European Review for Medical and Pharmacological Sciences 2018; 22: 8273-8280

MRFAP1 plays a protective role in neddylation inhibitor MLN4924-mediated gastric cancer cell death

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Abstract. – OBJECTIVE: MLN4924 is a second-generation small molecule inhibitor with anti-cancer activity that inhibits neddylation activation enzyme (NAE), subsequently blocking the neddylation-dependent activation of Cullin-RING E3 ligases (CRLs). Mof4 family associated protein 1 (MRFAP1) is a highly conserved, short half-life protein and one of the most up-regulated proteins in response to MLN4924 treatment. MRFAP1 has been identified as a novel cell cycle-related protein and a regulatory component monitoring and preventing genomic instability. However, whether MRFAP1 plays a role in MLN4924-mediated cancer cell death remains elusive.

PATIENTS AND METHODS: The expression of MRFAP1 in gastric cancer clinic samples was detected by Real-time PCR and Western blot. CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system was used to knockout MRFAP1 gene in both AGS and SGC-7901 cells. The proliferation of GC cells was measured by CCK8 assay. The cell cycle distribution of GC cells was determined by fluorescence-activated cell sorting (FACS) assay. Co-immunoprecipitation assay was used to determine the interaction between MRFAP1 and P27.

RESULTS: MRFAP1 was downregulated in clinic gastric cancer samples at post-translational level. Overexpression of MRFAP1 decreased gastric cancer cells proliferation. CRISPR-mediated knockout of MRFAP1 increased the cytotoxicity of MLN4924 by augmenting MLN4924-induced G2/M arrest and apoptosis against gastric cancer cells. At the molecular level, we found that MLN4924 induced the interaction between P27 and MRFAP1, the latter associated with P27, which was further stabilized in response to MLN4924 treatment.

CONCLUSIONS: We showed a protective role of MRFAP1 in gastric cancer cells with MLN4924 treatment and suggested the potential possibility to combine MLN4924 with MRFAP1 inhibition to treat gastric cancer.

Key Words:
MRFAP1, MLN4924, Cullin-RING E3 ligases, Ubiquitination.

Introduction

Gastric cancer (GC) is the fifth most common cancer and the second leading cause of cancer-related death worldwide, particularly in East Asia[1]. Despite the recent progresses of novel diagnostic and therapeutic interventions, the prognosis of patients with advanced GC remains poor[2]. GC is heterogeneous, which resulted from a combination of environmental factors and accumulation of specific genetic alterations[3]. An understanding of these alterations is essential for the improvements in the diagnosis and treatment of GC. Neddylation is an energy consuming process, which requires the NEDD8-activating enzyme (NAE) to transfer NEDD8 protein onto Ubc12, which then transfers NEDD8 onto the lysine of one of the seven cullins[4]. Cullins are subunits within the cullin-RING ligase (CRL) family of ubiquitin E3 ligases. Neddylation of the cullin is required to form an active CRL. Next, the active CRL was able to allow the associated ubiquitin E2 enzyme to polyubiquitinate and target its substrates to the proteasome for degradation[5]. MLN4924 is an investigational small molecule inhibitor of NAE that is currently under Phase I clinical trials[6,7]. The dynamics of the cullin interactome and substrates by MLN4924 treatment have recently been extensively studied by several quantitative proteomic analyses[8,9]. MLN4924 treatment leaded to the stabilization of a small subset of proteasome-degraded proteins, which were regulated by CRL[10]. Interestingly, some of the CRLs target

