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Original Research

The Deubiquitinating Enzyme USP4 Functions as an Oncoprotein in Gastric Cancer and Mediates NF-κB Signaling by Regulating PRL-3 Expression

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Abstract

Background: It has been reported that ubiquitin specific peptidase 4 (USP4) was functional in several tumors, but its function and mechanism in gastric cancer were still unknown. **Methods**: Bioinformatic tools were used to predict the prognosis of gastric cancer patients and the expression levels of USP4 in gastric cancer. Quantitative real-time polymerase chain reaction (qRT-PCR) and immunoblotting were carried out to detect the messenger RNA (mRNA) and protein levels. Cell viability of gastric cancer was evaluated by Cell Counting Kit-8 (CCK-8) assay. Cell line-derived xenograft models were established to evaluate the tumor growth of gastric cancer. Luciferase assay and immunoblotting were used to determine the activation of nuclear factor kappa B (NF-κB) signaling. **Results**: The public database Kaplan-Meier Plotter showed that gastric cancer patients with high USP4 expression had a shorter overall survival or post-progression survival than the patients with decreased USP4. Further studies indicated that USP4 was elevated in gastric cancer tumor tissues. In contrast, knockdown of USP4 markedly inhibited gastric cancer cell growth, and suppressed the tumor growth of gastric cancer. Further studies revealed that USP4 knockdown significantly suppressed NF-κB-driven luciferase activity, and inhibited the phosphorylation of NF-κB p65 in gastric cancer cells. Additionally, qRT-PCR analysis showed that USP4 knockdown significantly downregulated the expressions of cyclin D2 (CCND2) and B cell leukemia/lymphoma 2 (BCL2). We also found that USP4 knockdown decreased the expressions of phosphatase of regenerating liver-3 (PRL-3), in contrast, overexpression of PRL-3 attenuated the inhibitory effects of USP4 knockdown on NF-κB signaling and cell viability in gastric cancer cells. Finally, PR-619, which has been proven to inhibit the activities of USP4 and other deubiquitinases, could inhibit cell viability and NF-κB signaling in gastric cancer cells. **Conclusions**: This study indicated that elevated USP4 predicted a poor index for gastric cancer patients, and mediated gastric cancer cell growth by regulating PRL-3/NF-κB signaling, which suggested USP4 may be a novel therapeutic target for gastric cancer.

Keywords: USP4; gastric cancer; PRL-3; NF-κB; PR-619

1. Introduction

Gastric cancer is a common malignant tumor of digestive tract, which was reported to be the second leading cause of tumor-related mortality worldwide, especially in China [1,2]. In 2018, there were 1,033,701 new cases and 782,685 deaths of gastric cancer worldwide [3,4]. Gastric cancer has been proven to be a complex disease, which was reported to be related to many factors, including environmental exposure and genetic factors [1]. Despite the continuous development of medical strategies, the treatment of gastric cancer is still mainly limited to surgery, radiotherapy and chemotherapy [5]. Therefore, it is particularly important to find novel targets for the diagnosis and treatment of gastric cancer.

The ubiquitin-proteasome system (UPS) is an important regulator in cells, and dysregulation of UPS is closely related to the occurrence and development of diseases, including tumors [6]. The UPS is composed of at least 6 components, including ubiquitin, ubiquitin-activating enzyme, ubiquitin-conjugating enzyme, ubiquitin ligase, deubiquitinating enzyme (DUB) and 26S proteasome [7]. Among these, DUBs are widely expressed in cells, which are closely related to the modulation of tumorigenesis by removing ubiquitin chains from macromolecules involved in cancer regulation [8]. Thus, targeting DUBs has been considered as a potential strategy for anti-tumor drug discovery.

