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Suppressing SMURF1 to preserve GSTM2: An approach to reducing gastric cancer aggressiveness *in vitro* and *in vivo*

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Summary. Background. Smad Ubiquitination Regulatory Factor-1 (SMURF1) is implicated in promoting gastric cancer progression by enhancing cell proliferation, migration, and invasion. This study aims to elucidate how SMURF1 drives gastric cancer aggressiveness, with a focus on its interaction with Glutathione S-transferase mu 2 (GSTM2).

Methods. Bioinformatics analysis identified dysregulated SMURF1 and GSTM2 expression in stomach adenocarcinoma (STAD). The relation between GSTM2 and SMURF1 was predicted using Unibrowser. Functional assays, including cell counting kit-8, wound healing, and Transwell invasion, were conducted on gastric cancer cells to explore the effects of GSTM2 and/or SMURF1. The ubiquitination level of GSTM2 was measured using western blot and immunoprecipitation. *In vivo* tumorigenicity was assessed in a xenograft mouse model, alongside analysis of tumor growth and molecular markers of epithelial-mesenchymal transition (EMT).

Results. SMURF1 was highly expressed and the GSTM2 level was significantly downregulated in STAD. GSTM2 silencing activated the viability, migration, and invasion of gastric cancer cells, and these cell functions were inhibited by GSTM2 overexpression, which was reversed by SMURF1 overexpression. SMURF1 was predicted to be an E3 ubiquitin ligase for GSTM2. SMURF1 overexpression or Cyclohexanecarboxamide (CHX) addition suppressed GSTM2 levels in gastric cancer cells. Silencing of SMURF1 restrained GSTM2 ubiquitination. *In vivo*, GSTM2 overexpression suppressed tumor growth and EMT markers, such as Vimentin, while elevating E-cadherin, which was offset by SMURF1 upregulation.

Conclusion. This study reveals a novel oncogenic axis, where SMURF1 promotes gastric cancer progression by targeting GSTM2 for degradation. Inhibiting SMURF1 stabilizes GSTM2, leading to reduced cell proliferation, migration, and invasion both *in vitro* and *in vivo*.

Key words: SMURF1, Gastric cancer, GSTM2, Ubiquitination

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Introduction

Gastric cancer stands as one of the most prevalent and deadly cancers (Petryszyn et al., 2020), with its outcomes closely tied to the tumor stage at diagnosis (Aichler et al., 2014). That is, the earlier gastric cancer is diagnosed, the better therapeutic effect can be achieved, underscoring the significance of clinical tumor markers carcinoembryonic antigen (CEA), cancer antigen (CA) 19-9, and CA72-4 (Gao et al., 2013). With the application of tumor markers in diagnosing gastric cancer, clinical trials have made huge progress. Correspondingly, treatment methods like surgery, chemotherapy, chemoradiotherapy, and targeted and immune therapies have been widely used (Johnston and Beckman, 2019; Petryszyn et al., 2020). However, the molecular mechanism governing gastric cancer remains underexplored.

Glutathione S-transferase mu 2 (GSTM2), a member of a family of phase II detoxification enzymes with therapeutic implications (Hewawasam et al., 2016), has been widely studied in a variety of fields, such as pancreatic cancer (Peng et al., 2021), or hypertension (Zhou et al., 2007). For example, the higher GSTM2 expression is closely related to longer overall survival; therefore, GSTM2 becomes a possible prognostic factor for the chemosensitivity of pancreatic cancer (Peng et al., 2021). Meanwhile, the protein level of GSTM2 is decreased in SGC7901/DDP cells (Zou et al., 2019);

accordingly, it is reasonable to speculate that a low expression of GSTM2 may contribute to the malignant development of cells. These findings prompt an investigation into the relationship between the role of GSTM2 in gastric cancer.

Reportedly, changes in the ubiquitination of the protein contribute to the development of lung cancer (Jin et al., 2021). Besides, Ubiquitin-conjugating (E2) enzymes of the ubiquitination pathway are related to various cancers, including gastric cancer (Miyamoto and Saito, 2018), implying a possible connection between ubiquitination and gastric cancer. Bioinformatics analysis using Unibrowser predicted Smad Ubiquitination Regulatory Factor-1 (SMURF1) as an E3 ubiquitin ligase for GSTM2. Meanwhile, as an E3 ubiquitin ligase, SMURF1 regulates the proliferation, migration, and invasion of gastric cancer cells. In detail, SMURF1 knockdown markedly inhibits tumor growth (Tao et al., 2017). Additionally, downregulation of SMURF1 in gastric cancer enables miR-1254 to inhibit cell functions (Jiang et al., 2019). Previous findings hinted that SMURF1 expression may impact gastric cancer, while its role in GSTM2 ubiquitination in gastric cancer remains unexplored.

With regard to the fact that GSTM2 expression or SMURF1 expression is associated with gastric cancer, SMURF1 is an E3 ubiquitin ligase for GSTM2, and ubiquitination also influences gastric cancer; the current study explored whether and how SMURF1 could influence gastric cancer via mediating GSTM2 ubiquitination.

Materials and methods

Bioinformatics analysis

The expression of GSTM2 or SMURF1 in stomach adenocarcinoma (STAD) was demonstrated in The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) (Tomczak et al., 2015). Ubiquitin ligase enzymes for GSTM2 were predicted using Unibrowser (http://ubibrowser.ncpsb.org/) (Wang et al., 2022).

Cell culture

Gastric cancer-derived cell lines SNU-1 (CRL-5971) and AGS (CRL-1739) were provided by the American Type Culture Collection (ATCC) (Manassas, Virginia, USA), while MKN-74 (80104) and SNU-216 (00216) cell lines were obtained from the Korean Cell Line Bank (KCLB) (Chongno-gu, Seoul, Korea). A normal gastric epithelium cell line (GES-1) was purchased from Nanjing Cobioer Biosciences Co., Ltd (Nanjing, Jiangsu, China). Every cell line underwent mycoplasma identification and Short Tandem Repeat (STR) analysis. Cell thawing was performed first in a 37˚C water bath with gentle agitation for approximately 2 minutes. Subsequently, the cells were transferred into a centrifuge tube containing 9 mL of complete medium and then centrifuged at 1500 rpm for 5 minutes. Finally, the cells were resuspended with a complete medium for another 5 minutes at 1500 rpm. The complete medium comprised Roswell Park Memorial Institute (RPMI)-1640 medium (30-2001, ATTC, Manassas, Virginia, USA) and 10% fetal bovine serum (FBS) (30-2020, ATCC, USA), which was also employed to culture these cell lines up to their eighth passage when they could be used. Incubation was at 37°C with 5% CO_2.

Cell transfection

The full-length sequences of GSTM2 and SMURF1 were separately cloned into pcDNA3.1 vector (V79520, ThermoFisher, Waltham, Massachusetts, USA), thus generating the plasmids pcDNA3.1-GSTM2/SMURF1. Small interfering RNA (siRNA) targeting GSTM2 and SMURF1 were provided by Genepharma company (Shanghai, China) (siGSTM2 sense: 5'-AUCAUAAGC GAUGAAAUCCAC-3'; anti-sense: 5'-GGAUUUCAU CGCUUAUGAUGU-3'; siSMURF1 sense: 5'- GUUGU ACACUAAUGUUCUAUG-3'; anti-sense: 5'- UAGAA CAUUAGUGUACAACUU-3'). Lipofectamine 3000 Reagent (L3000001, ThermoFisher, USA) was used to facilitate transfection according to the manufacturer's protocol. Specifically, cells were seeded into 96-well plates until reaching 70-90% confluence for transfection. Ten μL Opti-MEM™ (31985062, ThermoFisher, USA) was added to two tubes of Lipofectamine 3000 reagent for dilution. Subsequently, pcDNA3.1-GSTM2/SMURF1 and siGSTM2/siSMURF, at a total volume of 0.2 μg, were diluted in 10 μL Opti-MEM™ to obtain the premix. This was then thoroughly mixed with 0.4 μL Lipofectamine 3000 reagent. Diluted DNA was added into two tubes of diluted Lipofectamine 3000 reagent. After a 10-minute incubation at room temperature, pcDNA3.1-GSTM2/SMURF1 and siGSTM2/siSMURF1 were separately transfected into cells. After a two-day incubation at 37°C, transfected cells were visualized and analyzed. The pcDNA3.1 vector or siNC (siN0000001-1-10, Ribobio, Guangzhou, China) was also transfected into cells as a negative control (NC).

