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Original Article

Downregulation of NLRP12 enhances the proliferation, migration and drug-resistance of colorectal cancer cells by modulating MEK/ERK/GLI1 signaling pathway

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Abstract: Objective: To investigate the role of inflammasome NLRP12 in the tumorigenesis of colorectal cancer and drug-resistance properties of colorectal cancer cell lines. Methods: NLRP12 protein expression in 57 CRC tissue specimens and surrounding normal tissues were investigated by immunohistochemical analysis. Western blotting and quantitative real-time polymerase chain reaction (QRT-PCR) were used to detect the protein and mRNA expression of NLRP12 in CRC tissues and cells. Later NLRP12 siRNA was transfected into HCT116 cells to explore the effects of NLRP12 on the proliferation, migration and drug-resistance in colorectal cancer cells in vitro. Results: NLRP12 protein levels were decreased in CRC tissues compared to surrounding normal tissues. This decrement in NLRP12 protein levels in CRC tissues was significantly associated with tumor size, Dukes stages, and TNM stages of CRC patients ($P<0.05$). Furthermore, downregulation of NLRP12 in HCT116 cells promoted cells growth and migration ($P<0.05$). And these HCT116 cells were shown to be more resistant to cisplatin ($P<0.05$). NLRP12 negatively regulated the expression of phosphorylation-extracellular signal-regulated kinase (p-ERK) and GLI1 in HCT116 cells, and restraining mitogen-activated protein kinases (MAPK) pathway by using the mitogen-activated extracellular kinases (MEK) inhibitor U0126 blocked the effect of NLRP12 on GLI1. Conclusion: Our preliminary results confirm that NLRP12 may play an important role in the tumorigenesis and drug-resistance of colorectal cancer. Thus, NLRP12 may be a potential marker to treat colorectal cancers.

Keywords: NLRP12, colorectal cancer, proliferation, drug-resistance, GLI1, siRNA

Introduction

Colorectal cancer (CRC) is a common health problem worldwide, which represents one of the leading causes of cancer-related mortality [1]. Although considerable progress in CRC diagnosis and therapy has been achieved in the past decades, majority of patients are diagnosed at an advanced stage, missing the optimal time for a radical operation [2]. The 5-year survival rate of CRC patients having distant metastasis is still significantly low [3]. Therefore, it is urgent to identify novel therapeutic targets for prevention of CRC and establish new biomarkers useful for its early detection of high-risk populations.

It is well known that CRC is a progressive disease and several factors such as epigenetic abnormalities, genetic mutations and inflammatory cytokines have been associated with the development of CRC [4, 5]. It has also been reported that several inflammasomes play important roles in the tumorigenesis of cancer. Thus, it may act as novel therapeutic strategies to cure CRC [6]. The nucleotide-binding domain and leucine-rich repeat containing (NLR) family of genes have been commonly characterized as the activators of inflammation [7]. In addition, NLRs have been considered to take part in the cancer progression [8]. For example, the NLRP3 is the most wellknown member of the NLR family, which has been shown to play duplex roles in

tumorigenesis. Specifically, NLRP3 activation induced by chemotherapeutic drugs can make contributions to cancer growth [9]. In contrast, NLRP3 deficient mice were shown to be more highly susceptible to colitis-associated colon cancer, suggesting NRLP3 might play a protective role against tumorigenesis [10, 11]. Similar to NLRP3, NLRP12 was also considered as a tumor suppressive molecule as displayed in tumorigenic animal models. NLRP12 deficient mice were found to be hypersusceptible to dextran sulfate sodium (DSS)-induced colitis and colitis-associated tumorigenesis [12]. The azoxymethane (AOM)-DSS mouse model also showed that mice lacking NLRP12 had more severe colon tumor compared with wild-type mice due to the activated canonical NF-κB signaling, noncanonical NF-κB signaling and ERK signaling [12, 13]. Although *in vitro* and *in vivo* data, and animal module demonstrated that NLRP12 has been associated with colorectal cancer [12, 13], it is essential to find more physiologic and clinical evidence to verify it.

Here, we examined the expressions of NLRP12 in colorectal cancer tissues and surrounding normal tissues of 57 CRC patients by immunohistochemical staining. Our study revealed the relationships between clinical significance and the expression of NLRP12. Furthermore, we found that the expression of NLRP12 was knocked down in CRC cells by siRNA and NLRP12 influenced on the proliferation, migration and drug-resistance properties of the CRC cells. Taken altogether, our results revealed that NLRP12 plays a vital role in the proliferation, migration and drug-resistance of CRC cells and might be a novel target to cure colorectal cancer.