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proteins are known to play critical roles in cancer development. For example, MLN4924 administration caused the stabilization of Cdt1, leading to DNA rereplication and accumulation of cells in S phase[11,12]. Rereplication leads to the activation of ATR and ATM-dependent DNA damage repair processes and deregulation which might finally result in cancer cell death[13]. However, accumulation evidence suggested several MLN4924 substrates could also affect the sensitivity of GC cells including Nrf2, p21 and p27[14-17]. The identification of novel MLN4924 substrates, which affected the cytotoxic of MLN4924, could provide critical insights into the mechanism of MLN4924-mediated GC cell death, and identify possible predictive biomarkers for the clinical utility of MLN4924. The MRFAP1 is highly conserved and only expressed in mammals with short half-lives[18]. MRFAP1 has been shown to be expressed in spermatogonia to maintain normal levels of histone modification by negatively regulating recruitment of the NuA4 complex to chromatin[18]. MRFAP1 is rapidly stabilized in MLN4924-treated cells[18,19]. However, the biological role of MRFAP1 remains largely unknown and it is still unclear whether MRFAP1 plays a role in MLN4924-mediated GC cell death.

Patients and Methods

Patients

A total of 20 patient-derived samples were obtained according to the guidelines approved by the Institutional Review Boards of our hospital. Procedures were executed according to the approved guidelines. None of these patients received any chemotherapy, radiotherapy, or other related antitumor therapies before surgery. At least two certified pathologists confirmed all diagnoses.

Cell Culture and Treatments

The human GC cell lines AGS and SGC-7901 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a 5% CO_2/95% air at 37°C. MLN4924 (HY-10484) was purchased from MedChemExpress (Monmouth, NJ, USA).

CRISPR/Cas9 KO

MRFAP1 CRISPR/Cas9 KO Plasmid (h): sc-410630 and MRFAP1 HDR Plasmid (h): sc-410630-HDR were purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). AGS and SGC-7901 cells were co-transected with both plasmids and selected by puromycin for about 2 weeks. The single clones were chosen and the knockout efficiency was verified by Western blot.

Western Blots

Cells were lysed by 2 × SDS loading buffer and the total protein was separated by 10-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% non-fat milk for 1 hour and incubated with the primary antibodies as indicated overnight at 4°C. After washed with Phosphate-buffered saline and Tween 20 (PBST) for 5 min three times, the membranes were incubated with secondary antibody for 1 hour at room temperature. The following primary antibodies were used: MRFAP1 antibody (Proteintech, Rosemont, IL, USA), Flag-M2 antibody (F1804, Sigma-Aldrich, St. Louis, MO, USA), NDRG2 antibody (Abcam, Cambridge, MA, USA), and GAPDH (Cell Signaling Technology, Danvers, MA, USA).

Immunoprecipitation (IP)

Cells were lysed in RIPA buffer for 45 min at 4°C. Lysates were subjected to IP with 20 µL FLAG M2 affinity resin (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. Resin-containing immune complexes were washed 8 times with RIPA buffer washes and eluted with 2 × SDS loading buffer by boiling at 95°C for 5 min.

CCK8 Assay

Cells were seeded at 1000 cells per well into 96-well plates for the CCK8 assay (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance at 450 nm was measured by using a microplate reader (SpectraMax Paradigm, Molecular Devices LLC, Sunnyvale, CA, USA).

FACS Assay

Cells were fixed by ice-cold 70% ethanol for 1 hour and then incubated with propidium iodide (PI) solution (50 µg/ml) in the presence of 0.2 mg/ml RNase A for 15 minutes, at 37°C. DNA content was measured on flow cytometry (Beckman Coulter, Brea, CA, USA).

Statistical Analysis

The GraphPad Prism6 software (La Jolla, CA, USA) was used for statistical analysis. Values were shown as mean ± SEM. The unpaired two-tailed T test was used for the comparison of parameters between two groups. Statistical significance is displayed as * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

Results

MRFAP1 was Downregulated in GC Samples at Protein Level

To investigate the potential role of MRFAP1 in GC, we firstly determined the mRNA expression levels of MRFAP1 in 20-paired GC tissues and the corresponding non-cancerous normal mucosa tissues. The Q-PCR data revealed that there were no significant changes of MRFAP1 mRNA in gastric cancer samples when compared to the non-cancerous normal mucosa tissues (Figure 1A). As MRFAP1 has been shown to be regulated mainly at post-translational level, we tested the protein levels of MRFAP1 in gastric cancer samples. The protein level of MRFAP1 in 12 paired GC and corresponding non-cancerous normal mucosa tissues has been determined by Western blotting assay. A significant decrease in MRFAP1 expression was found in GC samples compared with their matched adjacent normal ones (Figure 1B). These data suggested that MRFAP1 is significantly down-regulated only at protein level in GC.

