# **ORIGINAL ARTICLE**



# Chondroitin polymerizing factor (CHPF) promotes the progression of colorectal cancer through ASB2-mediated ubiquitylation of SMAD9

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Summary. Chondroitin polymerizing factor (CHPF) has been reported to play a pivotal role in the progression of multiple cancers, however, the relationship between CHPF and colorectal cancer (CRC) progression has not been fully understood. The current study revealed that CHPF expression was upregulated in patients with CRC and correlated with an unfavorable prognosis. Also, CHPF knockdown effectively suppressed the viability and mobility of CRC cells and the growth of xenograft tumors. Additionally, SMAD9 was identified as a downstream target of CHPF. SMAD9 knockdown successfully abrogated the promotion of CHPF overexpression in CRC progression, indicating that CHPF regulated the development of CRC through SMAD9. Mechanistically, SMAD9 is ubiquitinated by ASB2, and the regulatory effect of CHPF on SMAD9 activity was exerted via its mediation of ASB2. Collectively, CHPF functioned as a promising prognostic biomarker and tumor-promoter of CRC by regulating the ASB2-mediated ubiquitination of SMAD9.

Key words: HPF, SMAD9, Colorectal cancer

### Introduction

Colorectal cancer (CRC), a type of cancer that develops in the colon or rectum (Abuzar et al., 2020), is the third most common cancer worldwide and the second leading cause of cancer deaths (Song et al., 2021). CRC usually begins as a small growth or polyp on the lining of the colon or rectum and, over time, can become

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cancerous and spread to other parts of the body (Mojica et al., 2022). Common symptoms of colorectal cancer include changes in bowel habits, blood in the stool, abdominal pain, and weight loss (Thompson et al., 2022). Risk factors for developing CRC include a family history of the disease, age over 50, a diet high in red meat and low in fiber, and a history of inflammatory bowel disease (Liu et al., 2021). Despite treatment options including surgery, radiation therapy, chemotherapy, and targeted therapy (Woradulayapinij et al., 2022), the overall 5-year survival rate of CRC patients after surgery has been rising steadily, nevertheless, the survival rate of those diagnosed at advanced stages is only 10-30% (Zhou et al., 2019).

Chondroitin polymerizing factor (CHPF) is a protein that plays a critical role in the biosynthesis of chondroitin sulfate (CS) (Kitagawa et al., 2003), a major component of the extracellular matrix in many tissues, including cartilage and bone (Cheng et al., 2022). Some studies have shown that chondroitin sulfate is involved in the pathogenesis and progression of certain types of cancers, including breast cancer (Svensson et al., 2011), lung cancer (Oo et al., 2021), and colon cancer (Wu et al., 2021). Previous studies identified that CHPF is upregulated in CRC and is predictive of the prognosis of patients diagnosed with CRC (Wu et al., 2021; Liu et al., 2022). Nevertheless, the molecular mechanism and biological role of CHPF in CRC remain unclarified.

Therefore, the current study is designed to explore the biological function of CHPF in the progression of CRC, as well as the possible underlying mechanism involved. Our study uncovered that CHPF acted as a tumor promoter in CRC and the knockdown of CHPF effectively inhibited CRC progression. Moreover, CHPH was discovered to regulate the malignant behaviors of CRC cells by promoting the expression of SMAD9 through ASB2-mediated ubiquitylation. These findings suggest that CHPH is a promising therapeutic target in CRC treatment.



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#### Materials and methods

#### Clinical samples

Eighty samples of CRC tissues and 38 samples of adjacent normal tissues were obtained from patients during operations at Suzhou Integrated Traditional Chinese and Western Medicine Hospital. The tissue samples were stored in -80°C liquid nitrogen immediately after resection for future use. To evaluate CHPF expression, the tissues were fixed with formalin and embedded using paraffin to perform immunohistochemistry (IHC) staining following the previous steps (Li et al., 2014). Patient characteristics are shown in Table 2. None of these patients received preoperative chemotherapy or radiotherapy. This study was approved by the ethics committee of the Suzhou Hospital of Integrated Traditional Chinese and Western Medicine [Institution Review Board (IRB) No.2018SZTCWM011] and written informed consent was obtained from each patient.