Materials and methods

Cell lines and reagents

The human colon cancer cell lines (CCL224, HCT116, SW480 and RKO) were purchased from the Chinese Academy of Sciences (Shanghai, China), and were cultured in DMEM medium (Hyclone, Los Angeles, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA), 5% of penicillin/streptomycin (Gibco, Grand Island, USA). The cells were grown at 37°C containing 5% CO_2. U0126, a MEK inhibitor (U-400, Univ-bio, Shanghai, China) was dissolved in DMSO and stored at -4°C at the concentration of 20 mmol/L.

Patients and specimens

A total of 57 paired tumor specimens and matched normal tissues were obtained from CRC patients who underwent surgical resection between January 2014 and December 2015 at the Department of General Surgery, The First Affiliated Hospital of Soochow University. None of the patients received any anti-cancer therapy before surgery. All experiments involving human subjects were performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and the relevant guidelines and regulations of Soochow University. All experimental protocols were approved by research ethics committee of the first affiliated hospital of Soochow university.

Immunohistochemistry

Immunohistochemistry (IHC) was carried out using the Streptavidin-Peroxidase conjugate method to detect the expression of NLRP12 in 57 colorectal cancer tissues and matched adjacent normal tissues as well as 10 adenoma and polyps tissues. Briefly, 4 μm-thick sections which were embedded in paraffin were stored at 60°C for 2 h. Afterward, the sections were deparaffinazed in xylene twice and rehydrated using graded ethanol. Then sections were incubated with 3% hydrogen peroxide to block the endogenous peroxidase for 10 min. Then, the microwave was used to repair the antigen. The sections were incubated in endogenous peroxidase blockers for 10 min and incubated with NLRP12 antibody (1:200; Abcam, University of Cambridge, United Kingdom) overnight at 4°C. Slides were washed with distilled water three times each 10 min and incubated with normal goat serum. The slides were incubated with secondary antibodies for 10 minutes after washing. Finally, the sections were colored with diaminobenzidine (DAB) after washing. The expression of NLRP12 was assessed by the ratio of positive cells and the intensity of staining. The ratio of positive cells was graded on a scale of 0~3. The grades were as following: Grade 0 = (0~10)%; Grade 1 = (10~25)%; Grade 2 = (25~50)%; Grade 3 = ≥50% [14]. The intensity of staining was scored as following: Intensity 0 = -/no staining; Intensity 1 = +/weak staining; Intensity 2 = ++/moderate staining and intensity 3 = +++/strong staining. The total score was the product of the score for positive cell ratio and the intensity of