Cyclohexanecarboxamide (CHX) treatment

CHX was brought from Hubei Qifei Pharmaceutical & Chemical Co., Ltd (Tianmen, Hubei, China) and dissolved in distilled water to 0.2 mg/mL (Li et al., 2021). AGS or MKN-74 cells transfected with pcDNA3.1-SMURF1 or empty vector were then cultured in growth medium containing CHX solution for 8 hours (h), and the relative GSTM2 protein levels in AGS or MKN-74 cells were determined at 0, 2, 4, 6, and 8h.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

The expression of GSTM2 in AGS and MKN-74 cells was tested to determine the effect of GSTM2, and

the effect of overexpressing or inhibiting SMURF1 expression in AGS and MKN-74 cells was also measured. Total RNA was extracted using Trizol Reagent (T9424, Sigma-Aldrich, St. Louis, Missouri, USA) in strict accordance with the manufacturer's protocol. Afterward, the Revert Aid first-stand complementary DNA (cDNA) synthesis kit (K1622, Solarbio, Beijing, China) was employed to reverse transcribe total RNA into cDNA. The synthesized cDNA was subjected to qRT-PCR using synergy brands (SYBR) Green kit (QR0100, Sigma-Aldrich, USA) in a Light Cycle 480 machine (Roche Diagnostics, Basel, Switzerland, Germany). The reaction was performed at 95°C for 2 minutes, followed by 40 cycles at 95°C for 30 seconds and 72°C for another 30 seconds. All reactions were performed in triplicate to ensure the accuracy of the experiment. The relative expression levels of mRNA were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and normalized with GAPDH as the internal reference. The primer sequences used in this experiment are: GSTM2 forward primer: 5'-CCTTGAGAGAAACCAAGTAT-3'; GSTM2 reverse primer, 5'-ACAGCCATCTTTGTGAAC-3'; SMURF1 forward primer, 5'-TTTGCAAGGTTC TACAGG-3'; SMURF1 reverse primer, 5'-CTTCTC GTAGAGCTTCTCAT-3'; GAPDH forward primer, 5'-AGGGCTGCTTTTAACTCT-3'; GAPDH reverse primer, 5'-GGGTGGAATCATATTGGA-3'.

Immunoprecipitation (IP)

The Protein A/G Immunoprecipitation Kit (M2400, Solarbio, China) was used to detect the effect of SMURF1 inhibition on GSTM2 ubiquitination in AGS or MKN-74 cells. The growth medium of AGS or MKN-74 cells was removed, and cells were cultured in fresh growth medium. AGS or MKN-74 cells were collected under non-denaturing conditions. After the removal of growth medium, cells were washed once with 5 mL cold phosphate-buffered saline (PBS) (P1020, Solarbio, China). The culture plate was placed on ice. Cell Lysis Buffer (1 mL, 10×, #9803, Cell Signaling Technology, Shanghai, China) was diluted with 9 mL distilled water. Cold, diluted Cell Lysis Buffer was added to the plate (0.5 mL/well) after the PBS was removed and cultured on ice for 5 minutes. Cells were gently scraped, and the Cell Lysis Buffer was transferred into a microcentrifuge tube, which was placed on ice for 10 minutes and then centrifuged at 14000×g for 10 minutes at 4°C. The suspension was transferred into fresh tubes. Protein A/G beads for IP were washed with prepared Cell Lysis Buffer and resuspended. Twenty-five μL of the suspension was placed into a fresh microcentrifuge tube on a Magnetic Separation Rack (#7017, Cell Signaling Technology, China) for 10 seconds. When the solution turned clear, Cell Lysis Buffer (500 μL) was added to the precipitates after the supernatant was discarded. Again, the tube was placed in a Magnetic Separation Rack, and the Lysis Buffer was removed after the solution became clear. Subsequently, Cell Lysis Buffer (200 μL) was added to the prepared Protein A/G beads and incubated at room temperature for 20 minutes under rotation. A magnetic Separation Rack was used to separate beads and lysates, which were transferred into a new microcentrifuge tube, and the bead precipitates were removed. Anti-GSTM2 antibody (ab196503, 1/500, 26 kDa, Abcam, UK) was added to tubes containing 200 μL pre-prepared cell lysate and incubated overnight at 4°C. Beads received the same treatment as mentioned above, followed by the addition of lysate and antibody solution into tubes containing bead precipitates for a 20-minute incubation at room temperature under rotation. Beads and lysate were separated with the help of a Magnetic Separation Rack, and Cell Lysis Buffer (500 μL) was used to rinse precipitates 5 times on ice, which were resuspended in sodium dodecyl sulfate (SDS) loading Buffer. After thorough mixing, the sample underwent centrifugation to obtain precipitates and was then heated to 100°C for 5 minutes, followed by centrifugation at 5000×g for 1 minute at room temperature. A Magnetic Separation Rack was used to separate beads, and the suspension was transferred to new tubes. Next, the sample (20 μL) was subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and western blot analysis.

Cell counting kit-8 (CCK-8) assay

The CCK-8 assay kit (ab228554, Abcam, UK) was used to effectively determine the viability of AGS or MKN-74 cells under GSTM2 overexpression, GSTM2 inhibition, or SMURF1 overexpression. AGS or MKN-74 cells were grown in 96-well plates at a density of 2×10^3 cells/well (total volume of 100 μL). After AGS or MKN-74 cells were transfected with GSTM2 over-expression, SMURF1 overexpression or siGSTM2, 10 μL CCK-8 reagent was added for a four-hour incubation at 37°C with 5% CO_2. The optical density (OD) value was measured using a microplate reader (RNE90002, REAGEN, Shenzhen, China) at a wavelength of 450 nm.

Wound healing assay

Cells were seeded into six-well plates (2×10^6 cells/well) followed by three-day culture in RMPI-1640 Medium (30-2001, ATCC, USA) and 10% FBS (30-2020, ATCC, USA) under a humid atmosphere at 37°C with 5% CO_2. When the cell monolayer reached 100% confluence, a sterile pipette was applied to create a linear wound. Subsequently, the plates were washed with sterile PBS (P1020, Solarbio, China) three times to remove cellular debris, and the treated cells were incubated in serum-free RMPI-1640 medium at 37°C with 5% CO_2. The surface area of the scratch was measured at the time of scratching and after 24 hour incubation. The relative migration of cells under each condition was evaluated under a Leica INM 200 UV microscope (Leica, Wetzlar, Germany) at a fixed ×100

magnification, and representative images of the scratch area were obtained. ImageJ analysis software (National Institutes of Health, Bethesda, Maryland, USA) was used to analyze images and calculate data.

