning short hair RNAs were estabilshed. The shRNA oligos of targeted genes were designed at https://portals.broadinstitute.org/gpp/public.

Co-immunoprecipitation

The co-immunoprecipitation assays were performed as previously described [55]. In brief, transfect pcDNA3.1-FLAG-UCHL3 plasmids into HCT116 cells, after 48 h incubation, cells were lysed with NP40 lysis with 1 μM PMSF and then incubated with Anti-DYKDDDDK beads (Smart-lifesciences, SA042005, China) or Anti-HA affinity beads (Smart-lifesciences, SA068005) overnight at 4°C. Wash the beads with PBS 5 times and samples were used for mass spectrometry identification or Western blot.

Table 1. Primers used in the study

Primers for *P. gingivalis* detection	
P. gingivalis forward primer	5'-AGGCAGCTTGCCATACTGCG-3'
P. gingivalis reverse primer 1	5'-CCCCCGTCAATTCCTTTGAGT-3'
P. gingivalis reverse primer 2	5'-ACTGTTAGCAACTACCGATGT-3'
Primers for qPCR	
UCHL3 forward primer	5'-ATATGACACTTGACCTACGGC-3'
UCHL3 reverse primer	5'-TCATCTATACTTGGTGCCTCAGTC-3'
P. gingivalis forward primer	5'-TGCAAACCCATCACCTTCAAGAC-3'
P. gingivalis reverse primer	5'-TCCTTGCTTCTCTTCCTCGGT-3' [18]
PGT forward primer	5'-ATCCCCAAAG-CACCTGGTTT-3'
PGT reverse primer	5'-AGAGGCCAAGATAG-TCCTGGTAA-3' [18]
β-actin forward primer	5'-GGACCTGACAGACTACCTCA-3'
β-actin reverse primer	5'-GTTGCCAATAGTGATGACCT-3' [58]
GNG12 forward primer	5'-ACCGGCAGGCGGATTCATT-3'
GNG12 reverse primer	5'-GCTTGCTGTTTTGCTGGACA-3'
PD-L1 forward primer	5'-TGGCATTTGCTGAACGCATTT-3'
PD-L1 reverse primer	5'-A TGCAGCCAGGTCTAATTGTTTT-3'
TNFα forward primer	5'-TCTCTCAGCTCCACGCCATT-3'
TNFα reverse primer	5'-CCCAGGCAGTCAGATCATCTTC-3'
Primers for plasmid construction	
UCHL3 forward primer	5'-CATGAGAATTCAGATCCTGAACTCCTTAGCATGG-3'
UCHL3 reverse primer	5'-CATGAGCGGCCGCTGCTGCAGAAAGAGCAATC-3'
GNG12 forward primer	5'-CGCGGATCCTCCAGCAAAACAGCAAGCACC-3'
GNG12 reverse primer	5'-CCGGAATTC CTATAAGATGATGCAAG-3'
shUCHL3-1 forward primer	5'-CCGGCCCTGATGAACTAAGATTTAACTCGAGTTAAATCTTAGTTCATCAGGGTTTTTG-3'
shUCHL3-1 reverse primer	5'-AATTCAAAAACCCTGATGAACTAAGATTTAACTCGAGTTAAATCTTAGTTCATCAGGG-3'
shUCHL3-2 forward primer	5'-CCGGGTCTTACTTCTCTTTCCTATTCTCGAGAATAGGAAAGAGAAGTAAGACTTTTTG-3'
shUCHL3-2 reverse primer	5'-AATTCAAAAAGTCTTACTTCTCTTTCCTATTCTCGAGAATAGGAAAGAGAAGTAAGAC-3'
shUCHL3 (Mouse) forward primer	5'-CCGGCCTGTGGAACGATTGGACTAACTCGAG TTAGTCCAATCGTTCCACAGGTTTTTG-3'
shUCHL3 (Mouse) reverse primer	5'-AATTCAAAAACCTGTGGAACGATTGGACTAACTCGAGTTAGTCCAATCGTTCCACAGG-3'

Lentivirus package and stable cell line construction

To knock down UCHL3 or GNG12 in HCT116 cells by stably expressing short hairpin RNA target UCHL3 and GNG12, we generate pLKO.1-shUCHL3 and pLKO.1-shGNG12 constructs [56]. pLKO.1-shUchl3 was constructed to knock down Uchl3 in MC38 cells. Then we mix 4 μg target vector, 3 μg psAX2, 1 μg PMD2, and 24 μg PEI in 400 μl Opti-MEM at room temperature for 15 mins, and then add the mixture to the 293T cells for lentivirus package. Incubate for 48 h and harvest the lentivirus containing supernatant for cell infection. The shRNA lentivirus infected cells are selected with 2 mg/mL puromycin for 2 weeks, and then used for further experiments.