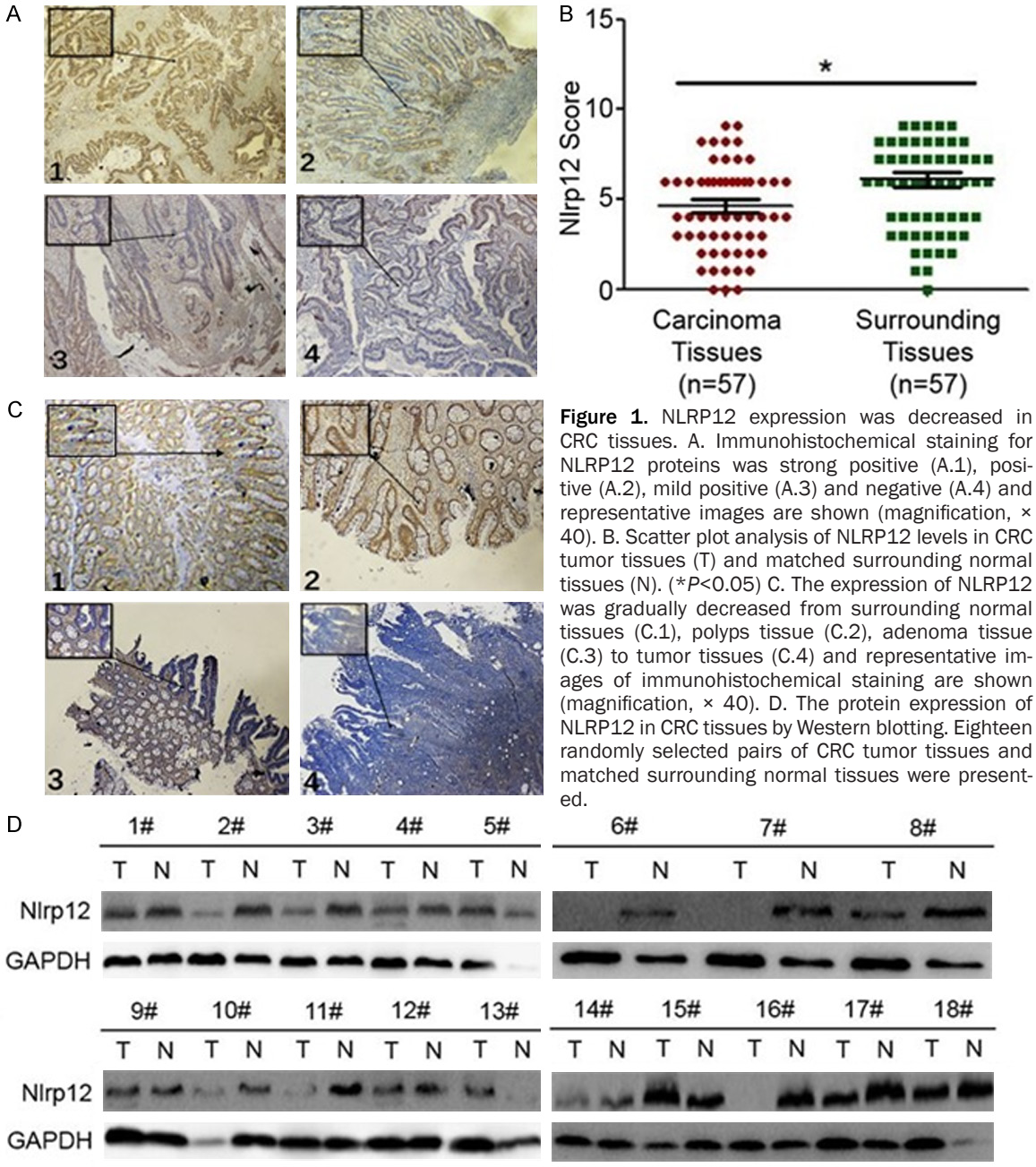

Figure 1. NLRP12 expression was decreased in CRC tissues. A. Immunohistochemical staining for NLRP12 proteins was strong positive (A.1), positive (A.2), mild positive (A.3) and negative (A.4) and representative images are shown (magnification, × 40). B. Scatter plot analysis of NLRP12 levels in CRC tumor tissues (T) and matched surrounding normal tissues (N). (*P<0.05) C. The expression of NLRP12 was gradually decreased from surrounding normal tissues (C.1), polyps tissue (C.2), adenoma tissue (C.3) to tumor tissues (C.4) and representative images of immunohistochemical staining are shown (magnification, × 40). D. The protein expression of NLRP12 in CRC tissues by Western blotting. Eighteen randomly selected pairs of CRC tumor tissues and matched surrounding normal tissues were presented.

the staining. IHC results were evaluated by two pathologists.

siRNA transfection

HCT116 cells were seeded in six-well plates. The cells were transfected with siRNA targeting NLRP12 (Gene Pharma, Suzhou, China) using lipofectamine 2000 (Invitrogen, CA, USA) after being incubated overnight. The sequences of three NLRP12-siRNA were as follows: siRNA#1 (Sense strand: 5'-GCAGGAAAUUCCGGCUCAUTT-3', Antisense strand: 5'-GCAGCAUGCAAGACCUCAUTT-3'); siRNA#2 (Sense strand: 5'-AUGAGGUCUUGCAUGCUGCTT-3'; Antisense strand: 5'-TCATCCTCCGTGAGTTCTCCA-3'); siRNA-#3 (Sense strand: 5'-GCGGGCCAAGUCUUCAAUUTT-3', Antisense strand: 5'-AAUUGAAGACUUGGCCCGCTT-3'). Cells were collected 48 h post-transfection and used for each experiment.

Western blot

Colorectal cancer tissues, surrounding normal tissues and HCT116 cells after washing with

phosphate buffered saline (PBS) were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (BeyotimeInc, Nantong, China). Cell lysates were centrifuged at 12000 rpm for 10 min at 4°C and then the proteins were quantified using Pierce bicinchoninic (BCA) assay kits (Thermo Scientific). The proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidenedifluoride (PVDF) membranes. The membranes were blocked with 5% fat-free milk in Tris-Buffered Saline (TBS) for 1 hour at room temperature and then incubated in rabbit anti-human NLRP12 antibody (1:1000, ab105409, Abcam, USA), rabbit anti-human ERK antibody (1:1000, ab17942, Abcam, USA), rabbit anti-human p-ERK antibody (1:1000, ab47339, Abcam, USA), rabbit anti-human GLI1 antibody (1:1000, ab151796, Abcam, USA) at 4°C overnight. Mouse monoclonal antibody against human GAPDH (1:2000, ab8245, Abcam, USA) was used as a loading control. The next morning, membranes were washed with TBST three times and then treated with secondary antibodies (1:1000, Santa Cruz, CA, USA). Analyses were performed with chemiluminescence. The western blotting results were quantified by Image J software. All experiments were performed three times.