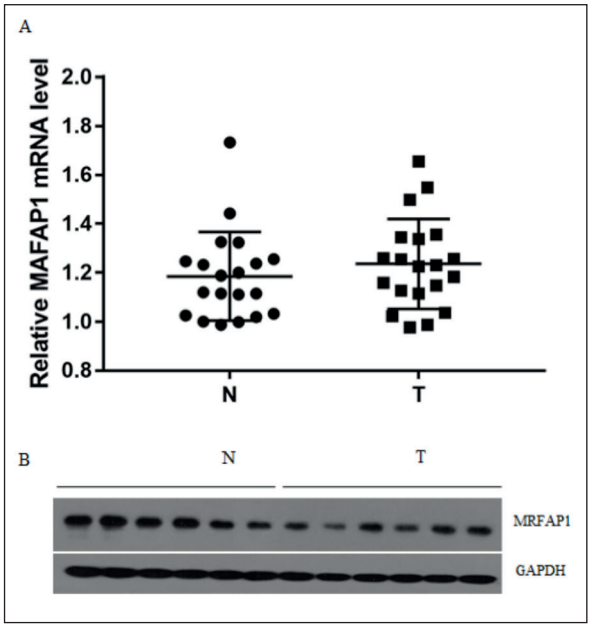

Figure 1. MRFAP1 was downregulated in GC samples at protein level. ***A.*** The mRNA levels of MRFAP1 in 20 paired GC tissues and the corresponding non-cancerous normal mucosa tissues were calculated by qRT- PCR. (N: normal mucosa tissues; T: tumor tissues). ***B.*** The protein levels of MRFAP1 in 10 paired GC tissues and the corresponding non-cancerous normal mucosa tissues were determined by Western blot.

MLN4924 Inhibited FBXW8-Mediated MRFAP1 Degradation in GC Cells

MRFAP1 has been found to be a cell cycle-regulated protein and targeted for ubiquitin-mediated degradation by CUL7/FBXW8 complex in HeLa cells[20]. Indeed, MRFAP1 was also significantly decreased in AGS cells with FBXW8 overexpression (Figure 2A). However, FBXW8-mediated MRFAP1 degradation in AGS cells could be efficiently reversed with MLN4924 administration (Figure 2A), without affecting its mRNA levels (Figure 2B), suggesting MLN4924 inhibited FBXW8-mediated MRFAP1 degradation in GC cells.

Overexpression of MRFAP1 Decreased AGS and SGC-7901 Cells Proliferation

To uncover the function of MRFAP1 in the progression of GC cells, MRFAP1 was overexpressed in both AGS and SGC-7901 cells, followed by CCK8 assay to measure the proliferation of these cells. Ectopic expression of MRFAP1 led to decreased growth rate of these two GC cells (Figure 3A-B). Furthermore, AGS and SGC-7901 cells transfected with MRFAP1 were subjected to cell cycle analysis by flow cytometry and we found that overexpressing MRFAP1 caused G1 arrested in both cells (Figure 3C-D). Together, these data suggested that MRFAP1 has a potential anti-proliferation role in both AGS and SGC-7901 cells.