Transwell assay

To investigate the effect of GSTM2 and/or SMURF1 expression on the invasion of AGS or MKN-74 cells, Transwell chambers (353097, Corning, Corning, New York, USA) in a 24-well plate containing 8-μm wells were used (Pijuan et al., 2019). Trypsin-EDTA solution (T1300, Solarbio, China) was used to trypsinize the AGS or MKN-74 cells transfected with GSTM2 overexpression, SMURF1 overexpression or siGSTM2, followed by centrifugation in serum-free RMPI-1640 medium three times to obtain the cell suspension. Matrigel (356234, Solarbio, China) was thawed at 4°C for 24h. After the Matrigel transformed into a liquid state, 300 μL serum-free RMPI-1640 medium was thoroughly mixed with 60 μL Matrigel. The upper compartment of the Transwell chambers was coated with 100 μL diluted Matrigel evenly at 4°C and then placed in an incubator at 37°C for 4h. Afterwards, 100 μL of the cell suspension was added to each well in the upper compartment. Meanwhile, 500 μL RMPI-1640 medium supplemented with 10% FBS was placed in the lower compartment. Transwell chambers were placed in an incubator at 37°C for 24h. Next, cells invading the lower chamber were fixed with 4% paraformaldehyde (P1110, Solarbio, China) for 30 minutes at 4°C, stained with 0.1% crystal violet (G1063, Solarbio, China), and incubated at room temperature for another 30 minutes. A cotton swab was used to carefully remove non-invading cells in the upper chamber after the chambers were washed twice with PBS. The invading cells in the lower compartment were photographed with an Olympus IX70 inverted microscope at ×250 magnification (Olympus, IX70, Tokyo, Japan) in five randomly selected visual fields. These were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, Maryland, USA) (Fu et al., 2019).

Animal treatment

Male BALB/C nude mice (n=30), six weeks old, weighing an average of 23 g, were bought (Cavens, Changzhou, China). Mice were acclimatized for one week before the experiment. Mice were housed in a controlled environment at 26°C, 60% humidity, and a 12/12-h light/dark cycle. Meanwhile, they received food and water freely. All procedures in the experiment were performed in strict accordance with Animal Care and Use Guidance in China. The ethical approval for the study protocols was acquired from the Ethics Committee of Zhejiang Baiyue Biotech Co., Ltd for Experimental Animal Welfare (approval number: ZJBYLA-IACUC-20231222).

To perform the *in vivo* experiment, mice were divided into six groups, with 5 mice per group. After transfection with/without GSTM2 or SMURF1 plasmids, AGS or MKN-74 cells were subjected to centrifugation in normal saline (IN9000, Solarbio, China) at 1000 rpm for 5 minutes to obtain a cell suspension (1×10^6 cells/well), which was used in the xenograft experiment. Mice received a subcutaneous injection of 0.2 mL cell suspension or normal saline in the right flank of the armpit (n=5) within 30 minutes. Mice in the Control (Con) groups were treated with normal saline, the GATM2+vector groups were subcutaneously injected with a suspension of AGS/MKN-74 cells with GSTM2 overexpression and empty vector, and the GSTM2+SMURF1 groups were treated with a suspension of AGS/MKN-74 cells containing GSTM2 and SMURF1 overexpression. Mice were under close observation, and at days 0, 7, 14, 21, and 28, the size of the tumor in mice treated with the AGS or MKN-74 cell suspension was measured using a vernier caliper. The volume of the tumor was calculated according to the formula: volume (mm^3) =0.5× (length × $width^2$). On the 28^{th} day, mice were sacrificed after receiving anesthesia with an intramuscular injection of ketamine (90 mg/kg) (SML1873, Sigma-Aldrich, USA) and xylazine (7.5 mg/kg) (X1126, Sigma-Aldrich, USA). The tumor was collected, photographed, and subsequently weighed.

Western blot analysis

AGS or MKN-74 cells were washed with PBS, and total protein was isolated with Radio Immunoprecipitation Assay (RIPA) Lysis Buffer (R0010, Solarbio, China). In addition, 250 mg of tumor tissues was collected and subjected to a homogenizer for 1 minute. The addition of the homogenate to 1× RIPA Lysis Buffer (R0010, Solarbio, China) was performed 30 minutes before centrifugation at 4°C for 15 minutes. The supernatant was collected as protein. The sample concentration was tested using a bicinchoninic acid (BCA) protein assay kit (23227, ThermoFisher, USA) following the manufacturer's instructions. Next, the sample proteins were subjected to 10% SDS-PAGE and then were transferred to polyvinylidene fluoride (PVDF) membranes (BSP0161, Solarbio, China). The membranes were blocked with 5% skim milk (D8340, Solarbio, China) for 2h at room temperature, and incubated with primary antibodies, including anti-GSTM2 (ab196503, 1/500, 26 kDa, Abcam, UK), anti-SMURF1 (ab57573, 1/1000, 86 kDa, Abcam) (Fang et al., 2020), anti-E-cadherin (ab76319, 1/10000, 97 kDa, Abcam), anti-Vimentin (ab92547, 1/1000, 54 kDa, Abcam), and anti-GAPDH (ab8245, 1/5000, 36 kDa, Abcam) at 4°C overnight. The membranes were then rinsed three times with Tris-Buffered Saline and Tween-20 (TBST) (T917680, Macklin, Shanghai, China) for 10 minutes each time. Similarly, the appropriate dose of horseradish peroxidase (HRP) labeled anti-mouse/rabbit secondary antibody (KC-MM-035/KC-RB-035, 1:1000, Aksomics, Shanghai, China) was added for further

incubation for 1h at room temperature. The membranes were then washed several times. Finally, the labeled proteins were visualized using an enhanced chemiluminescence (ECL) kit (ab65623, Abcam, UK), and a chemiluminescence imager (Tanon 5200, Tanon, Shanghai, China). The gray value and gray coefficient ratio of each protein were analyzed and calculated with ImageJ analysis software (National Institutes of Health, Bethesda, USA).

Statistical analysis

All data were presented as the mean ± standard deviation. Statistically significant differences among more than two groups were assessed using one-way analysis of variance (ANOVA). GraphPad Prism 8.0 (GraphPad Software, San Diego, California, USA) was used for statistical analysis. A significant variance was indicated when $p<0.05$.

Results

GSTM2 was lowly expressed and SMURF1 was highly expressed in STAD

The expression of GSTM2 and SMURF1 in STAD (n=415) and normal samples (n=34) was analyzed using the TCGA database. GSTM2 levels were lower in STAD than in normal tissues (Fig. 1A, p=6.64E-03). In contrast, SMURF1 levels were higher in STAD than in normal tissues (Fig. 1B, p=6.95E-11). Unibrowser predicted that SMURF1 was a ubiquitin ligase enzyme for GSTM2 (Fig. 1C).

GSTM2 regulated the malignant phenotype of AGS or MKN-74 cells

Compared with GES-1 cells, relative GSTM2 protein expression levels in SNU-1, AGS, MKN-74, or SNU-216 cells were reduced, and relative SMURF1 expression was augmented, especially in the AGS and MKN-74 cell groups (Fig. 2A-C, $p<0.001$). Due to the obvious expression change in AGS and MKN-74 cell groups, these two cell types were selected for further study. Relative GSTM2 mRNA levels were significantly enhanced in both AGS and MKN-74 cells transfected with plasmids overexpressing GSTM2 compared with those in vector-transfected cells (Fig. 2D-E, $p<0.001$); GSTM2 levels were markedly suppressed in AGS or MKN-74 cells with siGSTM2 relative to those in the siNC group (Fig. 2D-E, $p<0.01$). The relative cell viability was significantly decreased in AGS and MKN-74 cells transfected with the pcDNA3.1-GSTM2 plasmid compared with that in vector-treated cells (Fig. 2F-G, $p<0.01$). Inversely, the viability was enhanced in AGS and MKN-74 cells after siGSTM2 transfection relative to that in siNC-transfected cells (Fig. 2F-G, $p<0.01$). Compared with the vector group, migration rates in AGS and MKN-74 cells were downregulated after transfection