### Cell culture

Colorectal cancer cell lines (HCT116, SW480, HT29, and DLD-1) and human normal colonial epithelial cell lines (NCM460) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 (Gibco, CA) with 10% fetal bovine serum (FBS) (PAN, Germany) at 37°C, 5% CO<sub>2</sub>, in a humidified atmosphere.

### Cell transfection

Short hairpin RNA (shRNA) targeting CHPF (sh-CHPF), ASB2 (sh-ASB2), SMAD9 (sh-SMAD9), and sh-NC as control, overexpression plasmids containing CHPF (oe-CHPF), ASB2 (oe-ASB2), SMAD9 (oe-SMAD9), and empty pcDNA3.1 vector as control, were synthesized and provided by Shanghai Genepharm. Cell transfection was conducted using Lipofectamine 2000 Reagent. Cells were subjected to the subsequent experiments 48h after transfection.

#### RT-qPCR

Total RNA from HCT116 and DLD-1 cells was extracted using TRIzol reagent (Thermo Fisher, Waltham, MA, USA) and reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). qPCR was performed with the SYBR Green Master Mix (Vazyme, Nanjing, Jiangsu, China). The following conditions were used: activation: 50°C for 2 min; pre-soak: 95°C for 10 min; denaturation: 95°C for 15 sec, annealing: 60°C for 1 min; melting curve: 95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec. Gene expression quantification was performed using the  $2^{-\Delta\Delta Ct}$  method. GAPDH served as an endogenous control.

The primer sequences used are listed as follows: CHPF forward 'AGTTGGAGCGGGGCTTACAGTGA', reverse 'CAGCACCTCAAAGCGAGAGTGT'; SMAD9 forward 'GTGCTGTGAGTTCCCATTTGGC', reverse 'TTCACTGTGTCTTGGCACGAGC'; GAPDH forward 'GTCTCCTCTGACTTCAACAGCG', reverse 'ACCACCCTGTTGCTGTAGCCAA'.

#### Western blot (WB) and co-immunoprecipitation (Co-IP)

Total proteins were isolated using ice-cold RIPA lysis buffer and quantified using a BCA protein reagent kit (HyClone-Pierce, Logan, UT, USA). Total protein (20  $\mu$ g) was separated by 10% SDS-PAGE and transferred to PVDF membranes. The membrane was blocked with 5% BSA for 1h and was then incubated with primary antibodies at 4°C overnight, followed by HRP-labeled goat anti-rabbit secondary antibody. An Immobilon Western Chemiluminescent HRP Substrate (ECL-Plus<sup>TM</sup>) kit (Millipore, Schwalbach, Germany) was used for visualization, and proteins were detected with an X-ray imaging analyzer (Kodak, Rochester, NY, USA). GAPDH was used as the internal standard.

For the Co-IP assay, the total protein obtained from corresponding cells was used for immunoprecipitation with anti-CHPF or anti-ASB2, followed by the detection of protein expression by western blot with the indicated antibodies.

#### CCK-8 assay

The CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) was used to assess the proliferative ability of HCT116 and DLD-1 cells after designated treatments. The cells  $(1 \times 10^3)$  were plated in 96-well plates and treated with 10 µl of CCK-8 solution at indicated time points. The absorbance at 450 nM was analyzed by a microplate reader (Synergy4; BioTek, Winooski, VT, USA).

#### Transwell assay

The migrative and invasive abilities of treated HCT116 and DLD-1 cells were assessed using Transwell chambers (Corning, NY, USA) with Matrigel (BD Biosciences, San Jose, CA, USA) coating for invasion assay, or without the coating for migration assay. After incubation for 24h, the cells located on the lower chamber surfaces were fixed with methanol for 10 min, followed by staining with crystal violet. Then the stained cells were photographed and counted in five randomly selected fields.

