Effects of JAG-1 on the Proliferation and Migration of Gastric Adenocarcinoma Cells after TRAIP Knockout

Chongyang ZHU, Jiemin QI*

Department of Pathology, Chengde Medical University, Chengde 067000, China

Abstract [**Objectives**] To study the effects of JAG-1 on silencing TRAIP (tumor necrosis factor receptor associated factor interaction protein) after regulating Notch signaling pathway on the proliferation and migration of gastric adenocarcinoma cells. [**Methods**] Gastric adenocarcinoma cells were categorized into si-NC + DMSO (control + DMSO), si-TRAIP#1 + DMSO (transfected with TRAIP + DMSO), si-NC + JAG-1 (control + JAG-1), and si-TRAIP#1 + JAG-1 (transfected with TRAIP + JAG-1), and the proliferation of the cells was detected by CCK-8 assay and plate colony formation assay. Transwell assay was used to detect cell migration, and Western blot was adopted to detect the expression of proliferation-associated protein CyclinD1, migration-associated protein MMP2, and key proteins of Notch signaling pathway Notch1, Hes1 and Jagged1. [**Results**] Compared with siTRAIP#1 + DMSO, the gastric adenocarcinoma cells in si-TRAIP#1 + JAG-1 group showed increased proliferation and migration ($P<0.05$), and there was a significant increase in the expression of CyclinD1, MMP2, Notch1, Hes1, and Jagged1 ($P<0.05$). [**Conclusions**] After TRAIP knockdown, JAG-1 increased not only the proliferation and migration ability of gastric adenocarcinoma cells, but also the expression of key proteins of Notch signaling pathway Notch1, Hes1, and Jagged1.

Key words TRAIP, Gastric adenocarcinoma, Notch signaling pathway, Proliferation, Migration

1 Introduction

The statistical result of global cancer in 2020 show that gastric cancer ranks fifth in incidence and fourth in mortality, with more than 1 million new cases and an estimated 769 000 deaths[1] (among cancer patients worldwide, one cancer patient died of gastric cancer every 13 deaths). The occurrence and development of gastric cancer is a complex process, and its etiology and pathogenesis remain to be studied. Currently, protein ubiquitination is one of the important reasons leading to the occurrence and development of gastric cancer, and E3 ubiquitin ligase plays a crucial role in gastric cancer[2]. As an important member of the E3 ubiquitin ligase family, TRAIP is widely involved in the biological processes of various cells and the progression of cancer[3].

Previous experimental results show that at the tissue level, the expression of tumor necrosis factor receptor associated factor interaction protein (TRAIP) in gastric adenocarcinoma tissues was significantly higher than that in adjacent normal gastric mucosa tissues; at the cellular level, after TRAIP knockout, the proliferation and migration ability of gastric adenocarcinoma cells significantly declined, and the expressions of key proteins of Notch signaling pathway Notch1, Hes1 and Jagged1 also significantly dropped. It has been confirmed that TRAIP was involved in the occurrence and development of gastric adenocarcinoma, and it promoted the proliferation and migration of gastric adenocarcinoma cells through Notch signaling pathway. In order to better verify the accuracy of the previous experimental results, after TRAIP was knocked down, Notch signaling pathway agonist (JAG-1) was added, and the proliferation and migration ability of gastric adenocarcinoma cells were detected again by CCK-8 experiment, colony formation experiment and Transwell experiment. Besides, the expressions of key proteins of Notch signaling pathway Notch1, Hes1 and Jagged1 were detected by Western blot assay.

2 Materials and methods

2.1 Materials

2.1.1 Main instruments and equipment. The cell incubator, low-temperature refrigerator and pure water machine were manufactured by Thermo Scientific Company. The fluorescence microscope was produced by Olympu Company, Japan; the electrophoresis apparatus, film transfer apparatus and gel imager were manufactured by BIO-RAD Company; the ice maker was made by SANYO in Japan; the shaker was produced by Beijing Liuyi Biological Company.

