Silencing MASTL Inhibits Cell Proliferation and Migration of Gastric Cancer MGC-803 Cells Via Suprressing AKT, mTOR, p38 Signal Pathways

caixia An
Department of Gastroenterology, The First Hospital of Lanzhou University; Lanzhou 730000, P. R. China
hailong li
Gansu University of Traditional Chinese Medicine
Rong Niu
Department of External Chest, Gansu Provincial Cancer Hospital, Lanzhou 730030, P. R. China.
xiaoguang liu
Department of Gastroenterology, The First Hospital of Lanzhou University; Lanzhou 730000, P. R. China
Yonghua Hu
School of clinical Medicine, Gansu University of Chinese Medicine, Lanzhou 730000, P. R. China.
Zhuanxiong Wang
School of clinical Medicine, Gansu University of Chinese Medicine, Lanzhou 730000, P. R. China.
Xi Chen
School of clinical Medicine, Gansu University of Chinese Medicine, Lanzhou 730000, P. R. China.
Yongning Zhou (642101301@qq.com)
Department of Gastroenterology, The First Hospital of Lanzhou University; Lanzhou 730000, P. R. China.

Research

Keywords: MASTL, gastric cancer, migration, cell cycle, mRNA microarray

Posted Date: September 8th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-55295/v1

Abstract

Background: Microtubule-associated serine/threonine kinase (MASTL) functions to regulate chromosome condensation and mitotic progression. Emerging reports showed that aberrant MASTL expression is commonly implicated in various human cancers and act as an oncogene. This study aimed to discover the potential significance of MASTL in gastric cancer, and to uncover relevant mechanisms.

Methods: Lentivirus MASTL-shRNA was constructed and infected into MGC-803 cells to analysis its influences on cell proliferation by Green fluorescent protein (GFP)-based cellomics and colony formation assay, cell invasion and migration by transwell assay, apoptosis and cell cycle by flow cytometry detection, respectively. Nude mice and fluorescence imaging were used to characterize the regulation of tumor growth in vivo. Affymetrix mRNA microarray assay combined KEGG enrichment analysis were used to screen relevant molecules related to MASTL silencing. Finally several aberrantly expressed genes were validated by quantitative reverse transcription PCR(RT-qPCR)and western blot detection.

Results: Silencing MASTL significantly inhibited cell proliferation, migration and invasion, arrested cell cycle at G1 stage. Silencing MASTL reduced tumor growth in nude mice, and fluorescence imaging indicated that the total radiant efficiency of mice in the Lv-shMAST group was markedly reduced compared with in mice in the Lv-shCtrl group *in vivo*. Affymetrix mRNA microarray assay revealed that 124 genes upregulated, 167 genes downregulated. RT-qPCR and western blotting validation showed that cyclin dependent kinase 6(CDK6), bone morphogenetic protein 2(BMP2), snail family transcriptional repressor 2(SNAI2), phosphorylation-mechanistic target of rapamycin kinase (p-mTOR), phosphorylation-AKT serine/threonine kinase (p-AKT) and phosphorylation-p38 kinase (p-p38) are downregulated, and cyclin dependent kinase inhibitor 1A (CDKN1A) is upregulated.

Conclusions: Silencing MASTL could significantly inhibit cell growth, migration ability, induce apoptosis, arrest cell cycle at G1 stage, and the mechanisms of which were mediated via inactivation of mTOR, AKT, p38 signal pathways.

Background

Global cancer statistical data estimated 951,600 new gastric cancer cases and 723,100 cancer-related deaths in the world in 2012 [1, 2], indicating that gastric cancer is the fifth most common cancer and the third most common cancer-related death worldwide [3]. In China, gastric cancer has also shown increases in incidence and eventual mortality as a commonly diagnosed malignancy [1]. Gastric cancer risk factors include infection with Helicobacter pylori (H. pylori), which accounts for more than 60% of all gastric cancer cases [4, 5], tobacco smoking, dietary factors such as eating pickled vegetables, and obesity[6]. Despite recent advancements in the treatment and prevention of gastric cancer, the overall survival rate of gastric cancer patients remains low, which could be due to delayed diagnosis. For example, most gastric cancer patients have local and distant metastases at diagnosis [7], which, if diagnosed early, may allow more than 90% of patients to survive for at least five years post-detection or to be completely cured[6].

Thus, finding novel biomarkers for early detection and prediction of treatment responses, as well as prognosis and identification of novel targets for effective control of gastric cancer, could help medical oncologists reduce the gastric cancer burden clinically.

To this end, microtubule-associated serine/threonine kinase-like enzyme (MASTL, also known as Greatwall, or Gwl) is a serine/threonine kinase that is active during mitotic division that regulates chromosome condensation and mitotic progression through the phosphorylation of cyclinB-Cdk1 [8]. Previous studies have demonstrated that aberrant MASTL expression occurs in various human cancers, including breast [9, 10], gastric [11], and colorectal [12], and that MSATL overexpression is associated with poor patient survival [13], and resistance to chemotherapy [10, 11] and tumor recurrence [13]. In contrast, knockdown of MASTL expression using RNAi induced tumor cell cycle arrests and inhibits tumor cell invasion and metastasis in colon cancer [11]. However, to date, there is still no in vitro study of MASTL shRNA in gastric cancer and therefore, in this study, We assessed the effects of MASTL knockdown on regulation of gastric cancer cell viability, proliferation, apoptosis, and gene expressions in vitro. We provide novel insights regarding MASTL in the development and progression of gastric cancer, and whether the targeting of MASTL expression using MASTL shRNA is useful as a potential therapeutic or detection strategy of MASTL expression as a novel potential biomarker for gastric cancer patients.

Materials And Methods

Cell lines and cell preparation

Gastric cancer cell lines MGC-803 were provided by the Type Culture Collection of Cancer Institute and Hospital, Chinese Academy of Medical Sciences (CAMS) (Beijing, China). MGC-803 cells were maintained in DMEM supplemented with 100 IU/mL Penicillin and 100 μg/mL Streptomycin, and 10% heat-inactivated fetal bovine serum in a humidified cell incubator having an atmosphere of 5% CO_2 at 37˚C. All cells reaching to exponential growing stage were used for further experiments.