### Cell apoptosis by TUNEL staining

The apoptosis of treated cells was analyzed using a In Situ Cell Death Detection Kit (TUNEL fluorescence FITC kit, Roche). Briefly, HCT116 and HT29 cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 on ice for 5 min. Then, 50  $\mu$ l of TUNEL reaction mixture was added to the samples and incubated at 37°C for 60 min. The nuclei were stained with DAPI. TUNEL staining was assessed via fluorescence microscopy (Leica, Germany).

#### Mouse xenograft model

For the xenograft tumor model, 5-week-old female BALB/c nude mice were randomly divided into two groups (n=6 per group). HCT116 ( $5 \times 10^{6}/0.1$  ml PBS) cells with stable knockdown of CHPF or control HCT116 cells were subcutaneously inoculated into the rear flank of nude mice. Tumor volumes were measured every 5 days with digital calipers and were calculated by the following formula: tumor volume=1/2 (length× width<sup>2</sup>). Thirty-five days later, the mice were killed and the volume and weight of the tumors were measured.

#### Statistical analysis

Data are expressed as the mean  $\pm$  SD (n $\geq$ 3) in the current study. The chi-squared test was applied to evaluate CHPF expression differences between tumor and normal tissues. The association between CHPF expression and the characteristics of patients diagnosed with CRC was assessed using Mann-Whitney U analysis and Spearman rank correlation analysis. The differences between different treatment groups were analyzed using Student's t-test or one-way ANOVA. All statistics were analyzed using SPSS 17.0 software (Chicago, IL, USA), and P<0.05 was considered significant.

#### Results

# CHPF was upregulated in CRC and associated with poor prognosis

The expression of CHPF was first detected in clinical tissue samples. As indicated by RT-qPCR and western blot, CHPF expression level was significantly upregulated in CRC tissues compared with non-cancerous tissues (Fig. 1A,B). The HPA database (https://www.proteinatlas.org/) suggested that CHPF expression levels were higher in CRC tissues than in normal colon tissues (Fig. 1C). Also, it was confirmed that CHPF expression in CRC cell lines (HCT116, SW480, HT29, and DLD-1) was higher than in a human normal colon epithelial cell line (NCM460) (Fig. 1D,E). Statistical analysis also showed that CHPF mRNA was highly expressed in CRC tissues (Table 1), and high CHPF expression was correlated with the T status of the tumor (P=0.0017), lymph node metastasis (P=0.0016), distant metastasis (P < 0.001), and advanced TNM stage (P < 0.001) in patients diagnosed with CRC (Table 2). Moreover, the survival curve obtained from Kaplan-Meier Plotter (https://kmplot.com/analysis/) indicated that high CHPF expression was associated with a poorer prognosis in rectum adenocarcinoma and colon cancer patients (Fig. 1F). The survival analysis of clinical samples further proved the correlation between high CHPF expression and an unfavorable prognosis in CRC patients (Fig. 1G). These data implicated the potential role of CHPF as a prognostic biomarker and tumor promoter in the progression of CRC.

Table 1. Expression patterns in CRC tissues and normal tissues.

CHPF	Tumor tissue		Normal tissue		P value
expression*	Cases	Percentage	Cases	Percentage	
Low High	49 31	61.25% 38.75%	35 3	94.7% 5.3%	<0.001

\*: High and low expression was divided based on the median mRNA expression of CHPF in CRC patients.

 Table 2. Correlations between CHPF expression and clinical characteristics in CRC patients (n=80).