Quantitative real-time PCR

Total RNAs were isolated from cultured cells using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) as recommended by the manufacturer's instructions. The expression of NLRP12 mRNA was detected by QRT-PCR, which was performed using a SYBRGreen Real-time PCR MasterMix (Applied Biosystems, Foster City, CA, USA). The primers for NLRP12 were as follows: Forward: 5'-TGACCGACCTTTACCTGACC-3', Reverse: 5'-CCATCCCAAATAACCAGAGG-3'; 18 s (Forward: 5'-AGTTCCAGCACATTTTGCGAG-3'; Reverse: 5'-TCATCCTCCGTGAGTTCTCCA-3') was used as an internal control.

Cell viability assay

Colon carcinoma cells and si-NLRP12 cells were embedded in 96-well plates. MTT reagents were added into the wells every 24 h, 48 h and 72 h. Then the plates were incubated at 37°C with 5% CO_2 for 4 h. 150 μl DMSO was added and incubated at room temperature for 10 min. Optical densities (ODs) were detected at the absorbance of 490 nm.

Cell migration assay

Cell migration ability was studied using a transwell migration filter (Corning, NewYork, NY, USA). 48 h post-transfected HCT116 were resuspended in serum-free DMEM medium and were seeded in the upper chamber at the concentration of 2×10^4 cells per well. The lower chambers were added with 500 ul DMEM with 10% FBS. After incubated at 37°C for 24 h, cells on the upper side of the upper chamber were removed with a cotton swab and cells on the bottom of the upper chamber were fixed with methanol and stained with 0.1% crystal violet. Cells were visualized under an inverted microscope and five random fields were captured at 200 magnification. The experiments were performed at least three times.

Statistical analysis

Statistical analysis was performed with SPSS-17.0 software (SPSS Inc, Chicago, USA). Data are expressed as the mean ± standard deviation (SD). The student's t-test was used to compare means between two groups. The correlation between IHC results and clinical pathological characteristics was determined by chi-square statistical test. A 2-tailed $P<0.05$ was considered statistically significant.

Results

NLRP12 is downregulated in human CRC patients

To investigate the role of NLRP12 in CRC patients, immunohistochemical staining was used to test the NLRP12 expression in 57 CRC tissues and surrounding normal tissues. Immunohistochemical results showed that NLRP-12 was negative or weakly positive staining in tissue samples taken from CRC whereas NLRP12 was found positive in surrounding normal tissues (**Figure 1A** and **1B**). Interestingly, we also found that the expressions of NLRP12 were gradually decreased in surrounding normal tissues, polyps, adenoma, and tumor tissues (**Figure 1C**). To further confirm our results, western blotting was performed to detect the expression of NLRP12 in CRC patients. The western blotting results were similar to IHC

Table 1. Relationship between NLRP12 expression by IHC and clinicopathological factors in CRC patients

Clinicopathological parameters	Case no.	NLRP12 expression		*P* value
		Negative	Positive	
Gender				0.899
Male	32 (56.1%)	21 (65.6%)	11 (34.4%)	
Female	25 (43.9%)	16 (64.0%)	9 (36.0%)	
Age (years)				0.169
>60	27 (47.4%)	20 (74.1%)	7 (25.9%)	
≤60	30 (52.6%)	17 (56.7%)	13 (43.3%)	
BMI (kg/m^2)				0.192
≥18.5	35 (61.4%)	25 (71.4%)	10 (28.6%)	
<18.5	22 (38.6%)	12 (54.5%)	10 (45.5%)	
Tumor size				0.006**
≤5 cm	26 (45.6%)	12 (46.2%)	14 (53.8%)	
>5 cm	31 (54.4%)	25 (80.6%)	6 (19.4%)	
Dukes stages				0.005**
A-B	18 (31.6%)	7 (20.0%)	11 (80.0%)	
C-D	39 (68.4%)	30 (26.3%)	9 (73.7%)	
TNM stage				0.014*
I-II	17 (29.8%)	7 (41.2%)	10 (58.6%)	
III-IV	40 (70.2%)	30 (75.0%)	10 (25.0%)	
Tumor location				0.718
Colon	21 (36.8%)	13 (61.9%)	8 (38.1%)	
Rectum	36 (63.2%)	24 (66.7%)	12 (33.3%)	
Lymph node metastasis				0.249
Positive	20 (35.1%)	11 (55.0%)	9 (45.0%)	
Negative	37 (64.9%)	26 (70.3%)	11 (29.7%)	
Liver metastasis				0.432
Positive	18 (31.6%)	13 (72.2%)	5 (27.8%)	
Negative	39 (68.4%)	24 (61.5%)	15 (38.5%)	

Abbreviations: CRC, colorectal cancer; BMI, body mass index; TNM, tumor-lymph node-metastasis. *P<0.05, **P<0.01.

staining, and it showed a decreased expression of NLRP12 in CRC tissues compared to surrounding normal tissues (**Figure 1D**).