Nuclear factor kappa B (NF-κB) signaling can mediate the production of many regulators, such as growth factors, chemokines, cytokines, anti-apoptotic genes, etc., most of which are involved in the tumorigenesis of gastric cancer [9]. For example, cancer-associated fibroblasts of gastric cancer were reported to induce chemotherapy resistance and inhibitory immune cell infiltration by mediating NF-κB activation to secrete IL6, IL8 and other inflammatory factors [10]. ERCC6L was also reported to promote the cell growth and metastasis of gastric cancer by activating NF-κB signaling [11]. Targeting NF-κB signaling has been a promising strategy for anti-cancer drug discovery.

It has been also reported that phosphatase of regenerating liver-3 (PRL-3) was an upstream molecule of NF-κB signaling, and promoted the cell migation and invasion of gastric cancer [12]. In addition, in colorectal cancer, PRL-3 was proven to be a substrate of ubiquitin specific peptidase 4 (USP4), and USP4 could deubiquitinate and stabilize PRL-3 protein [13]. USP4 is a deubiquitinating enzyme, whose function was still unclear in gastric cancer, but it has been proven to play the oncogenic role in other types of cancer, including brain cancer [14], breast cancer [15], lung cancer [16] and melanoma cancer [17]. In the present study, USP4 was predicted as a negative indicator for gastric cancer patients, and knockdown of USP4 could significantly inhibit cell growth and suppress tumor growth of gastric cancer. Our further studies showed that knockdown of USP4 inhibited PRL-3/NF-κB signaling in gastric cancer cells. These results indicated that USP4 could be as a novel target for the treatment or diagnosis of gastric cancer.

2. Materials and Methods

2.1 Informatic Analysis

To predict the overall survival (OS) and post-progression survival (PPS) for gastric cancer patients with low or high USP4 (Affymetrix ID: 202681_at, 211800_s_at), the online database Kaplan-Meier Plotter was used (http://kmplot.com/) [18]. The expressions of USP4 in STAD based on individual cancer stages, tumor grade, H.pylori infection status or nodal metastasis status were analyzed by UALCAN online tool (http://ualcan.path.uab.edu/) [19,20]. The correlation analyses between USP4 and cyclin D2 (CCND2), CCND3, B cell leukemia/lymphoma 2 (BCL2) or XIAP in stomach cancer were analyzed by GEPIA 2 database online (http://gepia2.cancer-pku.cn/).

2.2 Cells, Chemicals and Tissues

Gastric cancer cell lines MKN-45 and SNU-1 were obtained from Cell Resource Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences. HEK293T cell line was purchased from American Type Culture Collection, Manassas, VA, USA. All cell lines were cultured in RPMI 1640 medium (Meilunbio, Dalian, China) with 10% FBS (Biochannel, Nanjing, China) and 1% penicillin/streptomycin (Beyotime, Beijing, China). The chemical PR-619 (Cat. No. HY-13814) was purchased from MedChemExpress, New Jersey, USA. The primary gastric cancer tissues were received from the First Affiliated Hospital of Soochow University, and the case information was listed in the **Supplementary Table 1**. The collection and use of human gastric cancer tissues for this study were approved by the Review and Ethics committee of the First Affiliated Hospital of Soochow University.

2.3 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The qRT-PCR was carried out to evaluate the messenger RNA (mRNA) levels of USP4, CCND2, BCL2 and GAPDH according to the previous study [21]. The primers used were as follows: USP4, forward 5′-AAGGAAGCCTGGGAGAAT-3′ and reverse 5′-GCAGTGGCAGCGTTAGAT-3′; CCND2, forward 5′-CTGTCTCTGATCCGCAAGCAT-3′ and reverse 5′-CCCACACTTCCAGTTGCGAT-3′; BCL2, forward 5′-GAACTGGGGGAGGATTGTGG-3′ and reverse 5′-CATCCCAGCCTCCGTTATCC-3′; GAPDH, forward 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse 5′-TGGTGAAGACGCCAGTGGA-3′.