2.1.2 Principal reagents. Gastric adenocarcinoma cell lines AGS and MGC-803 were provided by Shanghai Cell Bank of Chinese Academy of Sciences; fetal bovine serum, RPMI-1640 medium and F12 medium were offered by American Gibico Company; TRAIP siRNA was given by Guangzhou Ruibo Biological Company; transfection reagent LipofectamineTM3000 was provided by American invitrogen Company; protein quantification kit was offered by Beijing Solarbio Company; Notch signaling pathway agonist JAG-1 was given by American ANASPEC Company; GAPDH internal reference antibody was provided by Cell Signal Company; Goat Anti-Mouse IgG-HRP and Goat Anti-rabbit IgG-HRP were offered by American KPL Company; TRAIP, CyclinD1 and MMP2

Received: August 6, 2023 Accepted: September 8, 2023

Supported by the Chengde Medical University-National Natural Science Foundation Project Cultivation Fund (202114); Discipline Construction Fund of Chengde Medical College [(2023) No. 2]; Chengde Medical University-School-level Key Project Fund (201711).

* Corresponding author. E-mail: qijiemin@126.com

antibodies were given by Wuhan Sanying Biological Company; Notch1 antibody was provided by American CST Company; Hes1 and Jagged1 antibodies were offered by American immunowang Company.

2.2 Methods

2.2.1 Cell culture. MGC-803 cells were cultured in 10% 1 640 medium, and AGS cells were cultured in 10% F12 medium in an incubator containing 5% CO_2 at 37 ℃. When the cell length was more than 90%, the cells in the logarithmic growth period were taken for subsequent experiments.

2.2.2 Cell transfection and addition of JAG-1. Cells in the logarithmic growth period were inoculated into a 6-well plate and incubated in an incubator. Cell transfection was conducted when the cell density reached 50%. The transfection was performed on the next day according to the instructions of the LipofectamineTM3000 kit. The cells were slightly shaken and further cultured in the incubator. Notch signaling pathway was activated. After transfection for 36 h, JAG-1 was dissolved in dimethyl sulfoxide (DMSO), and 40 μmol/L JAG-1 was added to the cells after TRAIP knockdown. An equal amount of DMSO was added to the control group.

2.2.3 Cell counting. The cells were put into a super-clean table, and washed with 2 mL of PBS. 1 mL of pancreatic enzyme containing EDTA was added to them. After the pancreatic enzyme was discarded, and 1 mL of complete medium was used to terminate digestion. The cells were blown evenly to make cell suspension. The cell suspension was diluted 10 times and mixed well. 10 μL of cell suspension was added to both sides of the cell counting plate to count the number of the cells.

2.2.4 CCK-8 experiment. After cell counting, the cells were inoculated into five 96-well plates with a density of 1 000 cells, and 5 compound pores were set in each group. They were incubated in an incubator. The pore plates with the cells cultured for 0, 24, 48, 72 and 96 h were added with the corresponding CCK-8 working solution (basic medium: CCK-8 stock solution was 10 ∶ 1). After they were incubated at 37 ℃ for 2 h, the absorbance of each pore at 450 nm were detected by enzymoleter, and cell viability was calculated.

2.2.5 Experiment on plate colony formation. After cell counting, the cells were inoculated in a 6-well plate with a density of 200 AGS cells/pore. They were inoculated in a 6-well plate with a density of 150 AGS cells/pore, and 2 mL of 10% medium was added to each well. Cell fluid was changed every 5 d, and the cells were cultured in an incubator for 15 d. The cells were taken out for staining, fixed with ice methanol for 30 min, stained with crystal violet for 15 min, and photographed.

2.2.6 Transwell experiment. After cell counting, the cells were inoculated in the Transwell upper chamber of a 24-well plate with a density of 60 000 AGS cells/pore. They were inoculated in the Transwell upper chamber of a 24-well plate with a density of 40 000 MGC-803 cells/pore, and the lower chamber was added with 600 μL of 20% medium. After being cultured in an incubator for 24 h, they were taken out, and fixed with ice methanol for 30 min. They were stained with crystal violet for 15 min, and the unpenetrated cells in the upper chamber were gently wiped with cotton swabs. They were counted after taking photos.