CRISPR-Mediated Knockout of MRFAP1 Increased the Cytotoxicity of MLN4924 Against Gastric Cancer Cells

MRFAP1 is one of the most rapid stabilized proteins in response to MLN4924 administration, but the precise biological function of this event is still obscured[18]. To elucidate the functions of MRFAP1 in MLN4924-induced inhibition of proliferation of GC cells, we generated MRFAP1 KO AGS and SGC-7901 cells by CRISPR assay (Figure 4A-B). However, KO of endogenous MRFAP1 only has minor affection on the proliferation of both AGS and SGC-7901 cells, which could be ex-

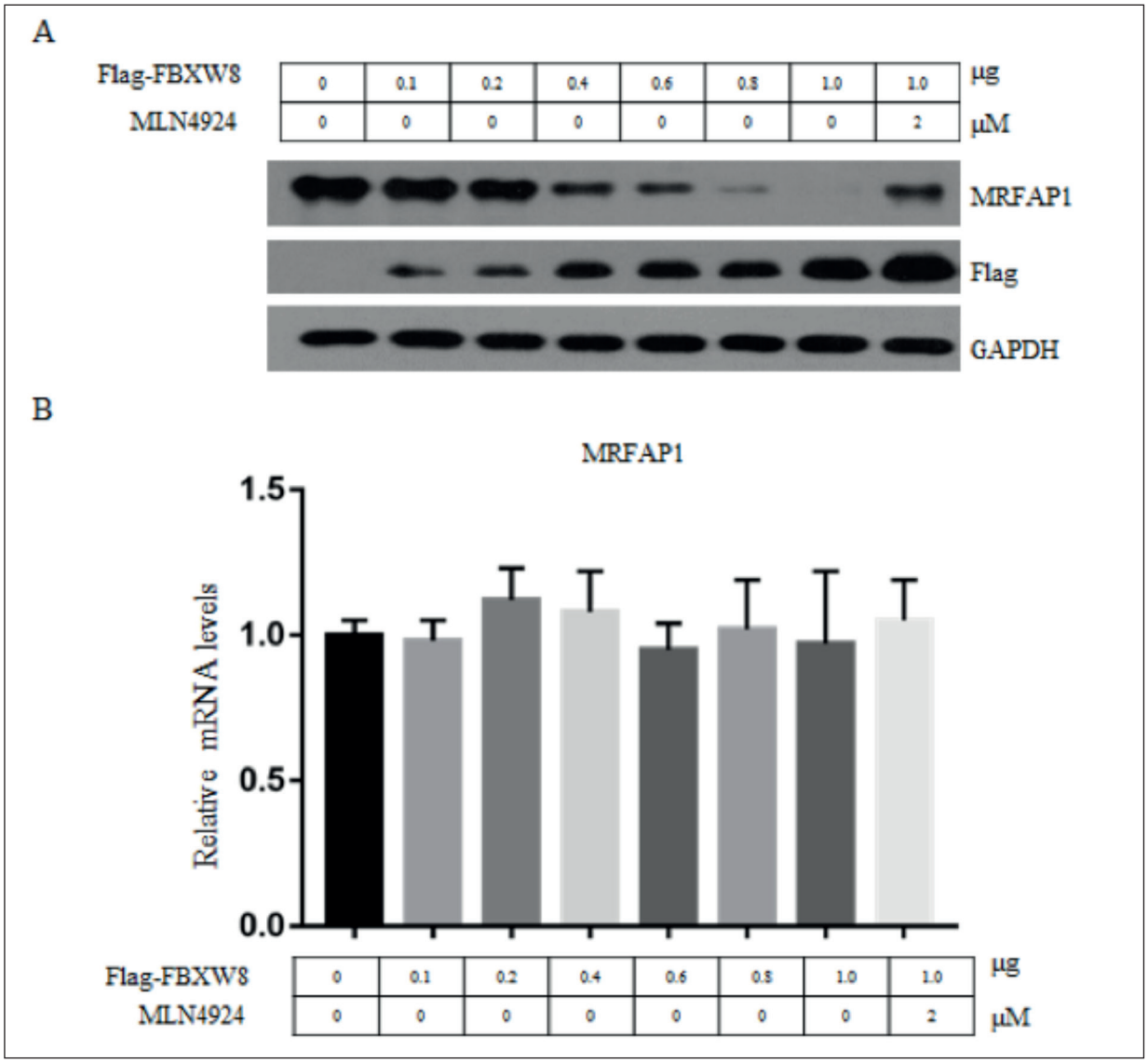

Figure 2. MLN4924 inhibited FBXW8-mediated MRFAP1 degradation in GC cells. A. AGS cells were transfected with increased dose of Flag-FBXW8 for 30 hours, 2 μM MLN4924 were added where indicated 4 hours before harvested. Western blot assay was performed with indicated antibodies. B. AGS cells were transfected with increased dose of Flag-FBXW8 for 30 hours, 2 μM MLN4924 were added where indicated 4 hours before harvested. The mRNA levels of MRFAP1 were measured by q-PCR assay.