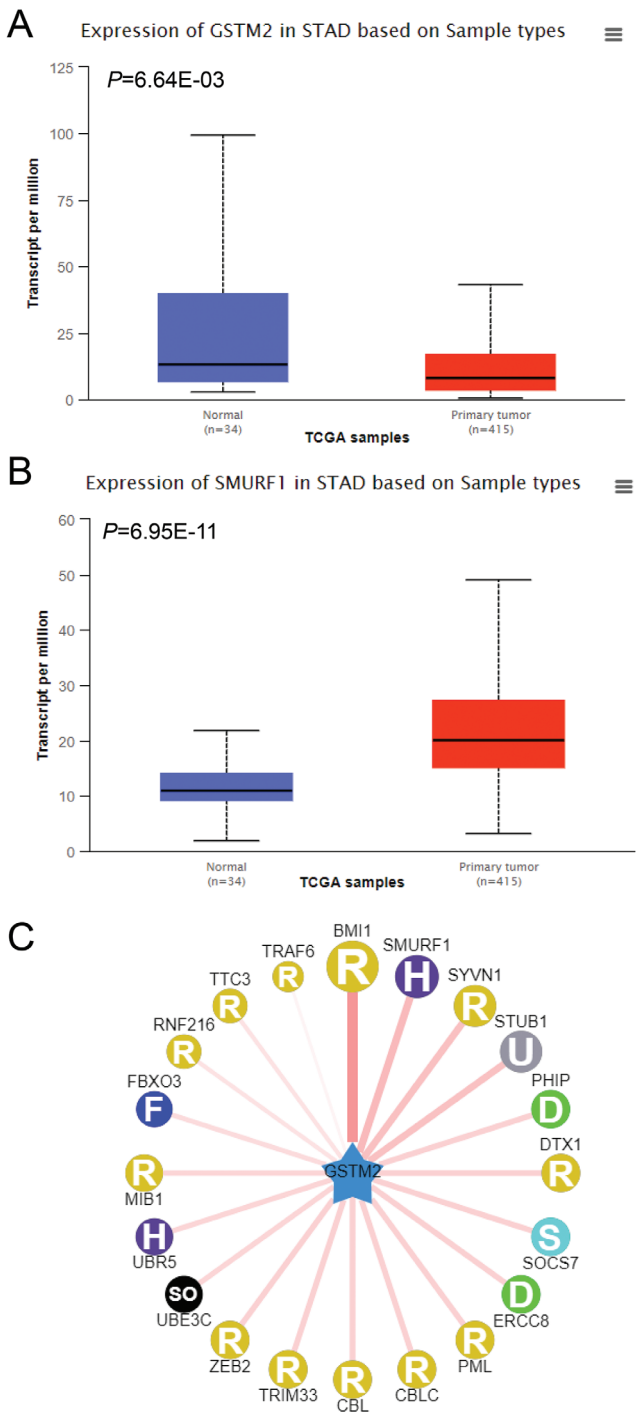

Fig. 1. Expression of GSTM2 or SMURF1 in primary tumor. **A.** The expression of GSTM2 in STAD was detected in TCGA (https://portal.gdc.cancer.gov/). **B.** The expression of SMURF1 in STAD was determined using TCGA (https://portal.gdc.cancer.gov/). **C.** Unibrowser (http://ubibrowser.ncpsb.org/) predicted the ubiquitin-ligase enzymes of GSTM2. The width of the pink edge reflects the confidence of the interaction; R, RING; H, HECT; U, UBOX; D, DWD; S, SOCS/VHL/BC-box; SO, SINGLE_OTHER; F, F-box. TCGA, The Cancer Genome Atlas; GSTM2, glutathione S-transferase mu 2; SMURF1, Smad Ubiquitination Regulatory Factor-1; STAD, stomach adenocarcinoma.

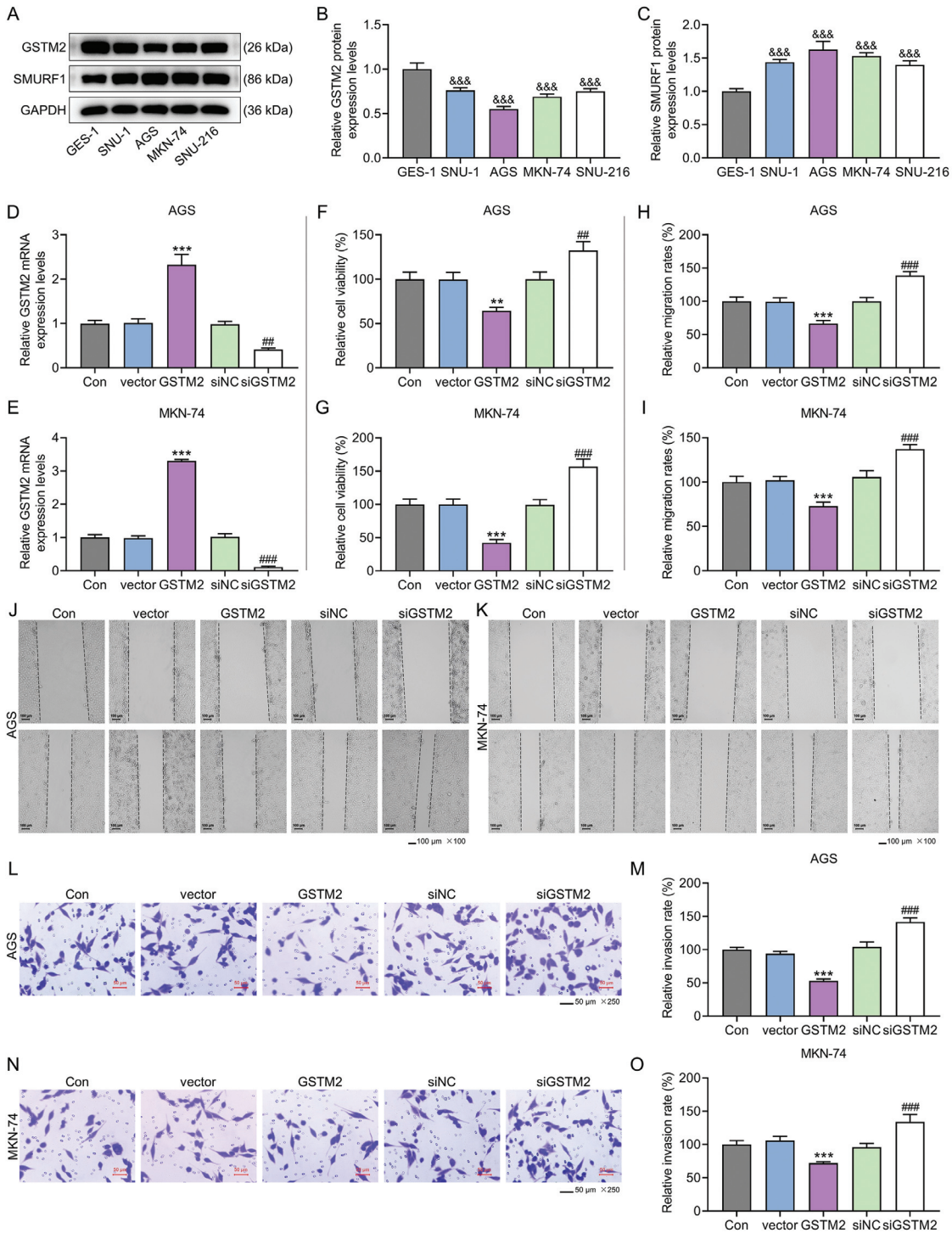

Fig. 2. Effects of overexpressing or silencing GSTM2 on viability, migration, and invasion of AGS and MKN-74 cells. **A-C.** GADPH was used as an internal reference in western blotting to test the expression of GSTM2 or SMURF1 in SNU-1, AGS, MKN-74, and SNU-216 gastric cancer-derived cell lines, as well as GES-1 cells. **D, E.** qRT-PCR was performed to determine GSTM2 expression in AGS and MKN-74 cells under the effect of GSTM2 overexpression or siGSTM2, with GAPDH as an internal reference. **F, G.** Viability of AGS and MKN-74 cells under the influence of GSTM2 overexpression or siGSTM2 was detected using CCK-8 assay. **H-K.** Migration rates in AGS and MKN-74 cells in the presence of GSTM2 overexpression or siGSTM2 were measured with the help of a wound healing assay after 24h at ×100 magnification on a scale of 100:1. **L-O.** Invasion rates in AGS and MKN-74 cells in the presence of GSTM2 overexpression or siGSTM2 were determined with a Transwell assay at ×250 magnification on a scale of 50:1 ($^{\&\&\&}p<0.001$, vs. GES-1; $^{**}p<0.01$, $^{***}p<0.001$, vs. vector; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, vs. siNC). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GSTM2, glutathione S-transferase mu 2; SMURF1, Smad Ubiquitination Regulatory Factor-1; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; CCK-8, cell counting kit-8, siGSTM2, small interfering glutathione S-transferase mu 2.