2.2.7 Detection of protein expression by Western blot. The treated gastric adenocarcinoma cells (RIPA lysate ∶ protease inhibitor = 100 ∶ 1) were lysated with lytic solution, and quantified by BCA protein quantitative kit. They were equipped with protein samples, and denatalized at 100 ℃ for 10 min. The electrophoresis was performed in a 30 μg/15 μL system with 10% lower glue and 5% upper glue, and the membrane was transferred at 150 V for 2 h. They were closed with 5% milk powder for 2 h, and the antibodies were diluted (the ratio of CyclinD1, MMP2, Notch1, Hes1 and Jagged1 was all 1 ∶ 1 000; that of GAPDH was 1 ∶ 10 000). The primary antibody was incubated, and stayed overnight at 4 ℃. It was taken out on the next day, and washed with TBST 5 times for 5 min. The antibody was diluted (the ratio of Goat Anti-Mouse/Rabbit IgG-HRP was 1 ∶ 10 000), and the secondary antibody was incubated at 37 ℃ for 1 h. It was washed with TBST 5 times for 5 min.

2.2.8 Statistical analysis. Graphpad 9.0 was used for statistics and mapping, and t test was used for comparison between two independent samples. Results were expressed as $\bar{x} \pm s$, and $P<0.05$ meant the difference was statistically significant. The experimental data were repeated 3 times.

3 Results and analysis

3.1 Effects of JAG-1 on the proliferation of gastric adenocarcinoma cells after silencing TRAIP CCK-8 assay showed that compared with siNC + DMSO group, the cell viability of siTRAIP#1 group decreased, indicating successful transfection of TRAIP. Compared with siNC + DMSO group, the cell activity of siNC + JAG-1 group enhanced, suggesting that JAG-1 could enhance the activity of gastric adenocarcinoma cells. Compared with siTRAIP#1 group, the cell viability of siTRAIP#1 + JAG-1 group enhanced, revealing that JAG-1 reversed the proliferation of gastric adenocarcinoma cells after TRAIP knockdown ($P<0.05$, Fig. 1).

From colony formation assay, it is found that compared with siNC + DMSO group, the number of cell colonies in siTRAIP#1 group reduced, suggesting that TRAIP transfection was successful. Compared with siNC + DMSO group, the number of cell colonies in siNC + JAG-1 group increased, revealing that JAG-1 could promote the proliferation of gastric adenocarcinoma cells. Compared with siTRAIP#1 group, there was an increase in the number of cell colonies rose in siTRAIP#1 + JAG-1 group, showing that JAG-1 reversed the proliferation of gastric adenocarcinoma cells after TRAIP knockdown ($P<0.05$, Fig. 2).

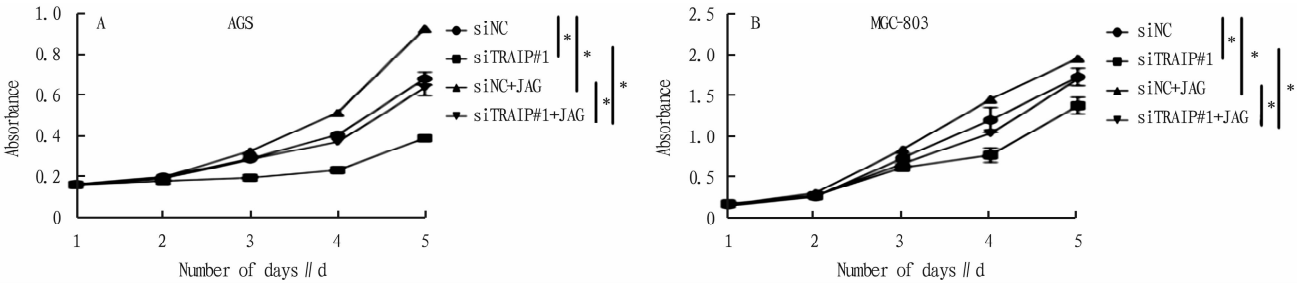

Fig. 1 Proliferation of gastric adenocarcinoma cells detected by CCK-8 assay after the use of JAG-1 (*: $P<0.05$)