Lentiviral infection of MGC-803 cells

Human gastric cancer MGC-803 cells were plated on six-well plates at 5×10^4 cells/well and incubated at 37℃ at 5×10^4 cells/well and incubated at 37℃ in 50 mL/L CO_2 until 30% confluence was reached. The study was designed as both groups: negative control (shCtrl, transfected with GFP lentivirus) and shMASTL group (shMASTL, transfected with shMASTL GFP lentivirus). An appropriate amount of lentivirus was added according to the multiplicity of infection (MOI). The cells were repeatedly cultured in normal culture medium after 12 h. GFP-tagged gene expression was observed under a fluorescence microscope at 3 d after transfection, and cells with a transfection efficiency > 80% using the Image J software (National Institute of Heath, Bethesda, MD, USA) were selected for subsequent analyses. Cells were harvested at 48h after post-transfection for further analysis.

Silencing effect detection by qPCR and western blot analysis

To detect silencing efficiency of MASTL knockdown in MGC-803 cells, RT-qPCR and western blot analysis were utilized. Briefly, Total RNA was extracted from tissues or cultured cells using RNAiso Plus reagent (Takara Bio, Dalian, China) and used to synthesize first-strand cDNA with the Prime ScriptTM RT Reagent Kit (Takara Bio, Dalian, China). For mRNA analysis, RT-qPCR was performed using a SYBR Master Mixture (Yeasen, Shanghai, China) in Bio-Rad CFX96 Real-time PCR system. and the following protocol: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec, in which fluorescence was acquired). GAPDH levels were measured as an internal control. The primer sequences (Takara Bio, Dalian, China) used were listed in Table1. At the end of all PCR cycles, melting curve were performed to analysis the specificity of PCR product. Each sample was run in triplicates for analysis. The level of MASTL mRNA was normalized to GAPDH mRNA using the $2^{-\Delta\Delta Ct}$ method. The experiment was run in triplicates and repeated at least once.

Gastric cancer of shMASTL and shCtrl group were collected and washed twice with PBS and lysed in ice-cold lysis buffer for 5 min (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM ethylene diamine tetraacetic acid, 1 mM PMSF, and 1% sodium deoxycholate). Total protein was isolated from the cell lysis buffer. Protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Shanghai, China). Protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes. Blots were incubated with the appropriate primary antibody (Mouse Anti-Flag, Sigma, 1:2000, China headquarters, Shanghai, China) at room temperature. After washing in 5% non-fat milk in TBST (composed of Tris-Hcl, NaCl and tween20) saline at room temperature for 1 h, blots were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Goat Anti-Mouse IgG, 1:2000, Santa-Cruz Biotechnology, Dallas, Texas, USA) for 1.5 h. Bands were evaluated using chemiluminescence (ECL, Thermo Scientific Pierce, Shanghai, China) and scanned images were quantified using ImageJ software (NIH Image for the Macintosh, USA). Western blotting experiments were performed in triplicate, with GAPDH (Mouse Anti-Flag, 1:2000, Santa-Cruz Biotechnology, Dallas, Texas, USA) used as a housekeeping control for normalization. The ratio of MASTL protein expression to GAPDH expression was used for semi-quantification and comparison between two groups.

Table 1
Primer sequences used in the study.

Gene	forward primer 5'-3'	reverse primer 5'-3'	Product size(bp)
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA	121
MASTL	AAGGACTCGTATGCCCTATGT	CCAATGCTTCATCACTTTCC	264
CDKN1A	CATGTGGACCTGTCACTGTCTTGTA	GAAGATCAGCCGGCGTTTG	249
BMP2	CATGCCATTGTTCAGACG	TGTACTAGCGACACCCACA	172
SNAI2	CAAGGACACATTAGAACTCACAC	CAAGGACACATTAGAACTCACAC	199
CDK6	CCTTAGCACAGCACCAC	GGGATTTCTCAGCCAGT	167

Cell counting assay by GFP-based Cellomics Array

The altered cell viability after shMASTL and shCtrl transduction was assayed using the GFP-based Cellomics Array Scan imaging assay. In brief, human gastric cancer MGC-803 cells infected with shMASTL and shCtrl 48 hr prior were seeded into 96-well plates at a density of 1,000 cells /well in five-replicate wells. Cells were grown for up to five days, during which growth rates were measured with the Cellomics Array Scan VT1 (Thermo Fisher Scientific) by monitoring the GFP florescence level.

Colony formation assay

MGC-803 cells after 48 hr of infection with shMASTL and shCtrl were re-seeded into six-well plates at 400-1000 cells/well and grown for up to 14 days with the medium being refreshed every three days. At the end of 14 days, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and subsequently stained with 500 µl of Giemsa solution (Dingguo Biotechnology Co., Ltd, Shanghai, China) for 20 min. The cell colonies with 50 cells or more were counted under an inverted microscope (Olympus Corporation, Tokyo, Japan). The assay was repeated three times.

Cell apoptosis assay

MGC-803 cells were grown and infected with a lentivirus carrying either the shMASTL or shCtrl transgene for 48 hr and harvested with 0.25% trypsin. Cells were then centrifuged at 1,300 rmp for 5 min, washed with 1 × binding buffer and resuspended in 200 µl of the binding buffer for 1 x 10^6 cells from the Annexin V-APC/7-AAD apoptosis kit (MultiSciences, Hangzhou, China). A volume of 10 µL of Annexin V-APC was added to the cells for every 100 µL of cell solution and incubated in the dark at room temperature for 15 min. Finally, the percentage of apoptosis was measured using a flow cytometer (Guava® easyCyte 6HT; EMD Millipore, Billerica, MA, USA). This assay was in triplicate and repeated at least once.