plained by the relatively low endogenous protein level of MRFAP1 protein in GC cells (Figure 4C-D). The cell cycle distribution was also identical in these cells (data not shown). However, MRFAP1 KO AGS cells showed enhanced cytotoxicity in response to MLN4924 treatment. As detected by the CCK8 assay, the cell viability of the MRFAP1 KO cells was significantly lower than wild-type cells treated by MLN4924 (Figure 4C). Similarly, this phenomenon was also observed in SGC-7901 cells (Figure 4D). Thus, these data suggested that MRFAP1 plays a critical role in the significant enhancement of MLN4924 cytotoxicity against GC cells.

Knockout of MRFAP1 Increased MLN4924-Induced G2/M Arrest and Apoptosis Against Gastric Cancer Cells

Given the important role of MRFAP1 in the regulation of mitosis transition in human cells[20], we performed further cell cycle analysis. As shown in Figure 5A-B, G2/M arrest was increased when

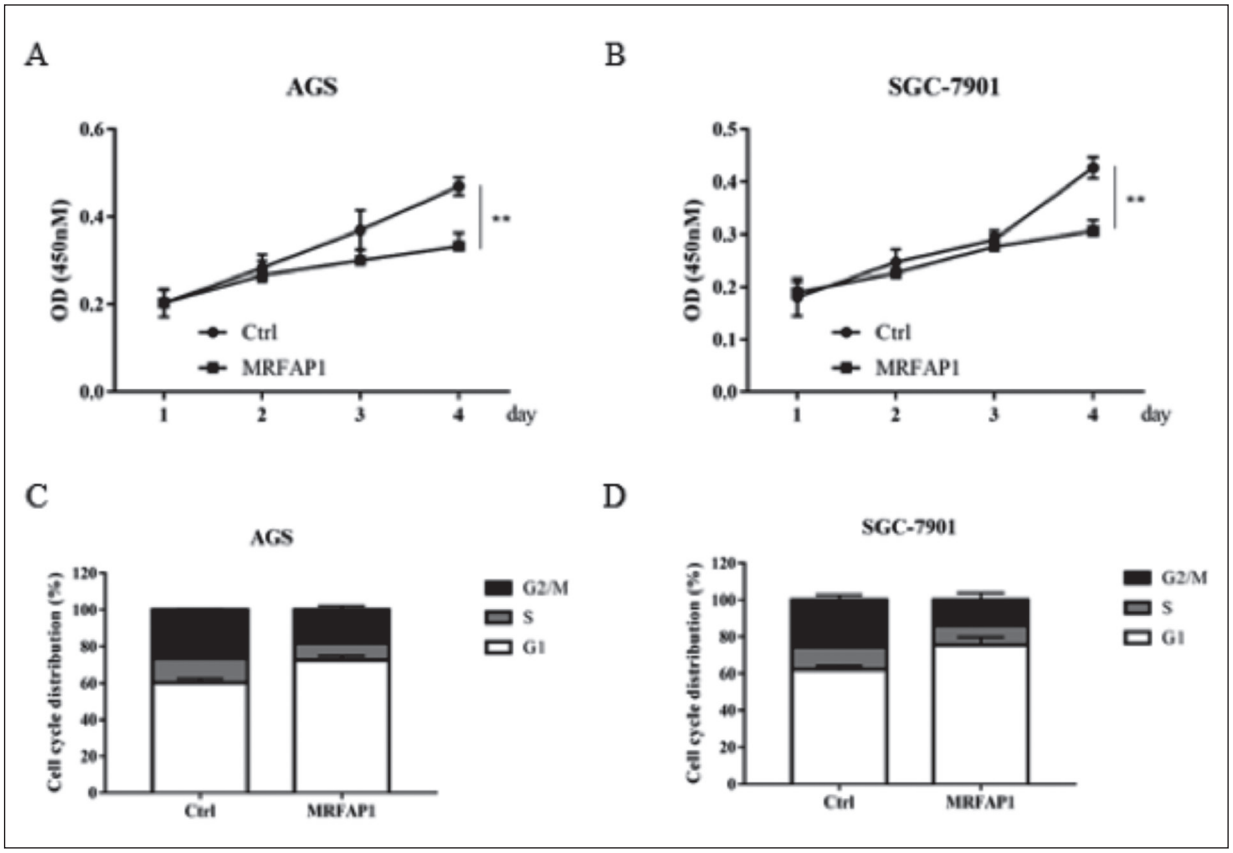

Figure 3. Overexpression of MRFAP1 decreased AGS and SGC-7901cells proliferation. ***A-B.*** Effect of overexpression of MRFAP1 in AGS and SGC7901 on cell proliferation. The number of cells was measured by means of CCK8 at the indicated time point. ***C-D.*** Effect of overexpression of MRFAP1 in AGS and SGC7901 on cell cycle distribution. Cells were transfected with MRFAP1 for 36 hours and analyzed by FACS. All experiments were replicated by at least three times. Statistical comparisons were performed using Student's *t*-tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