Parameters	Total (n=80)	CHPF expression*		P value
		Low (n=49)	High (n=31)	
Age (years) ≦65 >65	39 41	27 22	12 19	0.153
Gender Male Female	46 34	29 20	17 14	0.7014
Tumor size (cm) ≦5 >5	33 47	20 29	13 18	0.9203
Pathological T catego T1-T2 T3-T4	ory 17 63	14 35	3 28	0.0442
Lymph node metasta N0 N1-2	sis 24 56	20 29	4 27	0.0079
Distant metastasis M0 M1	46 34	43 6	3 28	<0.001
TNM stage I-II III-IV	37 43	30 19	7 24	<0.001
Differentiation Well Moderate Poor	10 58 12	7 36 6	3 22 6	0.613
Microsatellite instabili MSS MSI-L MSI-H	ty 29 30 21	15 20 14	14 10 7	0.4191

\*: High and low expression was divided based on the median mRNA expression of CHPF in CRC patients.

# CHPF knockdown regulated the proliferation, migration, invasion, and apoptosis of CRC cells

Next, CHPF was silenced to study the effect of CHPF on CRC cells by transfecting sh-CHPF or sh-NC into HCT116 and DLD-1 cells (Fig. 2A,B). The treated cells were subjected to the CCK-8 assay to evaluate cell proliferation, which revealed a remarkable inhibition of cell growth by CHPF knockdown (Fig. 2C). Transwell assay was employed to investigate the effect of CHPF knockdown on the migration and invasion of CRC cells. As presented in Fig. 2D,E, the sh-CHPF-transfected cells exhibited remarkably reduced migrative and invasive abilities. Moreover, the apoptosis of transfected HCT116 and DLD-1 cells was assessed using TUNEL staining, which indicated that sh-CHPF transfection substantially increased the apoptotic rate (Fig. 2F). The xenograft mouse model constructed further indicated that CHPF knockdown markedly reduced the volume and weight of xenograft tumors (Fig. 2G,H). Taken together, CHPF deficiency inhibited the growth and metastasis of CRC.

# CHPF was responsible for the dysregulation of SMAD9 expression in CRC

Considering that CHPF plays a pivotal role in CRC progression, we further explored its downstream mechanism. Using the ConsensusPathDB database,



**Fig. 1.** The expression pattern and prognostic significance of CHPF in CRC. **A.** RT-qPCR assessed the expression of CHPF in CRC (N=80) and noncancerous tissues (N=38). **B.** WB detected the level of CHPF in 4 pairs of tumor and non-tumor tissues. **C.** The representative images of CHPFstaining cells in CRC or normal colon tissues were obtained from the HPA database. **D.** Representative images of IHC staining using CHPF antibody in CRC and normal tissues. **E.** The mRNA level of CHPF in CRC cell lines (HCT116, SW480, HT29, and DLD-1) and human normal colon epithelial cell line (NCM460) was assessed using RT-qPCR. **F.** Protein expression was compared between CRC cell lines and the NCM460 cell line by WB. **G.** The correlation between CHPF expression and overall survival in patients with rectal adenocarcinoma and colon cancer was obtained from the Kaplan-Meier Plotter database. **H.** The correlation between CHPF expression and overall survival of patients diagnosed with CRC (N=80) was evaluated using Kaplan-Meier analysis. \**P*<0.05; \*\**P*<0.01.

SMAD9 was predicted to be a downstream target of CHPF (Fig. 3A). The STRING database also predicted that CHPF interacted with TGF- $\beta$ 1 (Fig. 3B). The HPA database indicated that SMAD9 protein levels were higher in CRC tissues than in normal colon tissues (Fig. 3C). Therefore, we hypothesized that CHPF may regulate the expression of SMAD9 in CRC. To verify

this hypothesis, SMAD9 expression was detected in clinical tissue samples and cell lines. RT-qPCR suggested that SMAD9 was upregulated in CRC tissues and cell lines in comparison with the non-cancerous tissues and cell lines (Fig. 3D,E). Additionally, the expression of CHPF and SMAD9 was positively correlated in CRC tissues (Fig. 3F), which was



consistent with the result obtained from TCGA (Fig. 3G). Moreover, the expression of SMAD9 was evaluated in CHPF-silenced HCT116 and DLD-1 cells, finding that SMAD9 expression was substantially decreased by sh-CHPF (Fig. 3H). In sum, CHPH positively regulated SMAD9 expression in CRC.