Cell migration and invasion assay in vitro

A Transwell chamber assay (BD Biosciences, Franklin Lakes, NJ, USA) was performed to observe cellular invasion in vitro. Briefly, the upper chamber was coated with 60 μL Matrigel (BD Biosciences) diluted with serum-free medium (1:50). Cells were then seeded into the upper chamber in 200 μL serum-free medium at a density of 4×10^4 cells/chamber. The lower chamber was filled with 750 μL medium containing 10% FBS. Following incubation at 37°C for 48 h, cells were fixed with 4% polyoxymethylene (Shanghai Macklin Reagent Co., Ltd., Shanghai, China) for 10 min, followed by staining with 0.5% crystal violet (Shanghai Macklin Reagent Co., Ltd.) for 30 min. Cells that did not invade through the pores were wiped away with a cotton swab. Invaded cells were counted using an inverted microscope. The experiment was repeated three times.

Cell Cycle Assay

MGC-803 cells after 48 hr of infection with shMASTL and shCtrl were harvested for cell cycle assay. Univariate cell cycle analysis was performed by using ethanol-fixed cells stained with propidium iodide in buffer containing RNase A (Fermentas, USA). DNA content was assessed by flow cytometry (Guava easyCyte 6HT, Millipore, USA), and cell cycle analysis was performed by using the ModFit software package (Verity Software House, San Diego, USA). This assay was repeated in triplicate.

Tumorigenesis in nude mice and in vivo imaging

The male BALB/c nude mice (age: 4 weeks, weight：15-19g, maintenance conditions: temperature: 22-24℃, humidity: 40-70%, food/water access: artificial feeding per 2-3 days, light/dark cycle: 12h/12h) were purchased from Shanghai Lingchang Biotechnology Co., Ltd. (Shanghai, China). The eligible nude mice were inoculated with MGC-803 cells infected by LV-shMASTL and Lv-shCtrl. To be brief, a total number of 20 mice were classified into two groups with 10 mice in each group randomly. The inoculated MGC-803 cells were resuspended in physiological saline solution at a density of $5x10^7$ cells/ml and a volume with 0.2 mL cell suspension was injected into the mice subcutaneously with a 6-gauge, 1 mL syringe. The mice were fed and survived until the tumors were visible, and tumor diameter and size were measured 8, 12, 15, 18, 21 and 24 days following inoculation. Tumor volume was monitored, recorded and calculated according to the following formula for hemi-ellipsoids routinely: Volume = length (cm) x width (cm) x height (cm) x 3.14/6. After the last measurement of tumor volume, the mice were injected with D-Luciferin 10 μL/g intraperitoneally (QianChen Biotechnology Co., Ltd, Shanghai, China). 15 min later, pentobarbital sodium with 70 mg/kg was injected intraperitoneally to anesthetize the mice for detecting the fluorescence emitted from mice by a small animal live imaging system (LT Lumina; PerkinElmer, Inc., Waltham, MA, USA). The mice were sacrificed and dissected to get the subcutaneous tumors, and photograph the mice body and tumor as well. The mice operations were conducted in strict accordance with international ethical guidelines and the National Institutes of Health Guide for the Care

and Use of Laboratory Animals. The experiments were authorized and approved by the Institutional Animal Care and Use Committee of Gansu University of Chinese Medicine (Lanzhou, China).

Screening aberrantly genes by Affymetrix mRNA microarray assay

Following successful transduction with Lv-shMASTL and Lv-shCtrl, MGC-803 cells of both groups were collected to perform mRNA microarray assay. Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) in compliance with the manufacturer's instructions. RNA integrity was determined by gel electrophoresis, and the concentration and purity of total RNA were assessed by A_{260} and A_{280}, using a nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA). Only the samples containing RNA with A260/A280 ratios >1.8 were used in this study. The mRNA microarray assay was performed using commercially available PrimeView™ Human Gene Expression Array (Genechem Corporation, Shanghai, China). The procedures of labeling, hybridization, washing, and scanning were performed according to the standard operating procedures provided by Affymetrix. Briefly, total RNA was used to synthesize cDNA in an in vitro transcription reaction, and the cDNA was labeled by biotin and T7 Enzyme Mix. After hybridized with gene expression array, non-specifically bound molecules were removed from the microarray with two washing buffers. Subsequently, the arrays were scanned with a GeneChip® Scanner 3000 (Affymetrix, Cleveland, USA) to gather hybridization data, and the data were analyzed using Gene Matrix cloud service (http://gcloud.taogene.com).

RT-qPCR and Western blotting validation of aberrantly expressed genes screened by microarray

Total RNA and protein was extracted from MGC-803 cells of shMASTL and shCtrl group. All procedures of RNA extraction, reverse transcription and amplification were same as the procedures mentioned above. The primer sequences (Takara Bio, Dalian, China) were provided in Table 1. Likewise, all procedures of protein isolation, gel electrophoresis, transferring to PVDF and immune blots were same as the procedures mentioned above too. The blots were incubated with the appropriate primary antibody against CDK6 (1:1000, #3136, CST, USA), BMP2 (1:500, ab6285, ABCAM, USA), SNAI2 (1:1000, #3879, CST, USA) and CDKN1A (1:500, #2947, CST, USA) at room temperature. After washed with 5% non-fat milk in TBST saline (20 mM Tris–HCl, pH 7.4, 137 mMNaCl, and 0. 1% Tween-20) at room temperature for 1h, the blots were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 1.5h. Bands were monitored using western blot chemiluminescence (ECL, Applygen, Beijing, China) detection reagents and scanned images were quantified using ImageJ software. In addition, in order to explore possible signal pathway involved in the effects induced by MASTL silencing, non-phosphorylation form of mTOR (1:500, #2983, CST, USA), AKT1 (1:500, ab124341, ABCAM, USA) and p38 (1:500, #8690, CST, USA) proteins, and phosphorylation form of p-mTOR (1:500, ab109268, ABCAM, USA), p-AKT (1:500, #4060, CST, USA) and p-p38 (1:500, #4631, CST, USA) proteins were detected by

western blotting analysis**.** Experiments were performed in triplicate with GAPDH (1:2000, sc-2004 or sc-2005, Santa Cruz, USA) used as a housekeeping control for normalization. The ratio of chosen protein to GAPDH was used for semi-quantification and comparison between two groups.