The expression of NLRP12 is significantly related with clinicopathologic parameters in CRC patients

To further estimate the relationship between NLRP12 expression and the clinicopathological parameters in CRC patients, clinical features of 57 CRC patients were analyzed. As shown in **Table 1**, we found that the low expression of NLRP12 was significantly correlated with tumor size (P = 0.006), Dukes stages (P = 0.005) and TNM stages (P = 0.014), whereas the NLRP12 expression level was not obviously associated with other clinical features such as gender, age, body mass index (BMI), tumor location, liver metastasis and lymph node metastasis (P>0.05). These data indicated that reduced NLRP12 expression is associated with the development of CRC.

Downregulation of NLRP12 enhances the proliferation and migration ability of HCT116 cells

To investigate the biological roles of NLRP12 in CRC, at first, Western blotting and QRT-PCR were performed to measure the expressions of NLRP12 using CRC cell lines including CCL224, HCT116, SW480 and RKO. Both protein and mRNA expression of NLRP12 were highest in HCT116 cells (**Figure 2A** and **2B**). However, other CRC cell lines showed relatively lower NLRP12 expression. Then, to reduce the NLRP12 levels in CRC cells, siRNA targeted NLRP12 was transfected into HCT116 cells and Control-siRNA was used as a negative control. The protein of NLRP12 in NLRP12-siRNA HCT116 cells was tested by western blotting (**Figure 2C** and **2D**). MTT assay was performed to detect the effect of NLRP12 on CRC cell proliferation. Our results showed that cell growth of HCT116 cells was significantly improved when NLRP12 was downregulated by the siRNA (**Figure 3A**). Transwell assay was performed to evaluate the influence of NLRP12 on CRC cells' migration. We found that NLRP12-siRNA HCT116 cells migrated faster than Control-siRNA HCT116 cells. And there was no change for control-siRNA compared to HCT-116 cells. These results indicated downregulation of NLRP12 promotes the proliferation and migration in HCT116 cells (**Figure 3B** and **3C**).

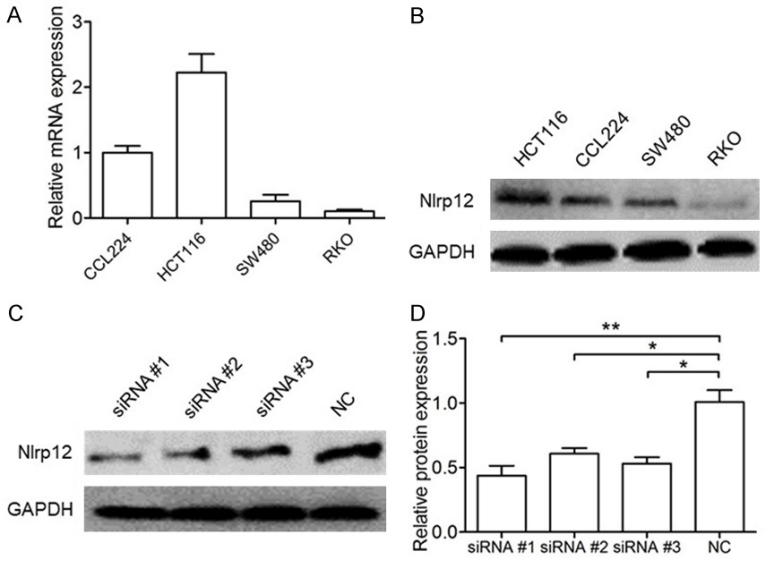

Figure 2. Knockdown efficiency of NLRP12 in HCT116 cells. (A) The mRNA expression of NLRP12 in CRC cell lines by QRT-PCR. (B) The protein expression of NLRP12 in CRC cell lines by Western blotting. The protein levels of NLRP12 in NLRP12-siRNA and Control-siRNA of HCT116 cells were tested by Western blotting (C and D). Error bars represent SD (n = 3) (*P<0.05, **P<0.01).