# SMAD9 knockdown attenuated the promotion of CHPF overexpression in CRC

Subsequently, sh-SMAD9 was transfected into CHPF-overexpressed HCT116 and DLD-1 cells to perform rescue experiments. Western blot showed that SMAD9 knockdown reversed the increase in SMAD9 protein levels in oe-CHPF-transfected cells (Fig. 4A). The CCK-8 assay indicated that CHPF overexpression dramatically facilitated cell growth while SMAD9 knockdown effectively abrogated the increase in proliferation (Fig. 4B). For cell migration and invasion, a Transwell assay revealed that CHPF supplementation substantially accelerated the migration and invasion of CRC cells, which was partially reversed by the introduction of sh-SMAD9 (Fig. 4C,D). Moreover, a TUNEL assay indicated that the suppression of cell apoptosis induced by CHPF abundance was annulled by SMAD9 depletion (Fig. 4E). Taken together, CHPF



**Fig. 3.** CHPF regulated the expression of SMAD9 in CRC. **A.** The ConsensusPathDB database predicted that CHPF might interact with SMAD9. **B.** The possible interaction between CHPF and TGF-β1 was forecasted by the STRING database. **C.** The representative images of SMAD9-staining cells in CRC or normal colon tissues were obtained from the HPA database. **D.** The mRNA level of SMAD9 in tumor and non-tumor tissues was assessed by RT-qPCR. **E.** The level of SMAD9 in tumor cells (HCT116, SW480, HT29, and DLD-1) and non-tumor colon epithelial cell line (NCM460) were compared by RT-qPCR. **F.** The relation between CHPF and SMAD9 expression in tumor tissues was analyzed using Pearson correlation analysis. **G.** Pearson correlation analysis result of CHPF and SMAD9 expression in Colon adenocarcinoma (COAD) and READ tissues. **H.** SMAD expression was detected in HCT116 and DLD-1 cells transfected with sh-NC or sh-CHPF by RT-qPCR and WB. \**P*<0.05; \*\**P*<0.001; \*\*\**P*<0.001.

promoted the growth and metastasis of HCT116 and DLD-1 cells by upregulating SMAD9.

### CHPF regulated SMAD9 expression through ASB2mediated ubiquitination

Studies show that the activity and stability of SMAD9 can be mediated by the ubiquitin-proteasome system (UPS) (Wagner et al., 2011; Chen et al., 2013). The ankyrin repeat and SOCS box containing 2 (ASB2) was revealed to be an E3 ligase of SMAD9, which specifically ubiquitylates SMAD9 and targets SMAD9 for proteasomal degradation in the embryo (Min et al.,

2021). It could be hypothesized that ASB2 could ubiquitinate SMAD9 and lead to the degradation of SMAD9 in CRC. ASB2 was silenced or overexpressed in HCT116 and DLD-1 cells to evaluate its regulatory effect on SMAD9. ASB2 knockdown caused a substantial decrease in the expression of ASB2 and an increase in SMAD9 protein expression (Fig. 5A). Oppositely, ASB2 overexpression increased the protein level of ASB2 but inhibited SMAD9 protein expression (Fig. 5B). Moreover, Co-IP was performed to validate the binding between ABS2 and SMAD9. As illustrated in Fig. 5C, ASB2 interacted with SMAD9 in HCT116 and DLD-1 cells. Besides, ASB2 depletion markedly



Fig. 4. The participation of SMAD9 in CHPF-mediated CRC progression. HCT116 and DLD-1 cells were divided into three groups: pcDNA3.1, oe-CHPF, and oe-CHPF + sh-SMAD9. A. The protein expression of SMAD9 in treated cells was detected. B-E. After the designated treatments, the cells were subjected to the CCK-8 assay to detect cell proliferation (B), Transwell assay to evaluate cell migration (C) and invasion (D) and Flow cytometry to observe cell apoptosis (E). \*P<0.05; \*\*P<0.01.