Statistical analysis

Data were expressed as the means ± standard deviation from at least 3 separate experiments. Statistical analyses were evaluated using Student's two-tailed t-test. Differences with P values of < 0.05 are considered statistically significant.

Results

Efficiency of shRNA-mediated MASTL knockdown in gastric cancer cells.

MGC-803 cells infected with Lv-shCtrl or Lv-shMASTL were observed under a fluorescence microscope to determine infection efficiency, and the infection rate was measured by monitoring GFP fluorescence emitted by cells. The results indicated that the evaluated infection efficiency was >80% (Fig. 1A). As determined by RT-qPCR analysis, the mRNA and protein expression of MASTL were significantly decreased in the Lv-shMASTL group, with a knockdown efficiency of 78.9% ($P<0.01$; Fig.1B) at the level of MASTL mRNA and protein level ($P< 0.01$; Fig.1B) compared with that of the shCtrl group.

Effects of MASTL knockdown on reduction of MGC-803 cell proliferation by Cellomics Array Scan assay.

To assess the effects of MASTL on gastric cancer cells in vitro, we knocked down MASTL expression using a lentivirus carrying MASTL shRNA and Lv-shCtrl in MGC-803 cells. We then assessed the alteration in cell viability using the GFP-based Cellomics Array Scan imaging assay and found that knockdown of MASTL expression make the number and fold-change in proliferation of cells in the Lv-shMASTL group reduced when compared with the Lv-shCtrl group at 4 and 5 days following transduction of MGC-803 cells significantly ($P<0.05$; Fig.1C). The GFP-based Cellomics Array Scan imaging assay showed that knockdown of MASTL expression dramatically reduced MGC-803 cell proliferation. These findings suggested that knockdown of MASTL may be associated with a reduction in cell proliferation.

MASTL silencing reduces gastric cancer cell colony formation.

A colony formation assay is used to assess the proliferative potential of cells. In the present study, the colony formation assay results demonstrated that the Lv- shMASTL group formed significantly fewer colonies in soft agar when compared with the Lv-shCtrl group (P<0.001; Fig. 1D). These results indicated that silencing MASTL may reduce the anchorage-independent proliferative potential of MGC-803 gastric cancer cells.

Effects of MASTL knockdown on induction of MGC-803 cell apoptosis

Knockdown of MASTL expression also significantly inhibited MGC-803 cell colony formation (P=0.0001, Figure. 2A). We then assessed the underlying mechanism of shMASTL-reduced cell proliferation using a flow cytometry apoptosis assay. Our data demonstrated that knockdown of MASTL expression induced MGC-803 cells to undergo apoptosis.

MASTL silencing induced cell cycle arrest at G1 stage.

To elucidate whether silencing of MASTL could alter cell cycle in MGC-803 cells further, cell cycle assay detection was designed to between MASTL silenced group and control group cells further. The results showed that the number of cells in G1 phase of shMASTL group increased and the number of cells in S and G2 phase of shMASTL group decreased compared with the cells in shCtrl group for 3 days of culturing (P<0.05, Figure 2B), indicating that silencing MASTL could arrest cell cycle at G1 phase.

MASTL silencing inhibited gastric cancer cell invasion and migration in vitro.

The present study determined whether MASTL overexpression inhibits gastric cancer cell migration and invasion. Gastric cancer MGC-803 cells motility was assessed using the transwell chamber assays. The transwell chamber assays demonstrated that MASTL silencing inhibited the migration capacity of MGC-803 compared with shCtrl group cells significantly (P<0.05, Fig. 3A). Cell invasion of MGC-803 cells was reduced significantly compared with the shCtrl group (P<0.05, Fig. 3B). These results indicate that MASTL silencing could inhibit gastric cancer cell migration and invasion in vitro.

MASTL silencing inhibited tumor growth and fluorescence imaging assay in vivo.

To detect the effects of MASTL knockdown *in vitro*, an *in vivo* mouse tumorigenesis model established by injecting MGC-803 cells solution from both Lv-shCtrl and Lv-shMASTL groups. Over the maintaining time of 24 days, the rate of tumor growth and the tumor volume were significantly reduced at 15, 18 and 24

days following inoculation with MGC-803 cells in Lv-shMASTL group compared with that in Lv-shCtrl group ($P<0.05$, Fig. 4A). The results of tumor weight analysis revealed that MASTL-silenced MGC-803 cells generated smaller subcutaneous xenograft tumors in nude mice compared with that in the Lv-shCtrl group. The results demonstrated that silencing MASTL using the Lv-shMASTL vector may inhibit the tumorigenicity of MGC-803 cells significantly in a xenograft nude mouse model.

The fluorescence imaging detection using a small animal live imaging system monitors the fluorescence signals emitted from MGC-803 cells infected with Lv-shMASTL and Lv-shCtrl with GFP in tumor xenografts which were triggered by specific fluorescence in the live imaging system. The recorded fluorescence signal was used to calculate the total radiant efficiency to reflect the number of xenograft tumor cells. The total radiant efficiency of the Region of Image (ROI) in xenografts from mice injected with Lv-shMASTL-infected MGC-803 cells and Lv-shCtrl-infected MGC-803 cells. Fluorescence imaging assay indicated that the total radiant efficiency of the ROI in xenograft tumors from the Lv-shMASTL-infected group was lower when compared with the Lv-shCtrl-infected group ($P<0.05$; Fig. 4B). The fluorescence imaging results demonstrated that the total radiant efficiency of mice in the Lv-shMASTL-infected group was markedly reduced compared with in the Lv-shCtrl-infected group. These results confirmed successful infection into MGC-803 cells with the lentiviral vectors and verified the effects of MASTL on cell proliferation *in vivo*.

Affymetrix mRNA microarray analysis of aberrantly expressed genes.

To investigate the modulation mechanism of MASTL in the tumorigenesis of gastric cancer, Affymetrix mRNA microarray were used to screen and analysis aberrantly expressed genes between Lv-shCtrl group and Lv-shMASTL group in MGC-803 cells. Knockdown of MASTL significantly induced 391 mRNAs were aberrantly expressed including 124 up-regulated genes, and 267 down-regulated genes between Lv-shMASTL group cells and Lv-shCtrl group cells (cut-off ≥ 1.5, Figure 5A).