MRFAP1 was knockout in both AGS and SGC-7901 cells following MLN4924 treatment. Moreover, the activation of cleaved caspase-3 was also increased in MRFAP1 KO AGS cells treated with MLN4924 when compared with control cells, suggesting the decreased cell viability was owing to increased apoptosis (Figure 5C). Taken together, our data demonstrated that MRFAP1 protected GC cells from MLN4924-mediated growth inhibition, and loss of MRFAP1 could result in the enhancement of the MLN4924 cytotoxicity.

MRFAP1 Inhibition Prevented MLN4924-Mediated Induction of p27 in Gastric Cancer Cells

It has been well documented that MLN4924 stabilizes many cell cycle-related proteins, including P27[10,21]. Indeed, P27 has been reported to play a protective role in MLN4924-treated GC cells[21]. We then tested whether MRFAP1 inhibition affected MLN4924-induced P27 accumulation. To this end, we treated AGS WT and MRFAP1 KO cells with MLN4924 and detected the expression of P27 by Western blot. We found that in WT cells, P27 was significant increased after MLN4924 administration (Figure 5C). However, in MRFAP1 KO cells, the protein lever of P27 was almost unchanged in response to MLN4924 treatment. Interestingly, the basic levels of P27 were slightly decreased in AGS cells in the absent of MRFAP1 (Figure 5C). Moreover, although both MLN4924 and MG132 caused MRFAP1 and p21 accumulation, we found that MRFAP1 did not interact with P27 unless in the present of MLN4924, but not MG132 (Figure 5D), suggesting MLN4924 specifically bridged the interaction between MRFAP1 and P27. Taken together, these data suggested that in response to MLN4924, MRFAP1 interacted with P27 to further augment the accumulation of P27.