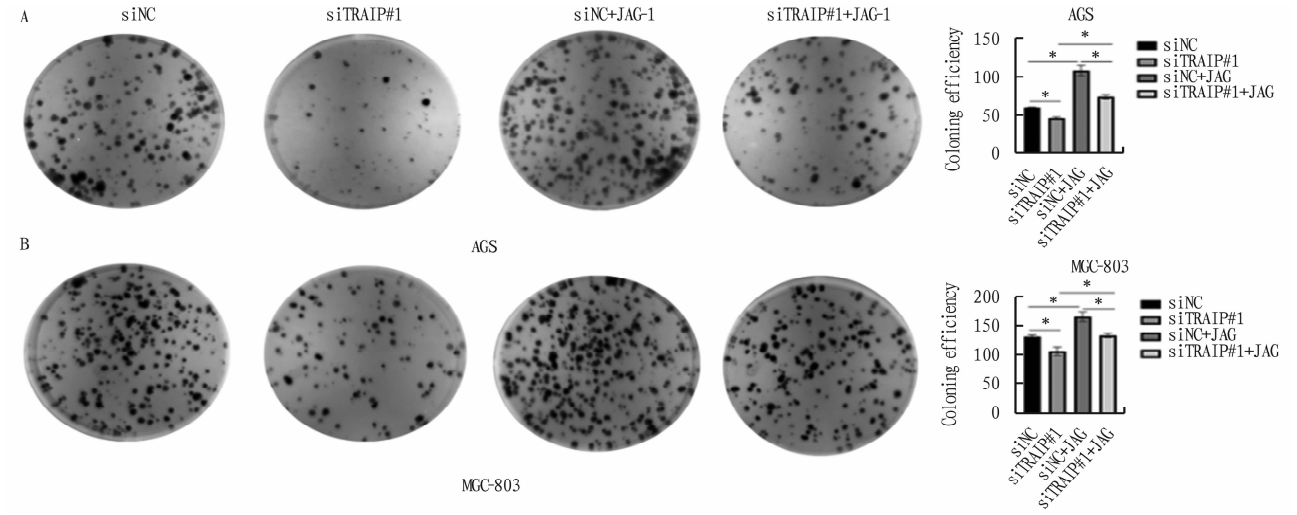

Fig. 2 Proliferation of gastric adenocarcinoma cells detected by colony formation assay after the use of JAG-1 (*: $P<0.05$)

3.2 Effects of JAG-1 on the migration of gastric adenocarcinoma cell after silencing TRAIP Transwell assay revealed that compared with siNC + DMSO group, the number of migrated cells in siTRAIP#1 group reduced, indicating that TRAIP transfection was successful. Compared with siNC + DMSO group, the number of migrated cells increased in siNC + JAG-1 group, suggesting that JAG-1 promoted the migration of gastric adenocarcinoma cells. Compared with siTRAIP#1 group, the number of migrated cells in siTRAIP#1 + JAG-1 group rose, showing that JAG-1 reversed the migration ability of gastric adenocarcinoma cells after TRAIP knockdown ($P<0.05$, Fig. 3).