Pathway analysis of aberrantly expressed genes with bioinformatical method.

In order to explore the potential mechanisms of aberrantly expressed genes related to tumorigenesis characterics of gastric cancer, the KEGG pathway analysis were conducted as following steps, a total of 391 aberrantly expressed genes were submitted to DAVID 6.8 online software (https://david.ncifcrf.gov/tools.jsp) to analysis relevant pathways. The results showed that 291 aberrantly expressed genes were enriched in 14 pathways, among which, 9 pathways were closely related to tumorigenesis were closely related to tumorigenesis including Small cell lung cancer, p53 signaling pathway, MAPK signaling pathway, Transcriptional misregulation in cancer, Ras signaling pathway, Pathways in cancer, Viral carcinogenesis, Focal adhesion, PI3K-Akt signaling pathway. In the end, several

genes including CDK6, BMP2, SNAI2 and CDKN1A in the pathway of hsa05200 were selected for RT-qPCR and western blotting validation, (Figure 5(B, C)). In summary, this kind of bioinformatical analysis facility subsequent molecular mechanisms to discover the effects of silencing MASTL in gastric cancer cells.

Any aberrantly expressed genes validation by RT-qPCR and Western Blotting.

In order to explore the molecular mechanisms of phenotype changes related to silencing MASTL, it is a wise choice to validate any aberrantly expressed genes screened by the microarray assay furtherly. Therefore, combing with bioinformatical analysis, we decided to choose several genes enriched in Pathways in cancer (Hsa05200) including CDK6, BMP2, SNAI2 and CDKN1A to validate their expression by RT-qPCR and western blotting method. The validation results discovered that CDK6, BMP2 and SNAI2 are downregulated, and CDKN1A are upregulated in Lv-shMASTL group cells (Figure. 6(A, B, C)). In addition, Western blotting analysis showed that phosphorylation form of p-mTOR, p-AKT and p-p38 proteins were downregulated significantly (Figure. 6(A, C)). This results showed that CDK6, BMP2, SNAI2, CDKN1A, p-mTOR, p-AKT and p-p38 which play important roles in the cell growth, migration and arresting cell cycle of MGC-803 cells following MASTL silencing which responsible for the mechanisms of MASTL in gastric cancer progression.

Discussion

MASTL played as a serine/threonine kinase during mitotic division actively, in this process, it has been founded that MASTL could regulates chromosome condensation and mitotic progression[8]. Emerged studies showed that MASTL participate multiple tumors progression [7, 9–12], however, fewer study was reported about the roe of MASTL in gastric cancer. Therefore, it is extremely needed to uncover the role and function of MASTL during gastric cancer tumorigenesis. In this study, Knocking down of MASTL by shRNA lentivirus infection in MGC-803 cells significantly suppressed cell proliferation, colony formation, inhibited cell migration ability, arrested cell cycle at G1 stage, and induced apoptosis significantly in gastric cancer cells. Tumorigenesis in nude mice and florescence imaging in vivo also confirmed that MASTL silencing could inhibit tumor growth in vivo.

In order to explore the potential mechanisms associated with silencing MASTL, Affymetrix mRNA microarray assay and subsequent enriched KEGG pathway analysis were used to screen and discover relevant molecules related to MASTL silencing. In this study, a total of 291 aberrantly expressed genes screened by mRNA microarray analysis make it possible that bioinformatical analysis will lead to deepening understand relevant mechanisms from those moleculars in a larger scale by high throughput screening. Bioinformatical analysis of KEGG pathway enrichment analysis showed that some of aberrantly expressed genes enriched in 14 pathways of were involved in gastric cancer tumorgenesis, among which, 9 pathways were closely related to tumorigenesis, which includes small cell lung cancer, p53 signaling pathway, MAPK signaling pathway, transcriptional misregulation in cancer, ras signaling

pathway, pathways in cancer, viral carcinogenesis, focal adhesion, PI3K-Akt signaling pathway. Therefore, it is an apparent sign that MASTL could participate in gastric cancer tumorigenesis which responsible for tumor biological phenotype, such as cell proliferation, cell cycle, cell migration, and also a good sign of gastric cancer biomarker for diagnosis and prognosis evaluation. In another hand, aberrantly expressed genes screened by mRNA microarray assay also provided significantly changed genes from the trivial perspective.

After Affymetrix mRNA microarray assay, any of those molecules related to cell growth, migration and cell cycle should be chosen from 291 aberrantly expressed mRNAs for more detection at mRNA and protein levels. In the end, 4 genes, CDK6, BMP2, SNAI2 and CDKN1A enriched in pathways in cancer were verified by the means of RT-qPCR and western blotting. In addition, p-mTOR, p-AKT and p-P38 were also detected by western blotting, which would make it easy to understand phenotype changes result from silencing MASTL by searching the potential upstream moleculars. The validation results showed that CDK6, BMP2, SNAI2, p-mTOR, p-AKT and p-P38 are downregulated, and CDKN1A are upregulated. CDK6, BMP2, SNAI2, and CDKN1A were classical phenotype molecular which responsible for tumor growth, cell cycle and metastasis, and overexpression of them could promote tumor invasiveness and metastasis.