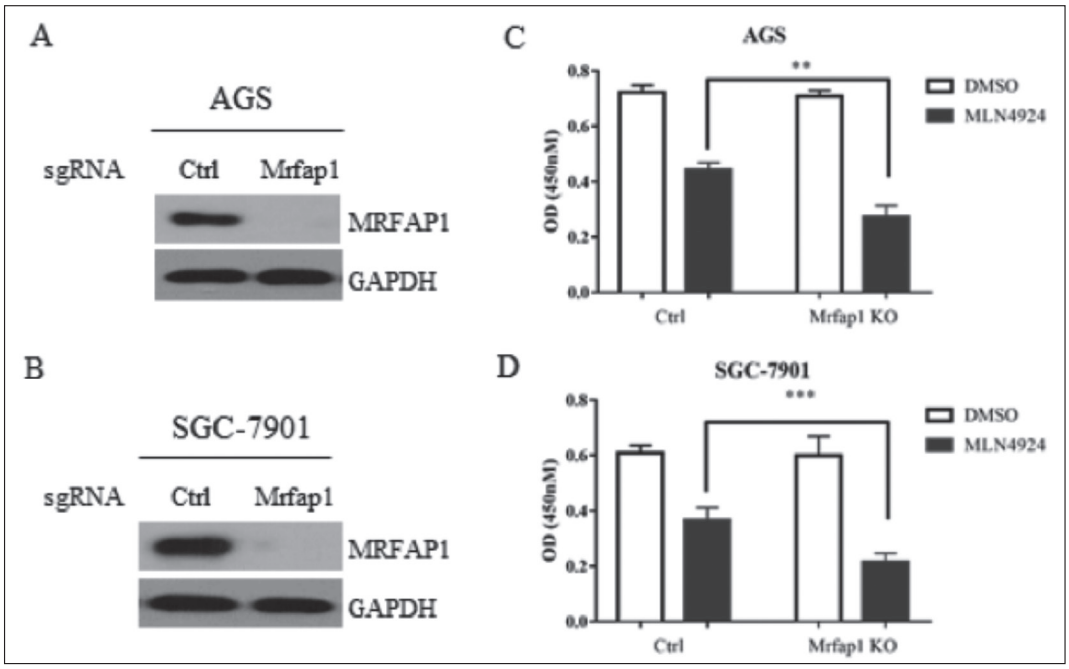

Figure 4. CRISPR-mediated knockout of MRFAP1 increased the cytotoxicity of MLN4924 against gastric cancer cells. ***A.*** MRFAP1 KO AGS cells were generated by CRISPR assay and detected by Western blot. ***B.*** MRFAP1 KO GC-7901 cells were generated by CRISPR assay and detected by Western blot. ***C.*** MRFAP1 KO AGS cells were treated with or without 2 μM MLN4924 for 24 hours, the cell proliferation was measured. ***D.*** MRFAP1 KO SGC7901 cells were treated with or without 2 μM MLN4924 for 24 hours, the cell proliferation was measured.