2.4 Immunoblotting and Co-Immunoprecipitation

Immunoblotting was carried out to evaluate the protein levels as described previously [22]. The anti-USP4 (Cat. No. sc-376000) and anti-PRL-3 (Cat. No. sc-130355) antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Anti-phospho-NF-κB p65 (p-p65) (Cat. No. #3033), p65 (Cat. No. #8242) and GAPDH (Cat. No. #97166) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Myc-tag antibody (Cat. No. M192-3) was received from MBL Beijing Biotech Co., LTD, Beijing, China.

Gastric cancer cells were also lysed for immunoprecipitation (IP) analysis. In brief, cells were lysed by using IP lysis (Beyotime, Shanghai, China, Cat. No. P0013J). Then, PRL-3 protein was purified by using an anti-PRL-3 antibody (Santa Cruz, Cat. No. sc-130355) and Protein A/G PLUS-Agarose (Santa Cruz, Cat. No. sc-2003). At last, the interacting protein USP4 was detected by using immunoblotting with an anti-USP4 antibody (Santa Cruz, Cat. No. sc-376000).

2.5 Cell Growth and Viability

The cell viability of gastric cancer cells was detected by Cell Counting Kit-8 (CCK-8) assay referenced to the manufacturer's instructions (Cat. No. B34302, Bimake, Houston, TX, USA).

2.6 Lentivirus Construction and Infection

Lentivirus-delivered shRNAs against USP4 (shUSP4) and a negative control shNC were synthesized from GeneChem Co., Ltd. (Shanghai, China). The target sequences of shUSP4#1 and shUSP4#2 were 5′-GGTCGCAGATGTGTATAAT-3′ and 5′-GCAGCCACTATTGCTTTCT-3′. Lentiviral particles were generated in HEK293T cells, and gastric cancer cells were infected by the lentivirus according to the previous study [23].

2.7 Plasmid Construction and Transfection

The luciferase reporter pNFκB-Luc, which was driven by NF-κB response elements, was purchased from Beyotime Biotechnology, Beijing, China. The human *PRL-3* gene was amplified by PCR and subcloned into pcDNA3.1 vector with a Myc tag. These plasmids were transfected into gastric cancer cells by using Lipofectamine® 2000 (Cat. No. 11668027, Invitrogen, Waltham, MA, USA) according to the manufacturer's instruction.

2.8 Xenograft Assay

SNU-1 and MKN-45 cells infected with shNC or shUSP4#1-derived lentivirus (3×10^6 cells/site) were inoculated on the right flank of nude mice (Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai, China). When tumors were palpable, tumor sizes were measured every two days for continuous two weeks using a caliper. At the end of the animal studies, tumors were excised and used for further analyses.

2.9 Luciferase Assay

SNU-1 and MKN-45 cells were transfected with luciferase reporter pNFκB-Luc, which was driven by NF-κB response elements, or empty vector. Twenty-four hours later, transfected cells were infected with shNC, shUSP4#1 or shUSP4#2-derived lentivirus for 48 hours, followed by luciferase assay (Promega, Madison, WI, USA) according to the manufacturer's instruction.

2.10 Statistical Analysis

In the experiments, to compare the differences between two groups, student's *t* test was used. One-way ANOVA with Tukey's Multiple Comparison Test was used to compare the differences among multiple groups. In this study, a *p* value less than 0.05 was considered to be statistically significant.

3. Results

3.1 USP4 Predicts a Negative Index for Gastric Cancer Patients

In order to examine the relationship between USP4 and clinical prognosis of gastric cancer patients, we tried Kaplan-Meier Plotter database for analysis. As shown in Fig. 1A,B, the Kaplan-Meier Plotter with Affymetrix microarray (ID: 202681_at) showed that the gastric cancer patients with high expression level of USP4 had the shorter overall survival (OS) and post-progression survival (PPS) than the patients with low expression. In addition, the Kaplan-Meier Plotter with Affymetrix microarray (ID: 211800_s_at) also showed that the gastric cancer patients with high expression level of USP4 had the shorter OS and PPS (Fig. 1C,D).