CDK6, as serine/threonine protein kinases controlled cell cycle G1 phase progression and G1/S transition. The expression of CDK6 protein was found upregulated in gastric cancer [14]. BMP-2 signaling pathway enhances tumor metastasis in gastric cancer by sequential activation of the PI3K/AKT or MAPK pathway followed by the induction of NF-κB and MMP-9 activity, indicating that BMP-2 has the potential to be a therapeutic molecular target to decrease metastasis[15]. BMP2 can induce AKT and ERK phosphorylation in a dose-dependent, Notably BMP2 alone treatment can induce the up-regulation of vimentin, snail, and N-cadherin in AGS cells, the down-regulation of E-cadherin also occurred. On the contrary, BMPR-II siRNA significantly prohibited BMP2-induced AKT and ERK phosphorylation[16]. BMP-2 may inhibit the proliferation of both normal and malignant gastric epithelial cells, down-regulate CDK4 expression in gastric cancer cells and arrest gastric cancer cells in G1-phase in cell cycle [17]. ShRNA-mediated silencing of SNAI2 suppressed the activation of Snail/Slug, whereby gastric cancer cell proliferation, invasion and migration, EMT, tumor growth, and lymph node metastasis were inhibited. High expression of miR-33a was a protective factor influencing the prognosis of gastric cancer. This study suggests that miR-33a inhibited EMT, invasion, and metastasis of gastric cancer through the Snail/Slug signaling pathway by modulating SNAI2 expression. MiR-33a targets and inhibits the expression of SNAI2, overexpression of SNAI2 activates the Snail/Slug signaling pathway, the Snail/Slug signaling pathway promotes gastric cancer cell proliferation, invasion, and metastasis[17]. P21WAF1/CIP1, a member of the cyclin dependent kinase inhibitor (CKIs) family, is a well-known cell cycle regulator. It can inhibit the activity of cyclin/cyclin dependent kinase complex. It can combine with almost every cyclin CDK complex and widely inhibit all kinds of cyclin CDK complexes. P21WAF1/CIP1 can go through many ways Participate in the regulation of cell cycle and induce cell apoptosis [13]. When the cells were exposed to DNA damage agent, p21WAF1/CIP1 gene expression was further induced by activating p53 to inhibit the activity of cyclin dependent kinase complex, so that the damaged cells were blocked in G1 phase and the cells had enough time for damage repair. Low expression of CDKN1A has independent prognostic

significance indicative of tumor progression and poor survival in patients with RGA. Evaluation of CDKN1A expression may assist in determining prognosis in patients with resected gastric adenocarcinoma.

A numbers of previous reports demonstrated that phosphorylation protein form of p-AKT [18–22], p-mTOR [23–26], p-p38 [27–30] in mTOR, AKT and p38 signal pathways were involved in the regulation of cell proliferation, cell cycle and cell migration in gastric cancer. In this study, western blotting validation confirmed that phosphorylation protein form of p-mTOR, p-AKT and p-P38 were downregulated significantly caused by MASTL silencing in gastric cancer cells. Collectively, Knockdown of MASTL in MGC-803 could inhibit cell proliferation, cell migration ability, and arrest cell cycle at G2 stage significantly. The phenotype change of silencing MASTL were associated with downregulation of CDK6, BMP2 and SNAI2, and upregulation of CDKN1A. Furthermore, knockdown of MASTL could lead to inactivation of phosphorylation form of p-mTOR, p-AKT and p-p38.

Conclusion

Silencing MASTL could significantly inhibit cell growth, migration ability, induce apoptosis and arrest cell cycle at G1 stage in MGC-803 cells, and the mechanisms of which were mediated by downregulation of CDK6, BMP2, SNAI2, p-mTOR, p-AKT and p-p38, and upregulation of CDKN1A. Therefore, it is suggested MASTL take part in proliferation, migration and cell cycle via inactivation of mTOR, AKT, p38 signal pathways. In conclusion, it is suggested MASTL take part in proliferation, migration and invasion, and which may be considered as a valuable target for gene therapeutic strategies in gastric cancer. Additionally, lots of pathways and aberrantly expressed mRNAs were still needed to study further to understand the role and function of MASTL in gastric cancer tumorigenesis. All of these studies would contribute to prove MASTL as a novel biomarker molecular in diagnosis and gene therapy target.

Abbreviations

Microtubule-associated serine/threonine kinase (MASTL), Green fluorescent protein (GFP),quantitative reverse transcription PCR(RT-qPCR),cyclin dependent kinase 6(CDK6), bone morphogenetic protein 2(BMP2), snail family transcriptional repressor 2(SNAI2), phosphorylation-mechanistic target of rapamycin kinase (p-mTOR), phosphorylation-AKT serine/threonine kinase (p-AKT) and phosphorylation-p38 kinase (p-p38), cyclin dependent kinase inhibitor 1A (CDKN1A), multiplicity of infection (MOI).

Declarations

Ethics approval and consent to participate

The animal experiments in this study were authorized and approved by the Institutional Animal Care and Use Committee of Gansu University of Chinese Medicine (approving NO: 2019-273).

Consent publication

The authors all agreed for publication of this paper.

Availability of data and material

All data and materials generated and analyzed during the present study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests in this paper.

Funding

This program was support by grants from Lanzhou Science and Technology Planning Project (#2016-3-113), University Research Project of Gansu Province (#2018A-049), and Natural Science Foundation of Gansu Province (#17JR5RA169).

Authors' Contributions

(I) Conception and design: Caixia An, Hailong Li, Yongning Zhou; (II) Administrative support: Yongning Zhou; (III) Provision of study materials or patients: Hailong Li, Yonghua Hu, Rong Niu; (IV) Collection and assembly of data: Hailong Li, Xiaoguang Liu; (V) Data analysis and interpretation: Caixia An, Hailong Li, Zhuanxiong Wang, Xi Chen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript.

Acknowledgments

This study was support in part by grants from Lanzhou Science and Technology Planning Project (#2016-3-113), University Research Project of Gansu Province (#2018A-049), and Natural Science Foundation of Gansu Province (#17JR5RA169).

References

1. Chen W: **Cancer statistics: updated cancer burden in China.***Chin J Cancer Res* 2015, **27:**1.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A: **Global cancer statistics, 2012.***CA Cancer J Clin* 2015, **65:**87-108.