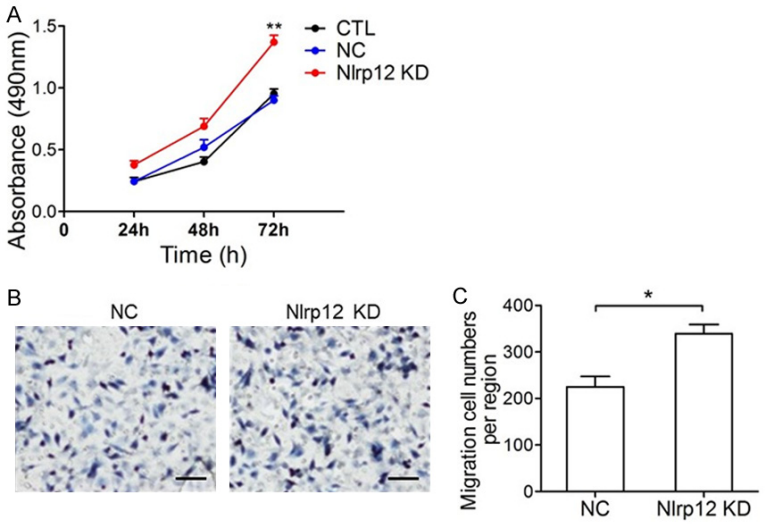

Figure 3. Knockdown of NLRP12 enhanced the proliferation and migration ability in HCT116 cells. A. MTT assays of HCT116 cells transfected with NLRP12-siRNA or Control-siRNA. Curves of HCT116 cells growth 24 h, 48 h and 72 h after transfection were presented (**P<0.01). B and C. HCT116 Cells migration transfected with NLRP12-siRNA or Control-siRNA was detected by Transwell assay and representative photographs were presented (Scale bar; 100 μm) (*P<0.05).

Downregulation of NLRP12 increases the drug-resistance of HCT116 cells to cisplatin

We further evaluated the effect of NLRP12 on the resistance of HCT116 cells to cisplatin by transfecting siRNA to HCT116 cells. HCT116 cells were transfected with NLRP12 siRNA and Control-siRNA. 48 h post-transfection different concentrations of cisplatin were added into the culture medium. MTT assays were used to detect the cell viability 24 h later. As shown in **Figure 4**, the downregulation of NLRP12 in HCT116 cells enhanced the drug-resistance to cisplatin. It has been confirmed that extracellular signal-regulated kinase (ERK) 1/2 and GLI1 signaling participated in the regulation of drug-resistance ability of cancer cells [15, 16], and it has also been reported that NLRP12 negatively regulate ERK signaling [12]. Therefore, this indicated that NLRP12 might mediate the proliferation and drug-resistance in CRC cells through ERK and GLI1.

NLRP12 negatively regulates the expression of ERK and GLI1 in HCT116 cells

It has been reported that abnormal activity of GLI1 could promote uncontrolled proliferation in human cancers [17]. So we checked whether NLRP12 influence GLI1 activity in CRC cells. And we found that the expression of GLI1 was significantly influenced by the altering expression of NLRP12 in HCT116 cells. As shown in **Figure 5**, downregulation of NLRP12 in HCT116 cells markedly increased the expression of GLI1 compared to control cells. Studies have shown NLRP12 deficient mice have enhanced the activity of phosphorylation-ERK (p-ERK) signaling pathway. Our study found that downregulated NLRP12 in HCT116 cells increased the expression of p-ERK (**Figure 5**). It is known that GLI1 activity is regulated by mitogen-activated

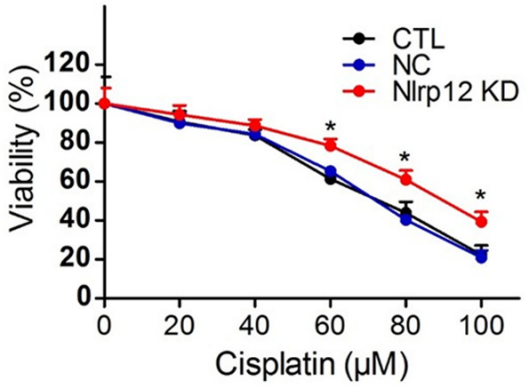

Figure 4. Knockdown NLRP12 increased the resistance to cisplatin in HCT116 cells. HCT116 cells were transfected with NLRP12 siRNA or Control-siRNA, different concentrations of cisplatin were added into culture mediums 48 h after transfection (*P<0.05).

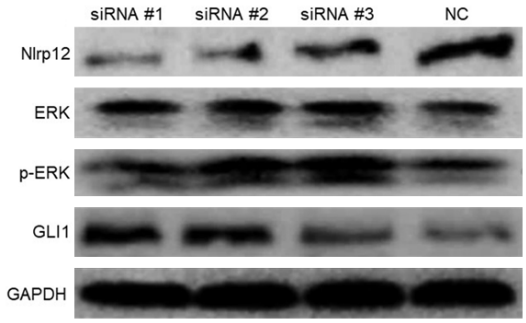

Figure 5. NLRP12 down regulated HCT116 cells by specific siRNA induced changes in some proteins that mediate proliferation of cells. Western blotting analysis of ERK, p-ERK and GLI1 in HCT116 cells transfected with NLRP12 siRNA and Control-siRNA. GAPDH was used as an equal loading.

protein kinases (MAPK) signaling in gastric cancer [18]. All these results indicated that NLRP12 might mediate the expression of GLI1 through the p-ERK signaling pathway.