Cell counting kit 8 (CCK8) assay

Cells were seeded in a 96-well plate with a density of 1000 cells per well. The cell proliferation was examined with CKK8 (Beyotime, C0037, China) according to the manual. Cells were cultured with the mixed liquid (10 μl CCK8 per 100 μl complete medium) at 37°C for 2 hours. And then we measured the absorbance of 450 nm by the microplate reader.

Colony formation

1000 cells were seeded per well in 6 well plates, then cells were cocultured with *P. gingivalis* (MOI=100) or PBS control for 14 days. Wash the cell with PBS and fix the cell with 4% paraformaldehyde. Stain the cells with Giemsa

for 30 mins, and then take imaging after washed with PBS. The colony numbers were calculated using ImageJ.

EDU cell proliferation assay

200 thousand cells per well are seeded in a 12-well plate. After 24 h incubation, cells were treated with control or *P. gingivalis* for 48 h. Then the EDU (10 μM) is added into the supernatant for another 4 h. Fix the cell with 4% paraformaldehyde for further dyeing. The process is conducted as described in the manufacturer's guideline of BeyoClick™ EDU-488 Cell Proliferation Kit (Beyotime, C0071L). The photos are taken with a fluorescence microscope and analysed by Photoshop.

Wound healing assay

12-well plates are seeded with 8×10^5 cells per well, and grown in the incubator overnight. When the cell confluence is up to 100%, scratch the cells with a 10 ul pipette. Wash the cells with PBS, and add DMEM medium without FBS for cell culture. Treated cells with control or *P. gingivalis* at MOI=100 for 72 h and take photos every 24 h. The area of the gap is analyzed by ImageJ.

Immunohistochemistry

The colonic neoplasm tissues are embedded in paraffin and sliced into 4 μm thick flakes. Immunohistochemistry staining is performed as previously described [57]. After the process of deparaffinage, antigen retrieval, and blocking, the sections are incubated with primary antibody UCHL3 (Proteintech, 12384-1-AP) at 4°C overnight. On the next day, HRP secondary antibody is added and incubated at room temperature for 1 h. The pictures are taken by a microscope and analyzed by ImageJ.

Animals and subcutaneous tumor mouse model

4-week-old male BALB/c nude mice were purchased from Shanghai Siple-Bikai Laboratory Animal Co. (Shanghai, China) and all mice were housed and cared for at the specified pathogen free (SPF) level animal facility at the Shanghai Tenth People's Hospital Clinical Medicine Science and Innovation Park (Shanghai, China). Subcutaneous tumor mouse model was carried out as previously described [18]. In brief, 5×10^6 MC38 cells with or without UCHL3 knock down were subcutaneously injected into both sides of the groin of a naked mouse to create a colon cancer mouse model. After five days of subcutaneous injection, the mice were separated into several groups randomly. *P. gingivalis* (10^6 CFU) was injected into the tumors twice weekly. After 2 weeks, the animals were sacrifced by cervical dislocation. The volume of tumors was calculated by the formula: Volume (mm^3) = width^2 (mm^2) × length (mm)/2. All animal studies were conducted in compliance with the guidelines of Shanghai Tenth People's Hospital and the National Institutes of Health guide for the care and use of Laboratory animals.

Statistic analysis

All experiments are repeated at least three times. Data is shown as mean± SD and analyzed with GraphPad Prism 8.0.2. All of the information is analyzed with Student's t-test between two groups. And the comparisons among more than two groups are evaluated by one-way ANOVA. $P<0.05$ is considered significant statistic.

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Disclosure of conflict of interest

None.