with plasmid overexpressing GSTM2 (Fig. 2H-K, $p<0.001$). On the contrary, migration rates were enhanced in AGS and MKN-74 cells after treatment with siGSTM2 when compared with siNC-transfected cells (Fig. 2H-K, $p<0.001$). Invasion rates in AGS and MKN-74 cells were reduced after treatment with plasmid overexpressing GSTM2 relative to that in vector-treated cells (Fig. 2L-O, $p<0.001$), and the rate was augmented

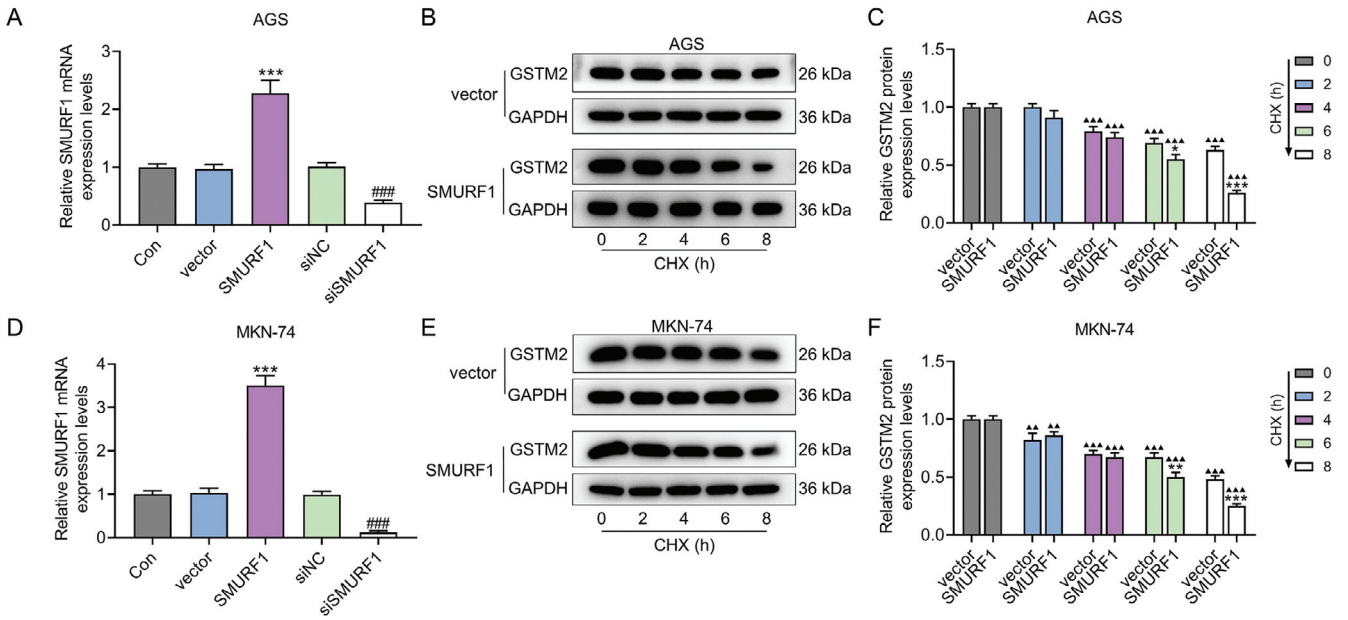

Fig. 3. Effects of overexpressing SMURF1 on GSTM2 level in AGS and MKN-74 cells. **A/D.** qRT-PCR was conducted to determine the SMURF1 mRNA expression in AGS and MKN-74 cells under the effect of SMURF1 overexpression or siSMURF1, with GAPDH as an internal reference. **BC/EF.** After upregulation of SMURF1 by cell transfection, AGS and MKN-74 cells were treated with CHX for 0, 2, 4, 6, 8h and then subjected to western blotting with GAPDH serving as the internal control (*$p<0.05$, **$p<0.01$, ***$p<0.001$, vs. vector; ##$p<0.01$, ###$p<0.001$, vs. siNC; ▲▲$p<0.01$, ▲▲▲$p<0.001$, vs. 0). qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; SMURF1, Smad Ubiquitination Regulatory Factor-1; CHX, Cyclohexanecarboxamide; GSTM2, glutathione S-transferase mu 2; siSMURF1, small interfering Smad Ubiquitination Regulatory Factor-1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

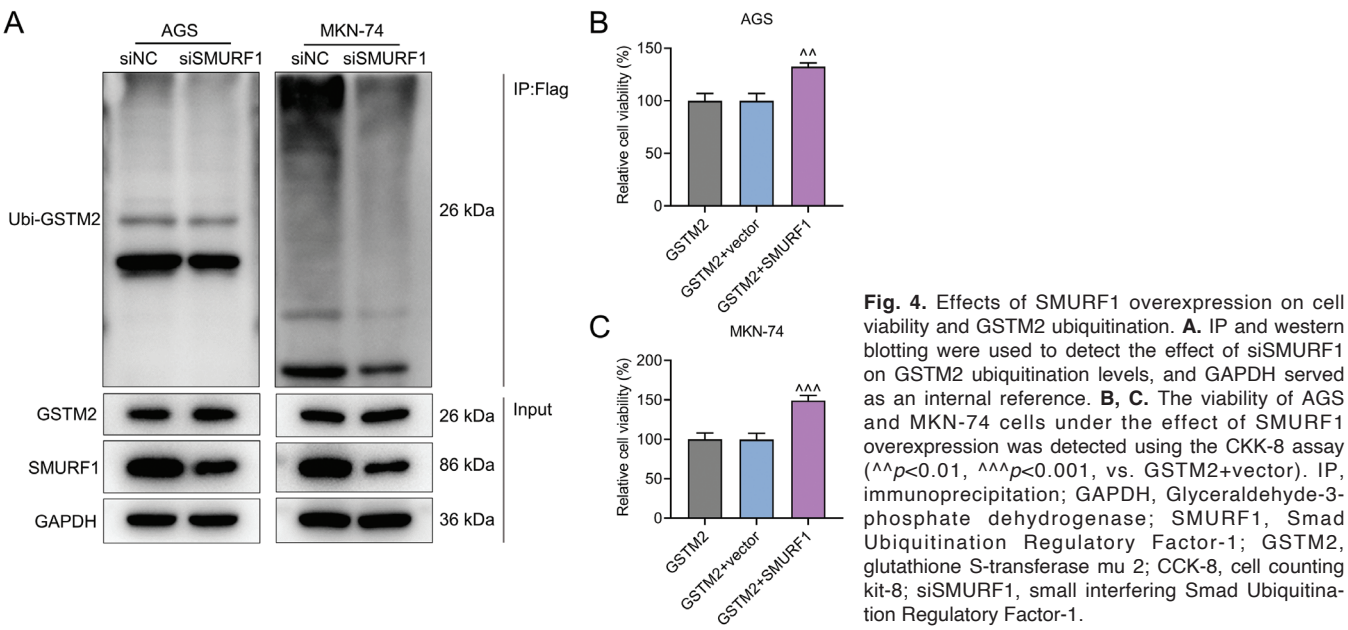

Fig. 4. Effects of SMURF1 overexpression on cell viability and GSTM2 ubiquitination. **A.** IP and western blotting were used to detect the effect of siSMURF1 on GSTM2 ubiquitination levels, and GAPDH served as an internal reference. **B, C.** The viability of AGS and MKN-74 cells under the effect of SMURF1 overexpression was detected using the CKK-8 assay (^^$p<0.01$, ^^^$p<0.001$, vs. GSTM2+vector). IP, immunoprecipitation; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; SMURF1, Smad Ubiquitination Regulatory Factor-1; GSTM2, glutathione S-transferase mu 2; CCK-8, cell counting kit-8; siSMURF1, small interfering Smad Ubiquitination Regulatory Factor-1.

in AGS and MKN-74 cells with siGSTM2 when compared with that in siNC-transfected cells (Fig. 2L-O, $p<0.001$).