NLRP12 regulates GLI1 activity via MEK/ERK/GLI1 signaling pathway

To further investigate how NLRP12 regulated the expression of GLI1, we used the canonical mitogen-activated extracellular kinases (MEK) inhibitor, U0126, to influence the MAPK signaling pathway. GLI1 has been confirmed to be regulated by MEK1 in gastric cancer cells [18]. MEK1/2 inhibitor U0126 was added into the culture medium. As shown in **Figure 6**, the MEK1/2 inhibitor U0126 suppressed the increased expression of GLI1 in cells transfected with siRNA NLRP12 along with the downregulation of p-ERK signaling pathway (**Figure 6**). These results suggested that p-ERK might be implicated in the mechanism of GLI1 activation by NLRP12.

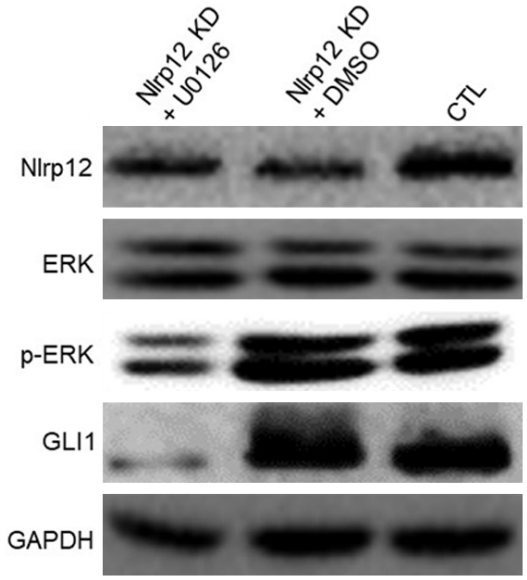

Figure 6. MEK inhibitor U0126 regulated the affection of NLRP12 on GLI1 in HCT116 cells. HCT116 cells were transfected with NLRP12 siRNA. The culture medium was added 10 μM U0126 or control reagent DMSO during the transfection for 48 h. Expressions of NLRP12, ERK, p-ERK and GLI1 were tested by Western blotting. GAPDH was used as an equal loading.

Discussion

Advanced colorectal cancer patients have a poor prognosis all over the world, especially in the Asian population. Chemotherapy for cancer patients is associated with numerous side effects and poor response due to the development of chemoresistance, which has resulted in a poor outcome in patient care. Hence, it's an urgency to find new biomarkers for early detection and identification of novel therapeutic approaches in order to decrease side effects and improve chemosensitivity of drugs.

Recently, NLR family of genes have been commonly characterized as the activators of inflammation [19]. For instance, NLRP3, NLRC4 and NLRP6 have been shown to suppress inflammation and tumorigenesis significantly in inflammasome-deficient mice model [20-22]. In contrast to inflammasome forming NLRs,

non-inflammasome forming NLRs have been reported to play a negative role in inflammation [23, 24]. Among them, NLRP12 was the first and the best characterized NLR protein. NLRP12 previously named as Monarch-1/PYPAF7, which was considered similar with NLRP6 in the structures and functions [25, 26]. Previously, overexpression of NLRP12 by transient transfection of NLRP12 was shown to induce the expression of NF-κB signal pathway [27], which suggests NLRP12 as a pro-inflammation NLR. It also had a positive effect on NF-κB signaling pathway. This *in vitro* result was compared with human data where mutations of NLRP12 were linked to hereditary periodic fever syndromes and increased secretion of IL-1β [28]. Thus, NLRP12 was previously considered as a pro-inflammatory protein despite contradictory recent reports. In other words, NRLP12 was regarded as a negative regulator of inflammation through canonical and noncanonical NF-κB signal pathway [12, 13, 29, 30]. Irving reported that in NLRP12-/-mice NLRP12 suppresses the inflammation and tumorigenesis of the colon by negatively regulating the noncanonical NF-κB signal pathway [13]. Zaki found that NLRP12 deficient mice were more susceptible to inflammation and tumorigenesis due to failure to dampen NF-κB and ERK activation in macrophages [12]. Thus, NLRP12 might play an important role during the progression of CRC.

In the present study, we found that the proteins of NLRP12 were significantly decreased in CRC tissues compared to surrounding normal tissues. Western blotting and IHC staining both showed NLRP12 was negatively expressed in CRC tissues and positively expressed in paratumor tissues. In addition, the degree of the NLRP12 expression was related to the size and the stages of the CRC patients. Since CRC develops from polyp and adenoma, we also compared the NLRP12 expression in surrounding normal tissues, polyps, adenoma and CRC tissues. Interestingly, we found that the expressions of NLRP12 were gradually decreased in surrounding normal tissues, polyps, adenoma, and tumor tissues. However, there was the limited number of samples of polyps and adenoma. Hence, further work is needed to strengthen this result. All these results suggested that NLRP12 might be a potential therapeutic target in colorectal cancer.