3. Zhong Y, Johnson AJ, Byrd JC, Dubovsky JA: **Targeting Interleukin-2-Inducible T-cell Kinase (ITK) in T-Cell Related Diseases.***Postdoc J* 2014, **2:**1-11.
4. Chang AH, Parsonnet J: **Role of bacteria in oncogenesis.***Clin Microbiol Rev* 2010, **23:**837-857.
5. Gonzalez CA, Sala N, Rokkas T: **Gastric cancer: epidemiologic aspects.***Helicobacter* 2013, **18 Suppl 1:**34-38.
6. Puthawala K, Hadjiangelis N, Jacoby SC, Bayongan E, Zhao Z, Yang Z, Devitt ML, Horan GS, Weinreb PH, Lukashev ME, et al: **Inhibition of integrin alpha(v)beta6, an activator of latent transforming growth factor-beta, prevents radiation-induced lung fibrosis.***Am J Respir Crit Care Med* 2008, **177:**82-90.
7. Wadhwa R, Taketa T, Sudo K, Blum MA, Ajani JA: **Modern oncological approaches to gastric adenocarcinoma.***Gastroenterol Clin North Am* 2013, **42:**359-369.
8. Castro A, Lorca T: **Greatwall kinase at a glance.***J Cell Sci* 2018, **131**.
9. Rogers S, McCloy RA, Parker BL, Gallego-Ortega D, Law AMK, Chin VT, Conway JRW, Fey D, Millar EKA, O'Toole S, et al: **MASTL overexpression promotes chromosome instability and metastasis in breast cancer.***Oncogene* 2018, **37:**4518-4533.
10. Yoon YN, Choe MH, Jung KY, Hwang SG, Oh JS, Kim JS: **MASTL inhibition promotes mitotic catastrophe through PP2A activation to inhibit cancer growth and radioresistance in breast cancer cells.***BMC Cancer* 2018, **18:**716.
11. Sun XJ, Li YL, Wang LG, Liu LQ, Ma H, Hou WH, Yu JM: **Mastl overexpression is associated with epithelial to mesenchymal transition and predicts a poor clinical outcome in gastric cancer.***Oncol Lett* 2017, **14:**7283-7287.
12. Uppada SB, Gowrikumar S, Ahmad R, Kumar B, Szeglin B, Chen X, Smith JJ, Batra SK, Singh AB, Dhawan P: **MASTL induces Colon Cancer progression and Chemoresistance by promoting Wnt/beta-catenin signaling.***Mol Cancer* 2018, **17:**111.
13. Wang L, Luong VQ, Giannini PJ, Peng A: **Mastl kinase, a promising therapeutic target, promotes cancer recurrence.***Oncotarget* 2014, **5:**11479-11489.
14. Li LP, Wu WJ, Sun DY, Xie ZY, Ma YC, Zhao YG: **miR-449a and CDK6 in gastric carcinoma.***Oncol Lett* 2014, **8:**1533-1538.
15. Kang MH, Oh SC, Lee HJ, Kang HN, Kim JL, Kim JS, Yoo YA: **Metastatic function of BMP-2 in gastric cancer cells: the role of PI3K/AKT, MAPK, the NF-kappaB pathway, and MMP-9 expression.***Exp Cell Res* 2011, **317:**1746-1762.
16. Liao A, Wang W, Sun D, Jiang Y, Tian S, Li J, Yang X, Shi R: **Bone morphogenetic protein 2 mediates epithelial-mesenchymal transition via AKT and ERK signaling pathways in gastric cancer.***Tumour Biol* 2015, **36:**2773-2778.
17. Zhang J, Ge Y, Sun L, Cao J, Wu Q, Guo L, Wang Z: **Effect of bone morphogenetic protein-2 on proliferation and apoptosis of gastric cancer cells.***Int J Med Sci* 2012, **9:**184-192.

18. Wei R, Xiao Y, Song Y, Yuan H, Luo J, Xu W: **FAT4 regulates the EMT and autophagy in colorectal cancer cells in part via the PI3K-AKT signaling axis.***J Exp Clin Cancer Res* 2019, **38:**112.
19. Ao R, Guan L, Wang Y, Wang JN: **Silencing of COL1A2, COL6A3, and THBS2 inhibits gastric cancer cell proliferation, migration, and invasion while promoting apoptosis through the PI3k-Akt signaling pathway.***J Cell Biochem* 2018, **119:**4420-4434.
20. Xie Y, Liu L: **Analysis of correlation between HP infection and activation of PI3K/Akt pathway in mucosal tissues of gastric cancer and precancerous lesions.***Oncol Lett* 2018, **16:**5615-5620.
21. Chiappini PBO, de Medeiros IUD, Lima LGC, Fregnani JH, Nonogaki S, da Costa WL, Jr., Coimbra FJF, Silva M, de Mello CAL, Pinto CAL, Begnami MD: **Prognostic implications of phosphatidylinositol 3-kinase/AKT signaling pathway activation in gastric carcinomas.***Arch Med Sci* 2017, **13:**1262-1268.
22. Cao F, Zhang C, Han W, Gao XJ, Ma J, Hu YW, Gu X, Ding HZ, Zhu LX, Liu Q: **p-Akt as a potential poor prognostic factor for gastric cancer: a systematic review and meta-analysis.***Oncotarget* 2017, **8:**59878-59888.
23. Cao GD, Chen K, Chen B, Xiong MM: **Positive prognostic value of HER2-HER3 co-expression and p-mTOR in gastric cancer patients.***BMC Cancer* 2017, **17:**841.
24. Cao GD, Xu XY, Zhang JW, Chen B, Xiong MM: **Phosphorylated Mammalian Target of Rapamycin p-mTOR Is a Favorable Prognostic Factor than mTOR in Gastric Cancer.***PLoS One* 2016, **11:**e0168085.
25. Riquelme I, Tapia O, Espinoza JA, Leal P, Buchegger K, Sandoval A, Bizama C, Araya JC, Peek RM, Roa JC: **The Gene Expression Status of the PI3K/AKT/mTOR Pathway in Gastric Cancer Tissues and Cell Lines.***Pathol Oncol Res* 2016, **22:**797-805.
26. Ying J, Xu Q, Liu B, Zhang G, Chen L, Pan H: **The expression of the PI3K/AKT/mTOR pathway in gastric cancer and its role in gastric cancer prognosis.***Onco Targets Ther* 2015, **8:**2427-2433.
27. Yang GF, Deng CS, Xiong YY, Luo J, Wang BC, Tian SF, Xu K: **[Expression of NFkappaB p65 and its target genes in gastric cancer and precancerous lesions].***Zhonghua Zhong Liu Za Zhi* 2004, **26:**551-553.
28. Seo JH, Lim JW, Kim H: **Differential Role of ERK and p38 on NF- kappa B Activation in Helicobacter pylori-Infected Gastric Epithelial Cells.***J Cancer Prev* 2013, **18:**346-350.
29. Fu ZH, Liu SQ, Qin MB, Huang JA, Xu CY, Wu WH, Zhu LY, Qin N, Lai MY: **NIK- and IKKβ-binding protein contributes to gastric cancer chemoresistance by promoting epithelial-mesenchymal transition through the NF-κB signaling pathway.***Oncology Reports*.
30. Ji HS, Lim JW, Kim H: **Differential Role of ERK and p38 on NF-??B Activation in Helicobacter pylori-Infected Gastric Epithelial Cells.***Journal of Cancer Prevention* 2013, **18:**346.