SMURF1 silencing suppressed GSTM2 ubiquitination levels in AGS and MKN-74 cells

The relative SMURF1 mRNA expression was elevated in AGS and MKN-74 cells after transfected with plasmid overexpressing SMURF1 (Fig. 3A,D, $p<0.001$). When compared with siNC-transfected cells, relative SMURF1 mRNA levels were greatly inhibited in AGS and MKN-74 cells with siSMURF1 (Fig. 3A,D, $p<0.01$). Compared with vector-transfected cells, relative GSTM2 protein levels in AGS and MKN-74 cells were markedly downregulated by SMURF1 overexpression at 6h and 8h after CHX addition (Fig. 3B,C,E,F, $p<0.05$). Relative GSTM2 protein levels in AGS and MKN-74 cells with or without SMURF1 overexpression were gradually reduced by CHX treatment at 2, 4, 6, and 8h (Fig. 3B,C,E,F, $p<0.01$). Also, the expression of GSTM2 was enhanced, and GSTM2 ubiquitination or SMURF1 levels were inhibited in AGS cells after transfection with siSMURF1. Similarly, the expression of GSTM2 was elevated, and GSTM2 ubiquitination or SMURF1 expression was suppressed in MKN-74 cells with siSMURF1 (Fig. 4A).

SMURF1 overexpression reversed the effect of overexpressed GSTM2 on the malignant phenotype of AGS and MKN-74 cells

Overexpressed SMURF1 increased the viability of AGS and MKN-74 cells compared with that of cells treated with empty vector in the presence of GSTM2 overexpression (Fig. 4B-C, $p<0.01$). Migration rates of AGS and MKN-74 cells were augmented by SMURF1 overexpression compared with that of cells treated with empty vector in the presence of GSTM2 overexpression (Fig. 5A-D, $p<0.001$). Likewise, overexpressed SMURF1 exerted a positive effect on the invasion rates of AGS and MKN-74 cells in comparison with that of cells transfected with empty vector in the presence of GSTM2 overexpression (Fig. 5E-H, $p<0.01$).

Overexpressed SMURF1 reversed the inhibitory effect of GSTM2 overexpression on tumor formation in gastric cancer

Compared with control cells, GSTM2 overexpression decreased the weight and volume of tumors from mice treated with the AGS or MKN-74 cell suspension (Fig. 6A-F, $p<0.01$). Overexpressed SMURF1 offset the effect of GSTM2 overexpression on tumor weight and volume (Fig. 6A-F, $p<0.001$).

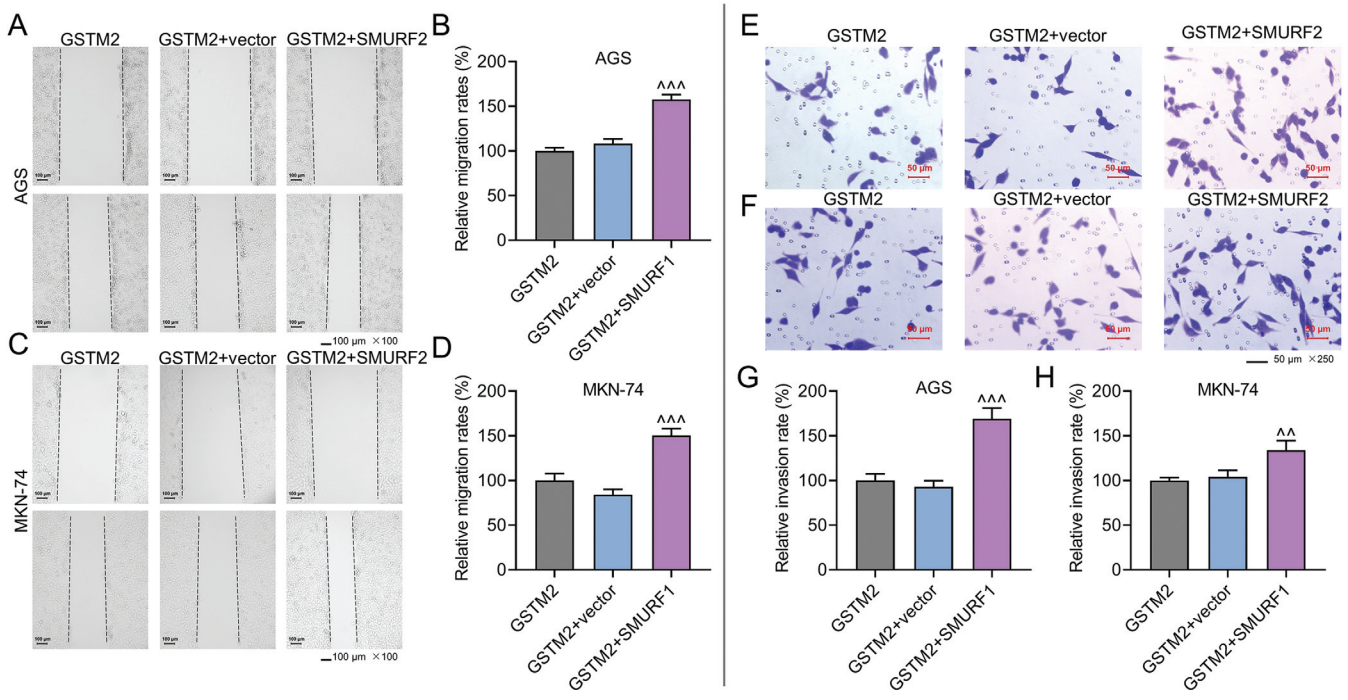

Fig. 5. Effects of SMURF1 overexpression on the migration and invasion rates of AGS and MKN-74 cells. **A-D.** The effect of SMURF1 overexpression on the migration rates of AGS and MKN-74 cells was measured via a wound healing assay at ×100 magnification on a scale of 100:1. **E-H.** The effect of SMURF1 overexpression on the invasion rates of AGS and MKN-74 cells were tested using a Transwell assay at ×250 magnification on a scale of 50:1 (^^$p<0.01$, ^^^$p<0.001$, vs. GSTM2+vector). SMURF1, Smad Ubiquitination Regulatory Factor-1; GSTM2, glutathione S-transferase mu 2.

The regulatory role of GSTM2 overexpression in the Vimentin and E-cadherin expression in gastric cancer was counteracted by overexpressing SMURF1

Relative SMURF1 protein expression levels were elevated in tumor tissues from mice in the GSTM2+ SMURF1 group in comparison with those in mice from the GSTM2+vector group (Fig. 7A,B,D,E, $p<0.01$). Relative GSTM2 protein levels were enhanced in tumor tissues from mice treated with the AGS or MKN-74 cell suspension with GSTM2 overexpression (Fig. 7A,C,D,F, $p<0.001$); SMURF1 overexpression completely reversed the positive role of GSTM2 overexpression (Fig. 1A-F, $p<0.001$). Moreover, GSTM2 overexpression enhanced relative E-cadherin protein expression levels and inhibited Vimentin protein levels in mouse tumors (Fig. 7G-L, $p<0.01$). Overexpression of SMURF1 increased the relative Vimentin protein expression levels and reduced E-cadherin protein levels in mouse tumors in the presence of GSTM2 overexpression (Fig. 7G-L, $p<0.01$).