**Fig. 5.** The involvement of ASB2-mediated ubiquitination in CHPF-regulated SMAD9 expression. **A.** The protein level of ASB2 and SMAD9 in HCT116 and DLD-1 cells treated with sh-NC or sh-ASB2. **B.** The protein level of ASB2 and SMAD9 in HCT116 and DLD-1 cells treated with pcDNA3.1 or oe-ASB2. **C.** Immunoprecipitation was performed using anti-ASB2, followed by WB using anti-ASB2 or anti-SMAD9 primary antibodies. **D.** ASB2-silenced cells were immunoprecipitated with SMAD9 or IgG antibodies and ubiquitination was assessed by WB. **E.** A Co-IP assay confirmed the interaction between CHPF and ASB2. **F.** Western blot assessed the impact of CHPF knockdown on ASB2 protein expression in CRC cells. **G.** The protein level of SMAD9 in HCT116 and DLD-1 cells transfected with sh-NC, sh-CHPF, or sh-CHPF + shASB2. \**P*<0.05; \*\**P*<0.01; \*\**P*<0.001.

attenuated the ubiquitination of SMAD9 (Fig. 5D). Furthermore, CHPF was directly bound to ASB2 and negatively regulated ASB2 expression (Fig. 5E,F). ASB2 knockdown effectively reversed the suppression of CHPF knockdown on SMAD9 protein level (Fig. 5G). These results suggest that the inhibitory effect of CHPF is exerted through ASB2-mediated ubiquitination of SMAD9 in CRC cells.

#### Discussion

The treatment strategy for CRC typically depends on several factors, such as the stage of the cancer, size and location of the tumor, and the overall health of the patient (Dekker et al., 2019). At present, surgical resection is an effective treatment for patients with earlystage CRC; for patients with advanced CRC, chemotherapy remains the main treatment strategy (Jiang et al., 2018). However, most CRC patients are diagnosed in the middle or advanced stages due to the lack of specific symptoms during the early stage (Ding et al., 2022). Therefore, it is essential to identify novel therapeutic targets to enhance the effects of chemotherapy. In recent years, numerous studies revealed that advancements in gene technology, such as high-throughput sequencing, have given novel insights into the research of molecular mechanisms underlying CRC, and various therapeutic biomarkers have been identified (Xu et al., 2018; Zhou et al., 2020; Kim et al., 2021). For example, Chen et al. reported that KDM5Cinduced METTL14 downregulation inhibited the metastasis of CRC in vitro and in vivo by mediating the m6A modification of SOX4 in a YTHDF2-dependent manner (Chen et al., 2020). In another case, circDDX17 was revealed to serve as a tumor suppressor in CRC (Li et al., 2018).

CHPF is located in the 2q35-q36 region of human chromosomes and plays an important role in cellular function (Li et al., 2022). Multiple studies have revealed that CHPF is dysregulated and plays a tumor-promotive role in different types of cancers. For instance, CHPF expression was marked upregulated and the knockdown of CHPF effectively suppressed gastric cancer tumorigenesis by suppressing the activity of E2F1 (Lin et al., 2021). Also, Liao et al. uncovered that high CHPF expression was associated with tumor metastasis, late stages, and short survival time of breast cancer patients, and CHPF facilitated the development of breast cancer through the modification of SDC4 activity and the tumor microenvironment (Liao et al., 2021). Moreover, the upregulation of CHPF was correlated with shorter overall survival in lung cancer patients and CHPF knockdown substantially suppressed the growth and metastasis of lung cancer cells (Hou et al., 2019; Cao et al., 2020). Nevertheless, the role of CHPF in CRC remains largely unclarified.