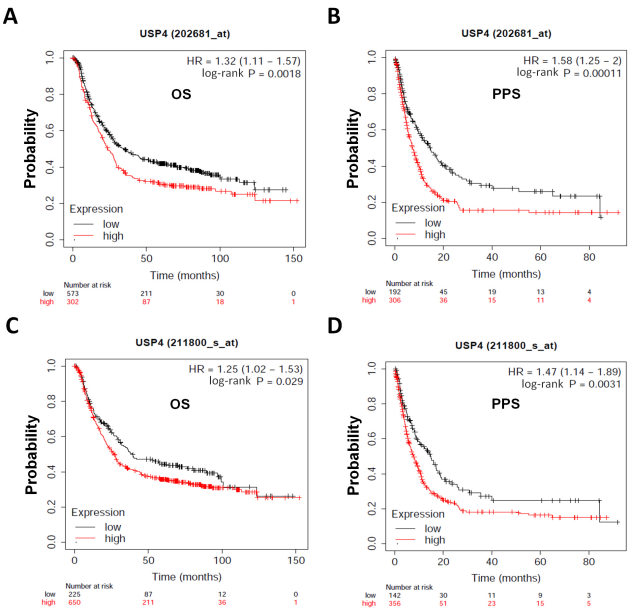

Fig. 1. USP4 predicts a negative index for gastric cancer patients. (A,B) The overall survival (OS) and post-progression survival (PPS) for gastric cancer patients with low or high USP4 (Affymetrix ID: 202681_at) were analyzed by Kaplan-Meier Plotter database online. (C,D) The OS and PPS for gastric cancer patients with low or high USP4 (Affymetrix ID: 211800_s_at) were analyzed by Kaplan-Meier Plotter.

3.2 USP4 Is Elevated in Gastric Cancer

Above results showed that USP4 predicted a poor prognosis for gastric cancer patients, which suggested that USP4 may have therapeutic significance for gastric cancer in clinic. Then, to further investigate the expression levels of USP4 in gastric cancer based on individual cancer stages, tumor grade, H.pylori infection status or nodal metastasis status, the public database UALCAN was used online. As shown in Fig. 2A, USP4 was significantly upregulated in patients with Stage 4 gastric cancer. USP4 was also significantly increased in patients with Grade 3 gastric cancer (Fig. 2B). It was also shown that gastric cancer patients without H.pylori infection had a higher expression of USP4 (Fig. 2C). In addition, the expression level of USP4 in gastric cancer was also related with the nodal metastasis status (Fig. 2D).

3.3 Knockdown of USP4 Inhibits Cell Growth of Gastric Cancer

Then, several gastric cancer tumor tissues were collected for qRT-PCR analysis, and it showed that USP4 was increased in tumor tissues compared with the adjacent normal tissues (Fig. 3A). To further investigate whether USP4 was functional in gastric cancer, USP4 was knocked down by shRNAs. As shown in Fig. 3B,C, the qRT-PCR analysis showed that USP4 was successfully knocked down in both of SNU-1 and MKN-45 cells. Then, infected cells were pre-

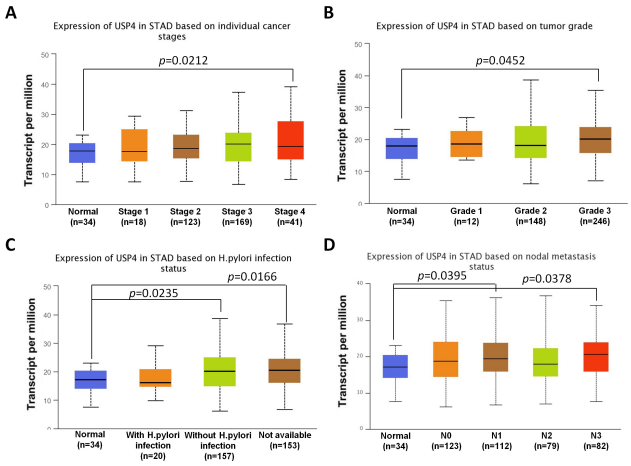

Fig. 2. The expression of USP4 in gastric cancer patients. (A–D) The expressions of USP4 in STAD based on individual cancer stages (A), tumor grade (B), H.pylori infection status (C) or nodal metastasis status (D) were analyzed by UALCAN database online.