Inflammation can act either way in disease. Modest inflammation can fight against infections and injury while aberrant inflammation increases the risk of the development of cancer [31]. It's a known fact that chronic inflammation leads to CRC. As a member of non-inflammasome forming NLRs, a variety of studies focused on NLRP12 showed NLRP12 suppresses inflammation and tumorigenesis by negative regulation of NF-κB and ERK signal pathway [12, 13]. The mechanism how NLRP12 suppresses the inflammation and tumorigenesis is still unclear. However, one study attributed it to the activated canonical NF-κB signal pathway and increased ERK signaling. The other study held the view that the enhanced inflammation and tumorigenesis in NLRP12 deficient mice resulted from the increased activation of noncanonical NF-κB signal pathway [12]. In spite of the present discrepancies between these two studies, our current results from CRC patients' specimens revealed the lower expression of NLRP12 in CRC tissues compared to surrounding normal tissues. Thus, it could provide us with some physiologic and clinical evidence to verify that NLRP12 as a tumor suppressor.

We also investigated the influence of NLRP12-siRNA on CRC cells. Interestingly, with the downregulation of NLRP12 in HCT116 cells, proliferation of the cells was significantly increased. In addition, migration of CRC cells was reduced compared to NC cells. Meanwhile, the drug-resistance to Cisplatin was also increased along with the downregulation of NLRP12. These *in vitro* data showed NLRP12 plays an important role in the tumorigenesis of CRC.

In order to understand the correlation and mechanism of NLRP12 and tumorigenesis, activities of signaling pathway as ERK1/2 and GLI1 were detected by western blotting. In fact, an activity of ERK signaling has beenfound to be enhanced in NLRP12 deficient mice and macrophage cells [12]. Several reports have shown that aberrant ERK1/2 activation appeared in different human cancers and played a vital role in the regulation of cell proliferation and metastasis of tumors [32, 33]. Hedgehog (HH) signaling has been considered to play critical roles, not only in differentiation and development but also in stem cell maintenance, cellular proliferation and tissue homeo-

stasis [34]. Among them, GLI1 was aberrantly activated in several cancers and the effect of targeted GLI1 therapy was very significant [35, 36]. GLI1 regulated cell proliferation and apoptosis in colon cancers cells. 48 h exposure to GLI1 inhibitors GANT61 resulted in cell death and DNA damage [37]. In addition, GLI1 could induce chemoresistance of cancer cells through multiple mechanisms. In high GLI1-expressed glioma cancer stem-like cells, there has been increased resistance to temozolomide and carboplatin whereas inhibiting this GLI1 signaling pathway has increased CD133 cells' sensitivity to temozolomide [38, 39]. In our study, we found NLRP12 knockdown cells have enhanced p-ERK and GLI1 activities, which explains that NLRP12 could attenuate colorectal tumorigenesis and the sensitivity of CRC cells to cisplatin. Montoko found that there was an important crosstalk between HH and RAS/MAPK signaling pathway in gastric cancer [18]. We also used the ERK inhibitor, U0126 to explore whether NLRP12 mediates GLI1 through the ERK-GLI1 axis. Interestingly, while we inhibited the activation of p-ERK, the inhibition of downregulated NLRP12 to GLI1 was significantly weakened. Collectively, these indicated that p-ERK might be the medium to join NLRP12 and GLI1.

There are still some limitations in our study. Firstly, the samples were not enough specifically for the polyps and adenoid tumor specimens. Therefore, the rules we summarized might be restricted and limited, and more experiments with adequate samples are needed to verify the conclusion. Secondly, we didn't conduct a tumor formation model to observe the influence of NLRP12 on CRC cells *in vivo*. Finally, our research was limited to phenotypic differences of CRC cells. There are varieties of MEK inhibitors via different mechanisms and we just used the MEK inhibitor U0126. Furthermore, inhibiting GLI1 might influence on NLRP12 in reverse. Thus, more research on NLRP12 should be undertaken to elucidate the relationship between NLRP12 and GLI1.

In summary, we reported that the downregulation of NLRP12 in CRC and the correlation between its expressions and the clinical significances. In addition, we detected that NLRP12 could suppress the tumorigenesis of CRC and inhibit the chemoresistance of CRC cells. The specific mechanism might be attributed to MEK/ERK/GLI1 signaling pathways. We believe this was the first study done on NLRP12 as a suppressive agent in CRC. Despite many types of researches being conducted on NLRP12 in nude mice and macrophages, NLRP12 was rarely studied in human cancers. The NLRP12 mediated suppression of ERK and GLI1 induced the inhibition effects on the progression in CRC. This finding might give us a new insight into the pathogenesis of CRC and provide us a novel therapeutic strategy in the treatment of CRC.

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Disclosure of conflict of interest

None.

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