This study is the first to explore the expression pattern, biological role, as well as molecular mechanism involved in CRC. By analyzing the public database as well as clinical samples, CHPF was identified to be significantly upregulated in CRC. Additionally, the high expression of CHPF was associated with the T status of the tumor, lymph node metastasis, distant metastasis, advanced TNM stage, and unfavorable survival in patients with CRC. It was noted that CHPF knockdown effectively inhibited the proliferative, migrative, and invasive abilities of CRC cells. Also, the deficiency of CHPF induced significant apoptosis in the treated cells. In addition, a xenograft mouse model was established to evaluate the suppressive effect of CHPF knockdown *in vivo*. It was illustrated that CHPF depletion suppressed tumor growth as indicated by the reduced weight and volume of xenograft tumors.

Furthermore, SMAD9 was predicted to be a potent downstream target of CHPF. SMAD9 is a member of the SMAD family of proteins, which has critical roles in signaling pathways that regulate cellular processes such as cell growth, differentiation, and apoptosis (Ten Dijke et al., 2002; Wei et al., 2002; Tsukamoto et al., 2014). SMAD9 specifically belongs to the receptor-regulated SMAD (R-SMAD) subgroup, which transduces signals from the TGF- $\beta$  superfamily of cytokines (Serralheiro et al., 2017; Esmaeili-Fard et al., 2021). Dysregulation of TGF- $\beta$  signaling, including SMAD9, can promote tumor growth, invasion, and metastasis (Batlle and Massagué. 2019; Tzavlaki and Moustakas, 2020). Studies have found that SMAD9 can act as both a tumor suppressor and a promoter, depending on the cellular context and type of cancer. For example, in non-small cell lung cancer, SMAD9 has been shown to work against the tumor-promoting gene BMP4 (Gao et al., 2021), while in MYCN-amplified neuroblastoma, SMAD9 promotes tumorigenesis (Tan et al., 2022). To verify the interaction between CHPF and SMAD9, we investigated the expression pattern of SMAD9, which showed that SMAD9 was upregulated in CRC tissues and cell lines and its expression was positively correlated with CHPF expression in CRC tissues. Besides, CHPF positively regulated SMAD9 expression in CRC cell lines. Further experiments were performed in the established cell model with SMAD9 knockdown following CHPF overexpression. The results demonstrated that silencing of SMAD9 substantially abrogated the promotion of CHPF abundance in the proliferation, migration, and invasion of CRC cells. Meanwhile, CHPF supplementation-induced suppression of cell apoptosis was effectively reversed by knocking down SMAD9. These data could lead to the conclusion that the tumorpromotive effect of CHPF in CRC was possibly exerted through the upregulation of SMAD9.

Since SMAD9 was reported to be specifically ubiquitylated by ASB2, we further investigated whether ASB2-mediated ubiquitination participated in CHPFregulated SMAD9 expression. Existing studies have proven that ASB2 may have essential roles in human cancer by targeting specific proteins. In acute promyelocytic leukemia, ASB2 might regulate the differentiation of hematopoietic cells by mediating cell spreading and actin remodeling by targeting filamins for degradation (Heuzé et al., 2005). Shin et al. also reported that loss of the aryl hydrocarbon receptor inhibited the ability of NK cells to migrate and infiltrate tumors via suppressing ASB2-mediated ubiquitination and degradation of Filamin A (Shin et al., 2021). Our data proved that ASB2 ubiquitinated SMAD9 protein and regulated SMAD9 protein expression in CRC cells. More importantly, the suppressive effect of CHPF knockdown on SMAD9 protein levels was annulled by silencing ASB2, suggesting that CHPF regulated SMAD9 protein expression via ASB2-mediated ubiquitination.

## Conclusion

Our study pinpointed the upregulation of CHPF and its positive correlation with an unfavorable prognosis in patients with CRC. CHPF knockdown impeded the malignant behaviors of CRC cells *in vitro* and suppressed the growth of xenograft tumors *in vivo*. Mechanistically, CHPF targeted ASB2 to regulate the ubiquitination of SMAD9 in CRC cells. To sum up, CHPF was identified as a potent oncogene, offering insights to improve targeted therapies for CRC treatment.

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