pared for CCK-8 assay to investigate whether USP4 knockdown affected cell growth of gastric cancer. As shown in Fig. 3D,E, knockdown of USP4 significantly suppressed cell growth in both of gastric cancer cell line SNU-1 and MKN-45 cells. These results further confirmed that USP4 was functional in gastric cancer.

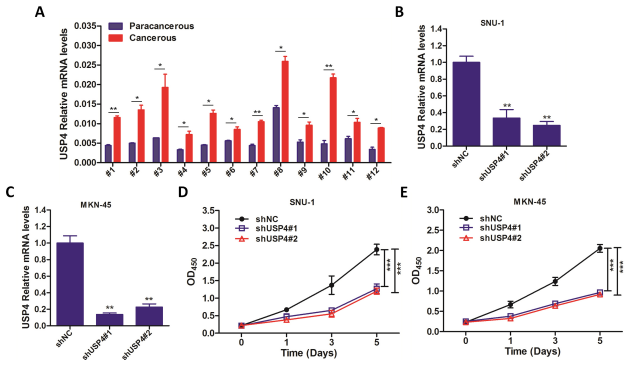

Fig. 3. Downregulation of USP4 suppresses cell growth of gastric cancer. (A) Twelve pairs of gastric cancer tumor tissues (cancerous) and adjacent normal tissues (paracancerous) were prepared for qRT-PCR against USP4. GAPDH was used as an internal control. (B,C) SNU-1 (B) and MKN-45 (C) cells were infected with shNC, shUSP4#1 or shUSP4#2 lentivirus for 3 days, followed by qRT-PCR against USP4. (D,E) SNU-1 (D) and MKN-45 (E) cells were infected with shNC, shUSP4#1 or shUSP4#2 lentivirus for indicated time, followed by CCK-8 assay at day 0, 1, 3 or 5. *** $p < 0.001$.

Moreover, we also establised the xenograft models to evaluate the function of USP4 *in vivo*. The animal studies showed that knockdown of USP4 markedly suppressed the tumor growth of gastric cancer (Fig. 4). These results further confirmed that USP4 was functional in gastric cancer.

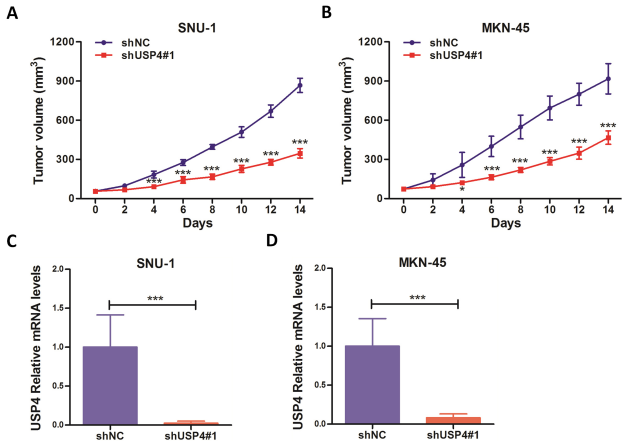

Fig. 4. Downregulation of USP4 suppresses tumor growth in gastric cancer cell line-derived xenografts. (A,B) The tumor volume curves. (C,D) The excised tumors were prepared for qRT-PCR analysis against USP4. GAPDH was used as an internal control. * $p < 0.05$, *** $p < 0.001$.