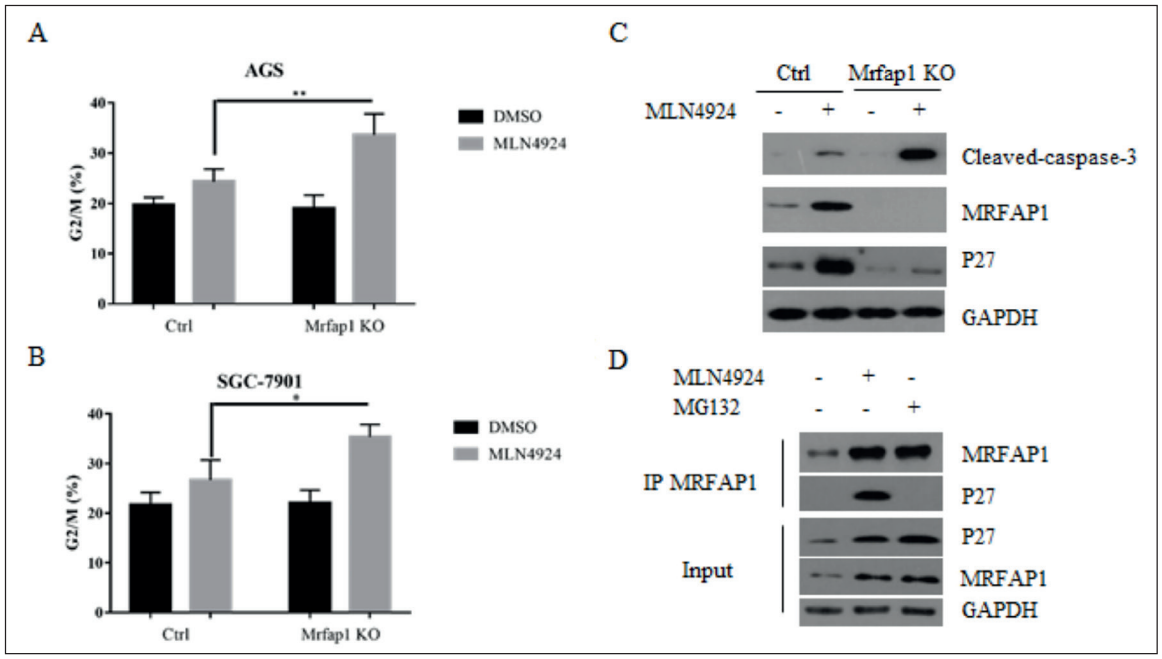

Figure 5. Knockout of MRFAP1 increased MLN4924-induced G2/M arrest and apoptosis against gastric cancer cells by preventing MLN4924-mediated induction of p27. ***A.*** MRFAP1 KO AGS cells were treated with or without 2 μM MLN4924 for 24 hours, cell cycle was analyzed by FACS. ***B.*** MRFAP1 KO SGC7901 cells were treated with or without 2 μM MLN4924 for 24 hours, cell cycle was analyzed by FACS. ***C.*** MRFAP1 KO AGS cells were treated with or without 2 μM MLN4924 for 24 hours, cells were harvested and subjected to Western blot with indicated antibodies. ***D.*** AGS cells were transfected with Flag-MRFAP1 for 30 hours and treated with or with 2 μM MLN4924 for additional 4 hours, cells were harvested and subjected to immunoprecipitation with Flag M2 beads and then detected with Western blot.

Discussion

MRFAP1 has been reported to be a cell cycle-related protein and has a critical role in mitosis transition. It is degraded by the Cul7/FBXW8 mediated ubiquitin-proteasome system and rapidly stabilized in response to MLN4924 treatment[20]. However, the functions of MRFAP1 as a critical MLN4924 substrate remain unclear. In this study, we uncover the novel functions of MRFAP1, which suggested that inhibition of MRFAP1 exerted a potential promising treatment when combined with MLN4924 against gastric cancer. The MRFAP1 is highly conserved and only expressed in mammals with short half-lives. Indeed, although we did not observe the significant mRNA level changes of MRFAP1, we do find the remarkable decline in its protein levels especially in clinic GC samples. The post-translational regulated of MRFAP1 was mainly governed by CUL7/FBXW8 and reversed by MLN4924 treatment. MLN4924 has been reported to suppress the growth and induce apoptosis of GC cells, driving us to consider whether MRFAP1 has a role in this process. By using CRISPR-mediated gene knockout assay, we found GC cells with MRFAP1 KO rendered great sensitivity to MLN4924, including decreased vitality, increased mitotic arrest and apoptosis. At the molecular level, we found MLN4924 could induce the interaction between P27 and MRFAP1, the latter might associate with P27 and further stabilize it in response to MLN4924 treatment. P27 has been considered as a tumor suppressor by binding with the three different CDK-Cyclin complexes, including CDK1-CyclinB1, CDK2-CyclinE and CDK4-CyclinD, impeding their kinase activities[22]. P27 suppresses the G1/S transition and prevents the cell cycle from entering subsequent phases[23]. It has been reported[21] that P27 played a protective role of in MLN4924-treated GC cells by suppressing ROS generation, modulating the profile of BCL-2 family members and maintaining the normal mitochondrial status. It will be interesting to test the ROS level as well as mitochondrial status in MRFAP1 KO GC cells treated with MLN4924 in the future.

Conclusions

We showed that MRFAP1 functions as a protective protein by interacting with and stabilizing P27 in GC cells treated with MLN4924. Importantly, the enhanced sensitization of MLN4924 by MRFAP1 inhibition in GC cells demonstrates that the potential possibility to combine MLN4924 with MRFAP1 inhibition to achieve better cancer treatment efficacy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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