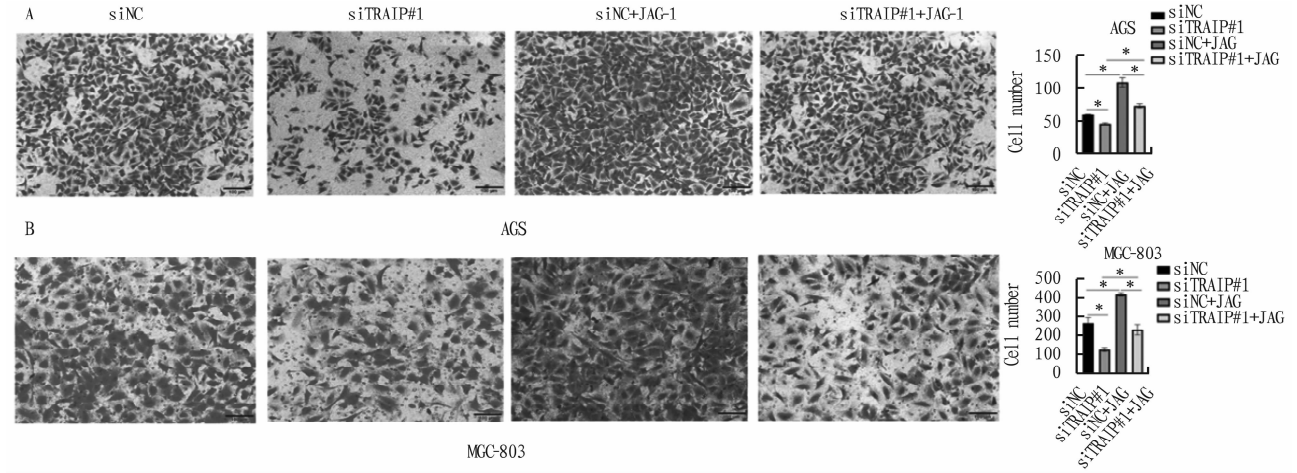

Fig. 3 Migration ability of gastric adenocarcinoma cells detected by Transwell assay after the use of JAG-1 (*: $P<0.05$)

3.3 Effects of JAG-1 on the proliferation and migration of gastric adenocarcinoma cells after silencing TRAIP Western blot assay showed that compared with siNC + DMSO group, the expression of proliferation-related protein CyclinD1 and migration-related protein MMP2 in siTRAIP#1 group decreased, indicating that TRAIP transfection was successful. Compared with siNC + DMSO

group, the expression of proliferation-related protein CyclinD1 and migration-related protein MMP2 in siNC + JAG-1 group enhanced, suggesting that JAG-1 could activate the downstream proliferation and migration-related proteins of Notch signaling pathway. Compared with siTRAIP#1 group, the expression of proliferation-related protein CyclinD1 and migration-related protein MMP2 in siTRAIP#1 + JAG-1 group enhanced, suggesting that JAG-1 could reverse the proliferation and migration of gastric adenocarcinoma cells after TRAIP knockdown ($P<0.05$, Fig. 4).

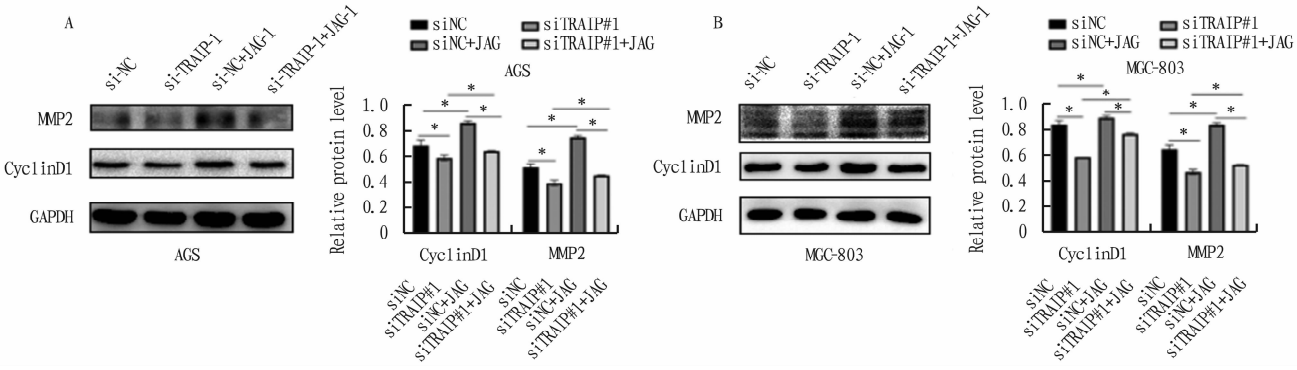

Fig. 4 Expression of proliferation- and migration-related proteins in gastric adenocarcinoma cells detected by Western blot assay after the use of JAG-1 (* : $P<0.05$)