3.4 Knockdown of USP4 Inhibits PRL-3 Expression and NF-κB Signaling in Gastric Cancer

Next, our further study showed that knockdown of USP4 decreased the expression of PRL-3 (Fig. 5A), which was previously reported to be a substrate of USP4 [13]. It has been also reported that PRL-3 was an upstream molecule of NF-κB signaling in gastric cancer [12], so we evaluated whether USP4 modulated NF-κB signaling in gastric cancer. As shown in Fig. 5B, the luciferase assay showed that silence of USP4 significantly decreased the luciferase activity driven by NF-κB in both of SNU-1 and MKN-45 cells. The immunoblotting assay also revealed that silence of USP4 downregulated the activation of NF-κB p65 in both of gastric cancer cell lines and tumor tissues (Fig. 5C,D).

In addition, our findings revealed that USP4 expression was positively correlated with the target genes of NF-κB signaling in gastric cancer, including *CCND2*, *CCND3*, *BCL2* and *XIAP* (**Supplementary Fig. 1**), which was analyzed by GEPIA 2 database online. Finally, the expression levels of some target genes of NF-κB signaling were also detected by qRT-PCR analysis, and the results showed that knockdown of USP4 significantly downregulated the expression levels of CCND2 and BCL2 in both of SNU-1 and MKN-45 cells (**Supplementary Fig. 2**).

In order to further confirm whether PRL-3 was a target for USP4 in gastric cancer, co-immunoprecipitation assay was firstly performed (Fig. 6A). In addition, PRL-3 was overexpressed by plasmid transfection (Fig. 6B). As shown in Fig. 6C,D, the luciferase assay showed that overexpression of PRL-3 could significantly attenuate the inhibitory

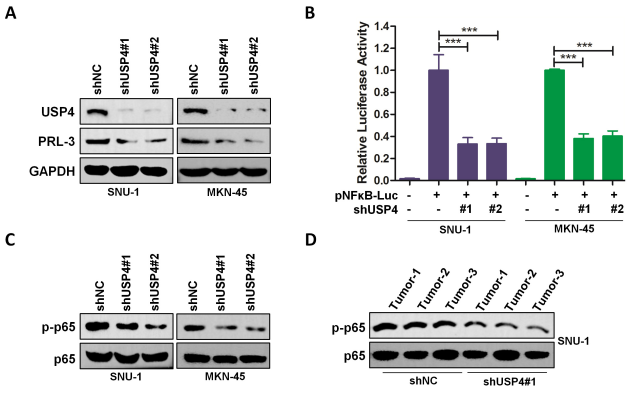

Fig. 5. Downregulation of USP4 inhibits PRL-3 expression and NF-κB signaling in gastric cancer. (A) SNU-1 and MKN-45 cells were infected with shNC, shUSP4#1 or shUSP4#2 lentivirus for 3 days, followed by immunoblotting against USP4, PRL-3 and GAPDH. (B) SNU-1 and MKN-45 cells were transfected with luciferase reporter pNFκB-Luc, which was driven by NF-κB response elements. Twenty-four hours later, transfected cells were infected with shNC, shUSP4#1 or shUSP4#2 lentivirus for 48 hours, followed by luciferase assay. (C) SNU-1 and MKN-45 cells were infected with shNC, shUSP4#1 or shUSP4#2 lentivirus for 3 days, followed by immunoblotting against p-p65 and p65. (D) The indicated excised tumors were lysed for immunoblotting against p-p65 and p65. *** $p < 0.001$.