Discussion

In this study, for the first time, we report the inhibitory effect of GSTM2 on gastric cancer and that SMURF1 deficiency reduces the ubiquitination degradation of GSTM2. This inhibited the development of gastric cancer, accompanied by suppressed cell proliferation, migration, and invasion *in vitro*, and inhibited tumor growth *in vivo*. SMURF1 has previously been reported to promote the proliferation, migration, and invasion of gastric cancer cells; however, this study found that it plays its role by regulating the degradation

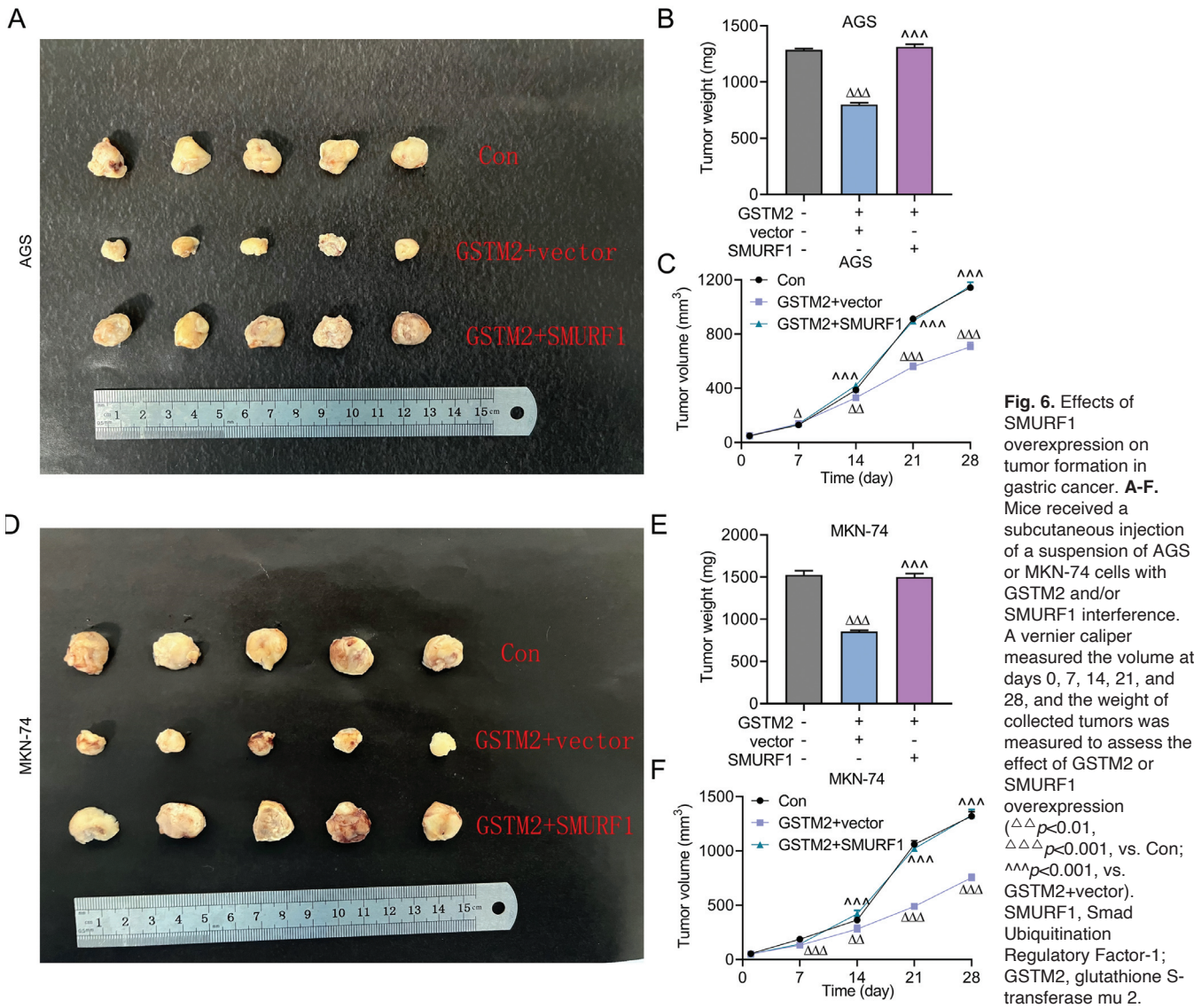

Fig. 6. Effects of SMURF1 overexpression on tumor formation in gastric cancer. **A-F.** Mice received a subcutaneous injection of a suspension of AGS or MKN-74 cells with GSTM2 and/or SMURF1 interference. A vernier caliper measured the volume at days 0, 7, 14, 21, and 28, and the weight of collected tumors was measured to assess the effect of GSTM2 or SMURF1 overexpression ($^{\Delta\Delta}p<0.01$, $^{\Delta\Delta\Delta}p<0.001$, vs. Con; $^{\wedge\wedge\wedge}p<0.001$, vs. GSTM2+vector). SMURF1, Smad Ubiquitination Regulatory Factor-1; GSTM2, glutathione S-transferase mu 2.

of GSTM2, revealing a new carcinogenic axis. This discovery enriches the understanding of the molecular mechanism of gastric cancer and provides a new potential target for its treatment.

GSTM2, a glutathione-S-transferase (GST) that belongs to a superfamily consisting of multifunctional and ubiquitous dimeric cytosolic enzymes (Bhattacharjee et al., 2013), is a phase II detoxification enzyme (Lan et al., 2022). It is involved in the detoxification of environmental pollutants and is of great importance in cell defense mechanisms against oxidative stress (Andonova et al., 2010). Meanwhile, GSTM2 shows a critical suppressive function and attenuates hepatic steatosis and inflammatory damage (Lan et al., 2022). Given its protective role in oxidative stress, the current study investigated the possible relationship between GSTM2 and gastric cancer. Intriguingly, TCGA analyses, a website collecting data on all types of cancers, revealed low expression of GSTM2 in primary tumors, indicating that GSTM2 expression may be related to the condition of cancer. Correspondingly, it was demonstrated that overexpression of GSTM2 downregulated the viability, migration rate, and invasion rates of AGS and MKN-74 cells, which are gastric cancer-derived cell lines. Moreover, tumor weight and volume were also suppressed by GSTM2 overexpression. These findings confirmed that GSTM2 expression is closely related to gastric cancer, implying that regulating GSTM2 expression may impact gastric cancer.

Additionally, the database analyses showed that GSTM2 has potential for ubiquitination, an essential posttranslational modification performed by ubiquitin-