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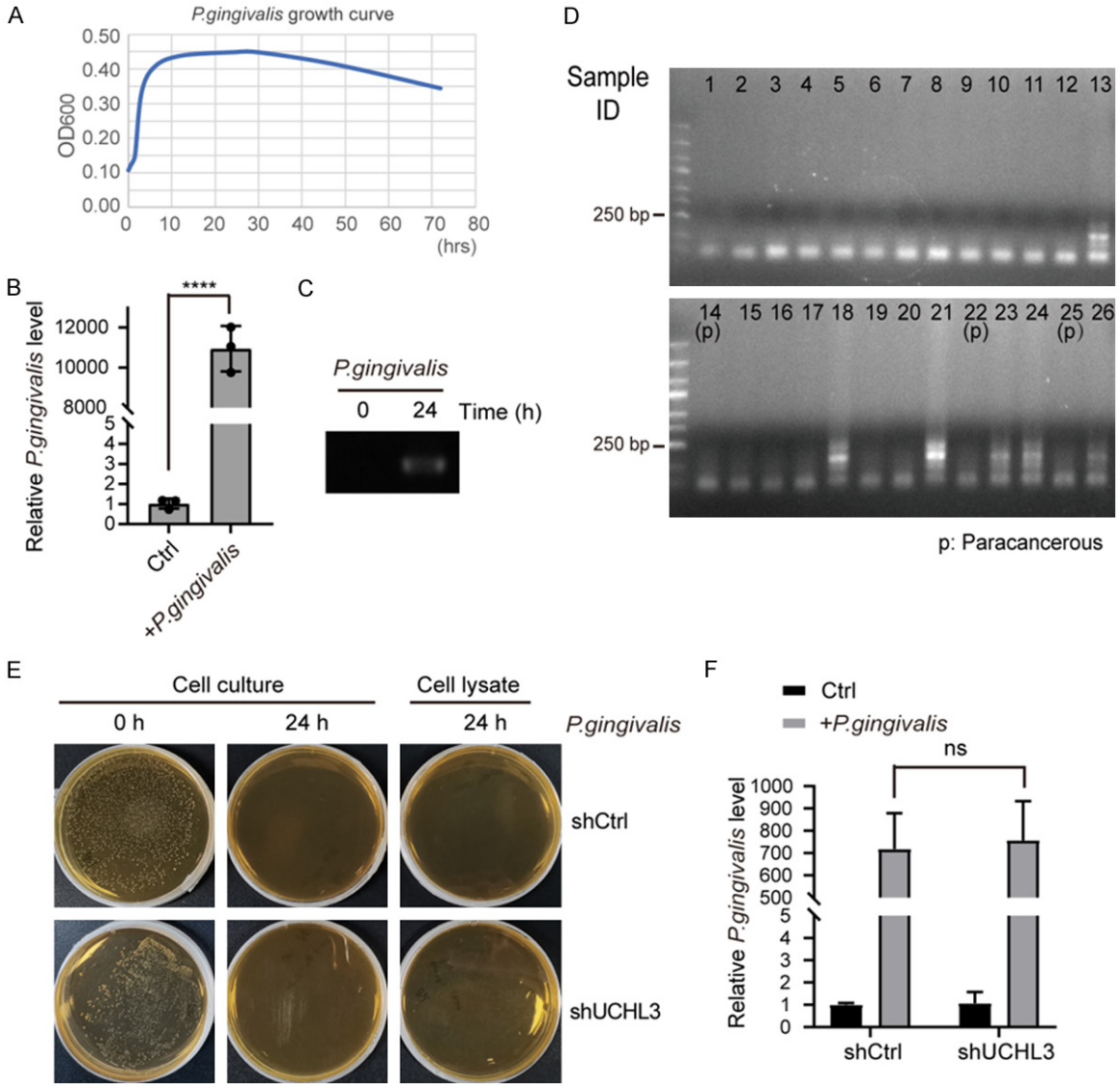

Figure S1. Infection of *P. gingivalis* in cells and mice. (A) Growth curve of *P. gingivalis*. (B, C) HCT116 cells were treated by *P. gingivalis* for 24 h, then cells were collected for PCR (B) and qPCR (C). (D) PCR identification of *P. gingivalis* infection in colon tissues. Extract DNA from colon tissue after grinding, and then detect the infection of pg bacteria through PCR. (E) CFU detection of shCtrl and shUCHL3 cells infected with *P. gingivalis*. shCtrl and shUCHL3 cells were treated with 500 MOI *P. gingivalis,* then cell supernatant sampled at 0 h, 24 h, and cells sampled at 24 h were collected for anaerobic cultivation to calculate the colony forming unit (CFU). (F) Detection of *P. gingivalis* infection in subcutaneous tumors of mice. The shCtrl and shUchl3 subcutaneous tumors in **Figure 4C** were used for DNA extraction and qPCR detection of the *P. gingivalis* level. The bar graphs were drawn by Graph Prism and analyzed with one-way ANOVA. ns: no significant; ****: P<0.0001.

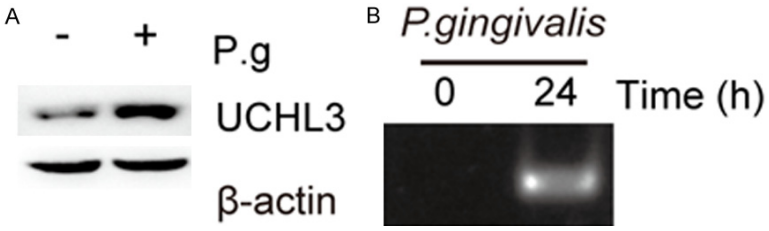

Figure S2. *P. gingivalis* infection upregulates UCHL3 expression in normal colon cells. (A, B) Western blot to detect UCHL3 expression level in *P. gingivalis* infected NCM460 (human normal colon epithelial) cells. NCM460 cells were treated by 500 MOI *P. gingivalis* for 24 h, and then cells were collected for western blot to detect the UCHL3 expression level (A), or PCR assay to confirm the infection of *P. gingivalis* (B).