3.4 Effects of JAG-1 on key proteins of Notch signaling pathway in gastric adenocarcinoma cells after silencing TRAIP Western blot assay showed that compared with siNC + DMSO group, the expression of key proteins of Notch signaling pathway in gastric adenocarcinoma cells in siTRAIP#1 group reduced, indicating that TRAIP transfection was successful. Compared with siNC + DMSO group, the expression of key proteins of Notch signaling pathway in siNC + JAG-1 group increased, suggesting that JAG-1 could activate Notch signaling pathway. Compared with siTRAIP#1 group, the expression of key proteins of Notch signaling pathway in siTRAIP#1 + JAG-1 group also enhanced, showing that JAG-1 reversed the expression of key proteins of Notch signaling pathway in gastric adenocarcinoma cells after TRAIP knockdown ($P<0.05$, Fig. 5).

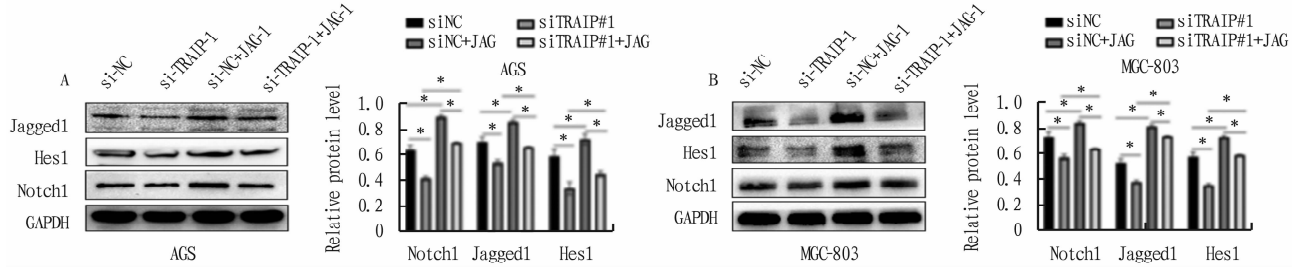

Fig. 5 Expression of key proteins of Notch signaling pathway in gastric adenocarcinoma cells(* : $P<0.05$)

4 Discussion

Tumor necrosis factor (TNF) receptor associated factor (TRAF) interaction protein (TRAIP) contains a ring structure, and belongs to the E3 ubiquitin ligase family[4]. It has been confirmed that TRAIP has biological functions such as promoting cell mitosis[5-6], embryonic development[7], and participating in DNA damage and repair[8-9]. TRAIP is associated with the development of various types of cancer, and it has been confirmed that it mainly promotes the occurrence and development of osteosarcoma[10], liver cancer[11], lung cancer[12], and breast cancer[13]. However, its biological function and mechanism of action in gastric adenocarcinoma have not been reported.

The previous results of the research group showed that at the tissue level, the positive expression of TRAIP in gastric adenocarcinoma tissues significantly increased through immunohistochemical SP detection, and the expression of TRAIP protein was related to the differentiation degree, TNM stage, depth of invasion and lymph node metastasis of gastric adenocarcinoma tissues. Western blot and qRT-PCR were used to detect the expression of protein and mRNA of TRAIP in fresh gastric adenocarcinoma tissues, and it was found that the expression of protein and mRNA of TRAIP in gastric adenocarcinoma tissues was significantly higher than that in adjacent normal gastric mucosa tissues. At the cellular level, Western blot was used to detect the expression of TRAIP in gastric adenocarcinoma cell lines MGC-803, SGC-7901, AGS, BGC-823 and normal gastric mucosal epithelial cell line GES-1, and the results showed that the expression of TRAIP in gastric adenocarcinoma cell lines was higher than that in normal gastric mucosal cell line. In order to further study the biological function of TRAIP in gastric adenocarcinoma cells, cell lines MGC-803 and AGS with high expression were selected for follow-up cell experiments. From CCK-8 assay, plate colony formation experiment and Transwell experiment, it is found that TRAIP knockdown resulted in the de-

creases in the activity of MGC-803 and AGS gastric adenocarcinoma cells, the number of cell colonies, and the migration ability of the cells. Moreover, after TRAIP knockdown, the expression of proliferation-related protein CyclinD1, migration-related protein MMP2 and key proteins of Notch signaling pathway Notch1, Jagged1 and Hes1 significantly reduced. Previous experiments confirmed that TRAIP was involved in the occurrence and development of gastric adenocarcinoma, and promoted the proliferation and migration of gastric adenocarcinoma cells through Notch signaling pathway.