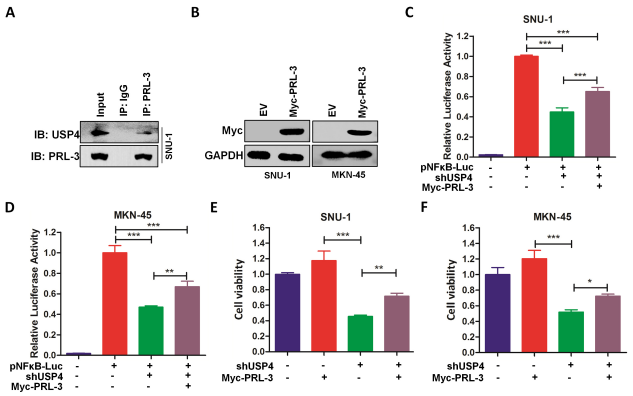

Fig. 6. Overexpression of PRL-3 attenuates the inhibitory effects of USP4 knockdown in gastric cancer cells. (A) SNU-1 cells were lysed for co-immunoprecipitation. IP, immunoprecipitation; IB, immunoblotting. (B) The overexpressing efficacy of Myc-PRL-3 plasmids was detected by immunoblotting in SNU-1 and MKN-45 cells. (C,D) SNU-1 (C) and MKN-45 (D) cells transfected with pNFκB-Luc plasmids were infected with shNC or shUSP4#1-derived lentivirus, or transfected with Myc-PRL-3 plasmids for 48 hours, followed by luciferase assay. (E,F) SNU-1 (E) and MKN-45 (F) cells were infected with shNC or shUSP4#1-derived lentivirus, or transfected with Myc-PRL-3 plasmids for 48 hours, followed by CCK-8 assay. *** $p < 0.001$, ** $p < 0.01$.

effects of USP4 knockdown on NF-κB-derived luciferase activity. The CCK-8 assay showed that PRL-3 overexpression could also significantly attenuate the inhibitory effects of USP4 knockdown on cell viability in gastric cancer (Fig. 6E,F). Therefore, we could get that knockdown of USP4 inhibited gastric cancer cell growth by suppressing PRL-3/NF-κB signaling.

3.5 Deubiquitinase Inhibition by PR-619 Inhibits Cell Viability and NF-κB Signaling in Gastric Cancer

It has been proven that PR-619, as a broad-range deubiquitinase inhibitor, inhibited the activities of USP4 and other deubiquitinases, and exerted its anti-tumor activity in several tumors [24,25]. Based on these, we finally evaluated whether the cell viability of gastric cancer could be inhibited by PR-619. As shown in Fig. 7A,B, the cell viability of gastric cancer cells was significantly inhibited by PR-619 treatment. In addition, the luciferase activity driven by NF-κB was also significantly downregulated by the treatment of PR-619 in gastric cancer cells (Fig. 7C,D). These results further indicated that targeting deubiquitinases could be a promising strategy for the anti-gastric cancer drug discovery.

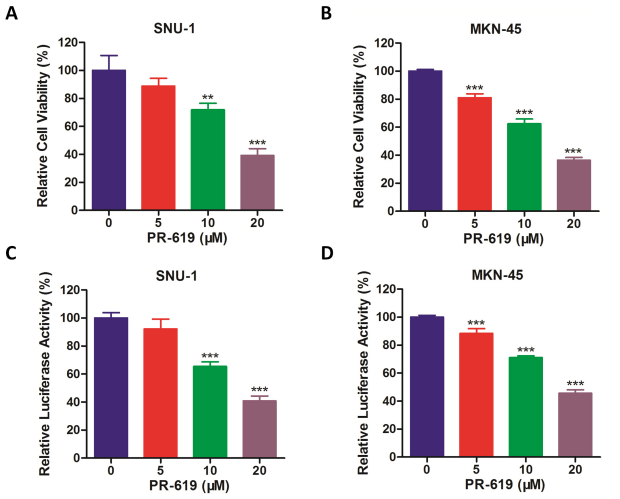

Fig. 7. Deubiquitinase inhibition by PR-619 inhibits cell viability of gastric cancer and NF-κB signaling in gastric cancer. (A,B) SNU-1 (A) and MKN-45 (B) cells were incubated with increasing concentrations of PR-619 for 24 hours, followed by CCK-8 assay to detect cell viability. (C,D) SNU-1 (C) and MKN-45 (D) cells transfected with pNFκB-Luc plasmids were incubated with increasing concentrations of PR-619 for 12 hours, followed by luciferase assay. *** $p < 0.001$, ** $p < 0.01$.