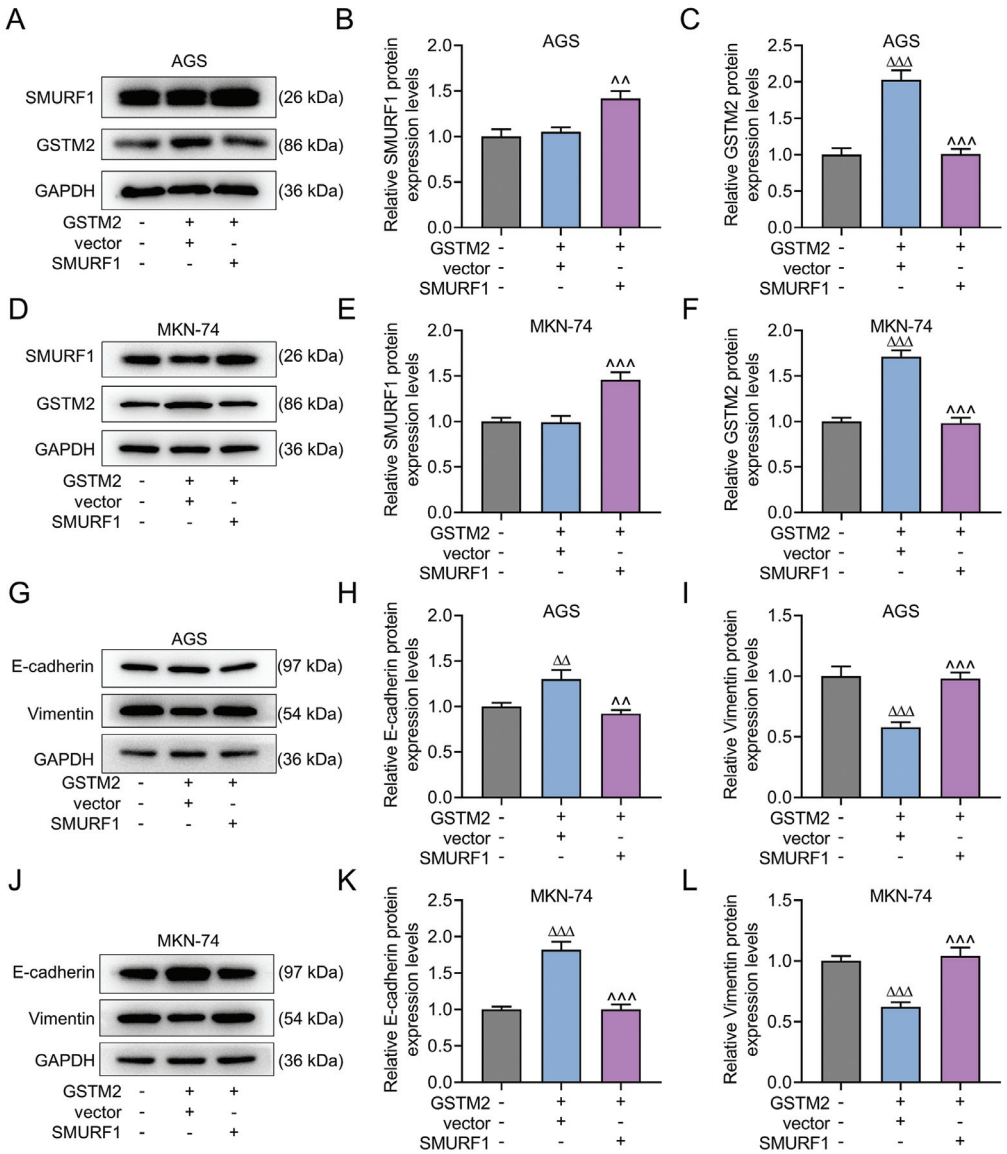

Fig. 7. Effects of GSTM2 or SMURF1 overexpression on the Vimentin and E-cadherin expression in gastric cancer. **A-F.** The effect of GSTM2 or SUMRF1 overexpression on the relative protein levels of SMURF1 or GSTM2 in mouse tumors was assessed using western blotting, with GAPDH as an internal reference. **G-L.** With GAPDH serving as an internal reference, western blotting was carried out to detect the effect of GSTM2 or SMURF1 overexpression on the relative protein expression levels of E-cadherin and Vimentin in mouse tumors ($^{\triangle\triangle}p<0.01$, $^{\triangle\triangle\triangle}p<0.001$, vs. Con; $^{\wedge\wedge}p<0.001$, $^{\wedge\wedge\wedge}p<0.001$, vs. GSTM2+vector). SMURF1, Smad Ubiquitination Regulatory Factor-1; GSTM2, glutathione S-transferase mu 2; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3 (Choo and Zhang, 2009). Different from E1 and E2, E3 ubiquitin ligase is a potential target in tumor therapy due to its characteristic substrate specificity. The ubiquitin-proteasome system is a main pathway for regulated protein degradation, whose dysregulation is crucial to human diseases (Hanna et al., 2019). Based on this, we inferred that the ubiquitination of GSTM2 may exert an effect on gastric cancer. According to the prediction carried out by the Unibrowser website, SMURF1, targeting substrate proteins for ubiquitination and proteasomal degradation (Yu et al., 2018), is an E3 ubiquitin ligase for GSTM2. SMURF1 is identified as a suppressor of cell growth, tumorigenesis, and drug resistance through mediating ubiquitination and degradation (Yu et al., 2018). SMURF1 also mediates the degradation of MEKK2 to activate the MEK1/2-ERK1/2-Snail pathway in gastric cancer cells (Ji et al., 2018). In line with these reports, this study carried out relevant assays and discovered that, by inhibiting SMURF1 expression, the ubiquitination level of GSTM2 was reduced, suggesting a correlation between SMURF1 expression and GSTM2 ubiquitination. Meanwhile, SMURF1 overexpression also suppressed the relative expression of GSTM2 protein and increased degradation levels. Therefore, this study conjectured that SMURF1 inhibition can suppress the level of GSTM2 degradation in cells, leaving a large amount of GSTM2 protein, which is conducive to a protective role.

Apart from influencing GSTM2 ubiquitination, SMURF1 expression itself may be linked to the development of gastric cancer (Tao et al., 2017). This study found that SMURF1 levels were elevated in primary tumors based on the TCGA results. It has been reported that SMURF1 plays a critical role in the development and progression of multiple tumors (Guo et al., 2020). Consistent with previous findings, this study also discovered that overexpression of SMURF1 significantly increased tumor weight and volume. Moreover, suppression of SMURF1 can reduce cell invasion of glioma cells (Chang et al., 2018). Overexpression of SMURF1 resulted in enhanced cell migration and invasion, whereas SMURF1 knockdown inhibited EGF-induced breast cancer cell migration and invasion (Kwon et al., 2013). The experimental data in this study demonstrated that overexpression of SMURF1 greatly increased migration and invasion rates. Cancer cell migration and invasion are an important initial step in cancer metastasis, a leading cause of cancer death (Duff and Long, 2017). Therefore, the positive effect of SMURF1 overexpression on invasion and migration rates supported the speculation that overexpressing SMURF1 potentially induces cancer, particularly gastric cancer. Finally, these findings establish a relationship between SMURF1, GSTM2, and gastric cancer.

In terms of tumor metastasis, it is necessary to examine the EMT-related proteins Vimentin and E-cadherin, molecular markers associated with tumor progression (Tian et al., 2013). The former is a mesenchymal marker associated with the acquisition of invasive properties, indicating invasive ability. The latter is a biological marker that can reduce cell mobility, invasion, and metastasis and maintain intact cell-cell interactions in human cancer (Luo et al., 2017; Zhang et al., 2020). Notably, western blotting was employed to detect the relative levels of Vimentin and E-cadherin proteins in AGS and MKN-74 cells. The results revealed that overexpression of GSTM2 significantly enhanced E-cadherin protein levels and inhibited Vimentin protein levels, suggesting a positive role of GSTM2 overexpression in maintaining cell stability. On the contrary, overexpression of SMURF1 decreased E-cadherin protein levels and suppressed Vimentin protein levels, indicating that overexpressed SMURF1 was detrimental to metastasis maintenance.

In this study, we found differences in the protein expression of GSTM2 and SMURF1 in different cell lines, which may be attributable to different Lauren classifications of gastric cancer patients. AGS is a Lauren intestinal-type gastric adenocarcinoma cell line, and MKN-45 is a Lauren diffuse-type gastric adenocarcinoma cell line. The expression profiles of GSTM2 and SMURF1 in AGS and MKN-45 cells need to be further sequenced to better understand the commonalities and specificities of GSTM2 and SMURF1 in different gastric cancer cells. In addition, further efforts should be made to detect the expression levels of GSTM2 and SMURF1 in clinical samples of gastric cancer patients, as well as a survival analysis based on Lauren's classification to evaluate the clinical significance of GSTM2 and SMURF1 in different histological subtypes of gastric cancer.

In summary, by leveraging TCGA, this study has demonstrated that GSTM2 expression is low and SMURF1 expression is high in STAD. The high GSTM2 expression exerts an anti-cancer effect, and SMURF1 silencing suppressed GSTM2 ubiquitination levels. In this way, the relation between SMURF1 and GSTM2 in gastric cancer is established. Moreover, as SMURF1 overexpression enhances the migration rate, invasion rate, and tumor weight, it is reasonable to conclude that inhibiting SMURF1 might help suppress gastric cancer. According to the role of GSTM2 degradation under the effect of SMURF1 inhibition, this study concludes that SMURF1 inhibition attenuates gastric cancer by suppressing GSTM2 degradation.

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Conflict of Interest. The authors declare that they have no conflicts of interest.

Availability of Data and Materials. The analyzed data sets generated during the study are available from the corresponding author upon reasonable request.

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