To further confirm that TRAIP promoted the proliferation and migration of gastric adenocarcinoma through Notch signaling pathway, Notch signaling pathway agonist (JAG-1) was added on the basis of TRAIP knockdown. From CCK-8 assay, it is found that the activity of gastric adenocarcinoma cells significantly enhanced. Plate colony-forming assay showed that the colony-forming ability of gastric adenocarcinoma cells significantly improved. Through Transwell assay, the migration ability of gastric adenocarcinoma cells increased significantly. Western blot assay revealed that the expression of proliferation-related protein CyclinD1, migration-related protein MMP2, key proteins of Notch signaling pathway Notch1, Hes1 and Jaggde1 increased significantly. It was confirmed that after TRAIP knockout, JAG-1 not only increased the proliferation and migration ability of gastric adenocarcinoma cells, but also enhanced the expression of key proteins of Notch signaling pathway Notch1, Hes1 and Jagged1.

References

[1] SUNG H, FERLAY J, SIEGEL RL, *et al.* Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries[J]. CA: A Cancer Journal for Clinicians, 2021, 71(3): 209 – 249.

[2] WANG M, DAI W, KE Z, *et al.* Functional roles of E3 ubiquitin ligases in gastric cancer[J]. Oncology Letters, 2020, 20(4): 22.

[3] WU RA, PELLMAN DS, WALTER JC. The ubiquitin ligase tRAIP: Double-Edged sword at the replisome[J]. Trends in Cell Biology, 2021, 31(2): 75 – 85.

[4] ZHENG N, SHABEK N. Ubiquitin ligases: Structure, function, and regulation[J]. Annual Review of Biochemistry, 2017(86): 129 – 157.

[5] PRIEGO MS, JONES RM, POOVATHUMKADAVIL D, *et al.* Mitotic replisome disassembly depends on TRAIP ubiquitin ligase activity[J]. Life Science Alliance, 2019, 2(2): e201900390.

[6] WU RA, SEMLOW DR, KAMIMAE-LANNING AN, *et al.* TRAIP is a master regulator of DNA interstrand crosslink repair[J]. Nature, 2019, 567(7747): 267 – 272.

[7] CLERCQ D, KATRIEN, VRIENS J. Establishing life is a calcium-dependent TRIP: Transient receptorpotential channels in reproduction[J]. Biochimica et Biophysica Acta-Molecular Cell Research, 2018, 1865(11): 1815 – 1829.

[8] HARLEY ME, MURINA O, NüRNBERG P, *et al.* TRAIP promotes DNA damage responseduring genome replication and is mutated in primordial dwarfism[J]. Nature Genetics, 2016, 48(1): 36 – 43.

[9] SOO LN, JIN CH, KIM HJ, *et al.* TRAIP/RNF206 is required for recruitment of RAP80 to sites of DNA damage[J]. Nature Communications, 2016, 19(7): 10463.

[10] LI M, WU W, DENG S, *et al.* TRAIP modulates the IGFBP3/AKT pathway to enhance the invasion and proliferation of osteosarcoma by promoting KANK1 degradation[J]. Cell Death & Disease, 2021, 4, 12(8): 767.

[11] GUO Z, ZENG Y, LIU M, *et al.* TRAIP promotes malignant behaviors and correlates with poor prognosis in liver cancer[J]. Biomedicine & Pharmacotherapy, 2020(124): 109857.