4. Discussion

It has been reported that USP4 was functional in several tumors mostly as an oncogene. For example, in glioblastoma multiforme (GBM), USP4 was found markedly upregulated in GBM tissues and patients with high USP4 had a poor prognosis [26]. Moreover, knock-

down of USP4 significantly suppressed cell growth of GBM by inhibiting ERK signaling both *in vitro* and *in vivo* [26]. Additionally, in glioblastoma, USP4 could induce temozolomide (TMZ) chemoresistance by suppressing cell apoptosis in a p53-dependent manner [27]. USP4 was also reported elevated in both of melanoma tumor tissues and cell lines, and knockdown of USP4 could increase the sensitivity to cisplatin as well as attenuating the migratory and invasive abilities of melanoma cells by suppressing epithelial-mesenchymal transition (EMT), which suggested that USP4 displayed an oncogenic role in melanoma [17]. However, the function of USP4 has not been investigated in gastric cancer. In our present study, USP4 was also found upregulated in gastric cancer tumor tissues, and knockdown of USP4 significantly inhibited cell growth and tumor growth of gastric cancer, which indicated that USP4 was a novel oncogene in gastric cancer.

At present, targeting DUBs, especially ubiquitin-specific proteases, for the development of anticancer drugs has become an effective strategy. Take USP4 as an example, neutral red (NR) was identified as an uncompetitive inhibitor of USP4 through the *in vitro* deubiquitinating activity assays, and it showed that NR markedly reduced cell migration and colony formation of colorectal cancer cells as well as inhibiting tumor growth of colorectal cancer in a mouse xenograft model [28]. Thus, this study further supported the possibility of developing USP4 inhibitors as therapeutic agents in gastric cancer, and our future work will focus on this aspect.

So far, many studies have proven that NF-κB signaling is closely related to the occurrence of gastric cancer [29]. And our further studies in this paper showed that knockdown of USP4 could significantly inhibit NF-κB signaling in gastric cancer, which further explained the activation mechanism of NF-κB signaling in gastric cancer. Similarly, several reports in other tumors also focused on the studies between USP4 and NF-κB signaling. A paper showed that USP4 suppressed p53 and NF-κB signalings by deubiquitinating and stabilizing HDAC2 [28]. Another paper reported that USP4 inhibited TNFα-induced cancer cell migration by targeting TRAF2 and TRAF6 for deubiquitination [30]. USP4 was also reported to target TAK1 to downregulate TNFα-induced NF-κB activation [31]. USP4 was also reported to regulate the deubiquitination and stabilization of PRL-3 in colorectal cancer [13]. Consistently, our study confirmed that USP4 knockdown markedly decreased the expression of PRL-3 and downregulated the activation of NF-κB signaling in gastric cancer. Moreover, overexpression of PRL-3 in gastric cancer partly attenuated the inhibitory effect of shUSP4 on NF-κB signaling, which meant that the modulation of NF-κB signaling mediated by USP4 was not entirely dependent on PRL-3. So, our future studies will try to identify other additional participating elements.

5. Conclusions

Collectively, our present study indicated that targeting USP4/PRL-3/NF-κB axis could be as a novel therapeutic strategy for gastric cancer.

Data Availability Statement

All data generated or analyzed during this study are included in this article.

Author Contributions

WDY participated in the conception and design of the study. YYT and WDY performed the experiments. WDY interpreted the data and produced the main document. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Review and Ethics committee of the First Affiliated Hospital of Soochow University (Ethic Approval Number: 2019, 127).

Acknowledgment

Not applicable.

Funding

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2710286.

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