[12] LI J, YU T, YAN M, *et al.* DCUN1D1 facilitates tumor metastasis by activating FAK signaling and upregulates PD-L1 in non-small-cell lung cancer[J]. Experimental Cell Research, 2019, 15, 374(2): 304 – 314.

[13] ZHENG Y, JIA H, WANG P, *et al.* Silencing TRAIP suppresses cell proliferation and migration/invasion of triple negative breast cancer via RB-E2F signaling and EMT[J]. Cancer Gene Therapy, 2023, 30(1): 74 – 84.

(From page 21)

[2] LIU L, CUI ZX, YANG XW, *et al.* Simultaneous characterisation of multiple *Mahonia fortunei* bioactive compounds in rat plasma by UPLC-MS/MS for application in pharmacokinetic studies and anti-inflammatory activity *in vitro*[J]. Journal of Pharmaceutical and Biomedical Analysis, 2020(179): 113013.

[3] LIU YY, YI CX, XIE SH, *et al.* Research progress of clinical application of *Mahoniae caulis*[J]. Chinese Journal of Clinical Rational Drug Use, 2019, 12(25): 180 – 181. (in Chinese).

[4] SONG HP, ZHANG H, HU R, *et al.* A strategy to discover lead chemome from traditional Chinese medicines based on natural chromatogram-effect correlation (NCEC) and natural structure-effect correlation (NSEC): *Mahonia* bealei and *Mahonia fortunei* as a case study[J]. Journal of Pharmaceutical and Biomedical Analysis, 2021 (1181): 122922.

[5] KAKAR MU, LI J, MEHBOOB MZ, *et al.* Purification, characterization, and determination of biological activities of water-soluble polysaccharides from Mahonia bealei[J]. Scientific Reports, 2022, 12(1): 8160.

[6] DAMJANOVIĈ A, KOLUNDŽIJA B, MATIĈ IZ. Mahonia aquifolium extracts promote doxorubicin effects against lung adenocarcinoma cells *in vitro*[J]. Molecules (Basel, Switzerland), 2020, 25(22): 5233.

[7] TANG HF, QUAN XR, LI JH, *et al.* Efficacy observation of Gonglaomu solution combined with Blue-red LED phototherapy in treatment of moderate-severe acne vulgaris[J]. Acta Medicinae Sinica, 2018, 31(2): 86 – 88. (in Chinese).

[8] NIU RT, HUANG LY, LIANG QJ, *et al.* Effects of AFP and AFB1 on the proliferation, migration and invasion of HepG2 and Bel-7404 cells [J]. Journal of Youjiang Medical University for Nationalities, 2023, 45(1): 28 – 32. (in Chinese).

[9] MATTIUZZI C, LIPPI G. Current cancer epidemiology[J]. Journal of Epidemiology and Global Health, 2019, 9(4): 217 – 222.

[10] Fang M, WANG DG, LIU PQ. Research progress on anti-tumor mechanism of natural products[J]. Food and Drug, 2022, 24(2): 167 – 171. (in Chinese).

[11] HUANG Y, WANG T, JIANG Z. Fast analysis of alkaloids from different parts of *Mahonia bealei* (Fort.) Carr. studied for their anti-Alzheimer's activity using supercritical fluid chromatography[J]. Journal of Separation Science, 2021, 44(9): 2006 – 2014.

[12] HUANG Y. Simultaneous determination of nine compounds in *Mahoniae caulis* by HPLC[J]. China Pharmaceuticals, 2022, 31(4): 79 – 82. (in Chinese).

[13] YAN GY, ZHANG M, LU K, *et al.* Influences of total alkaloids in caulis mahoniae on proliferation and apoptosis of cervical cancer cells and the Caspase-3 Expression[J]. Cellular and molecular Biology (Noisy-le-Grand, France), 2022, 68(6): 161 – 166.

[14] LIU S, PENG LY, ZHU MY, *et al.* Screening of anti-tumor active components from extracts of *Solanum solanum in vitro* and study on their mechanism of action[J]. Journal of Youjiang Medical University for Nationalities, 2016, 38(2): 157 – 159, 167. (in Chinese).