Figures

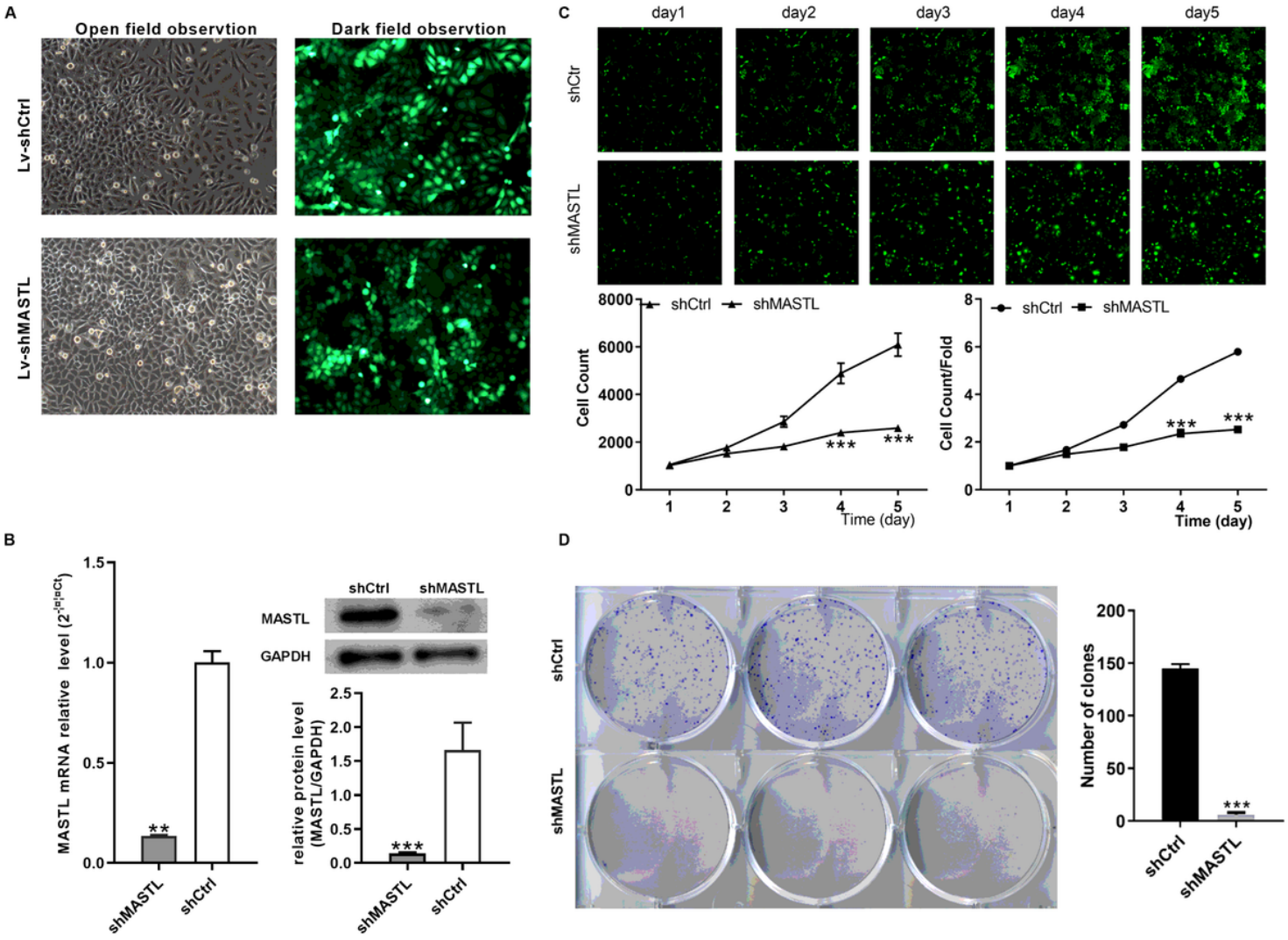

Figure 1

(A, B, C, D): Effect of Lv-shMASTL infection efficiency and gene knockdown efficiency on cell proliferation in MGC-803 cells. The mRNA expression level of MASTL were significantly downregulated ($p < 0.01$) in Lv-shMASTL group, compared with Lv-shCtrl group with a significant knockdown efficiency amounts to 78.9% ($p < 0.01$), as shown in Figure 1(A). MASTL mRNA and protein expression was greatly reduced in the MASTL -shRNA transefected MGC-803 cells detected by western blotting, indicating effective knockdown of MASTL ($p < 0.01$, as shown in Figure 1(B). The detetion result of GFP-based Cellomics Array Scan VTI imaging assay showed the number of cells and the fold-change of proliferation were markedly reduced in the MASTL-shRNA-silenced gastric cancer cells on the fourth and fifth day following MASTL silenced significantly in MGC-803 cells compared with that of Lv-shCtrl group (*P<0.05 or **P<0.01 or **P<0.001 vs Lv-shCtrl group at the same day), as shown in Figure 1(C).The colony number of MGC-803 cells of Lv-shMASTL infection group decreased, when comparing with that of Lv-shCtrl group as shown in Figure 1(D), suggesting that the MASTL gene was significantly related to cell proliferation ability of MGC-803 cells.

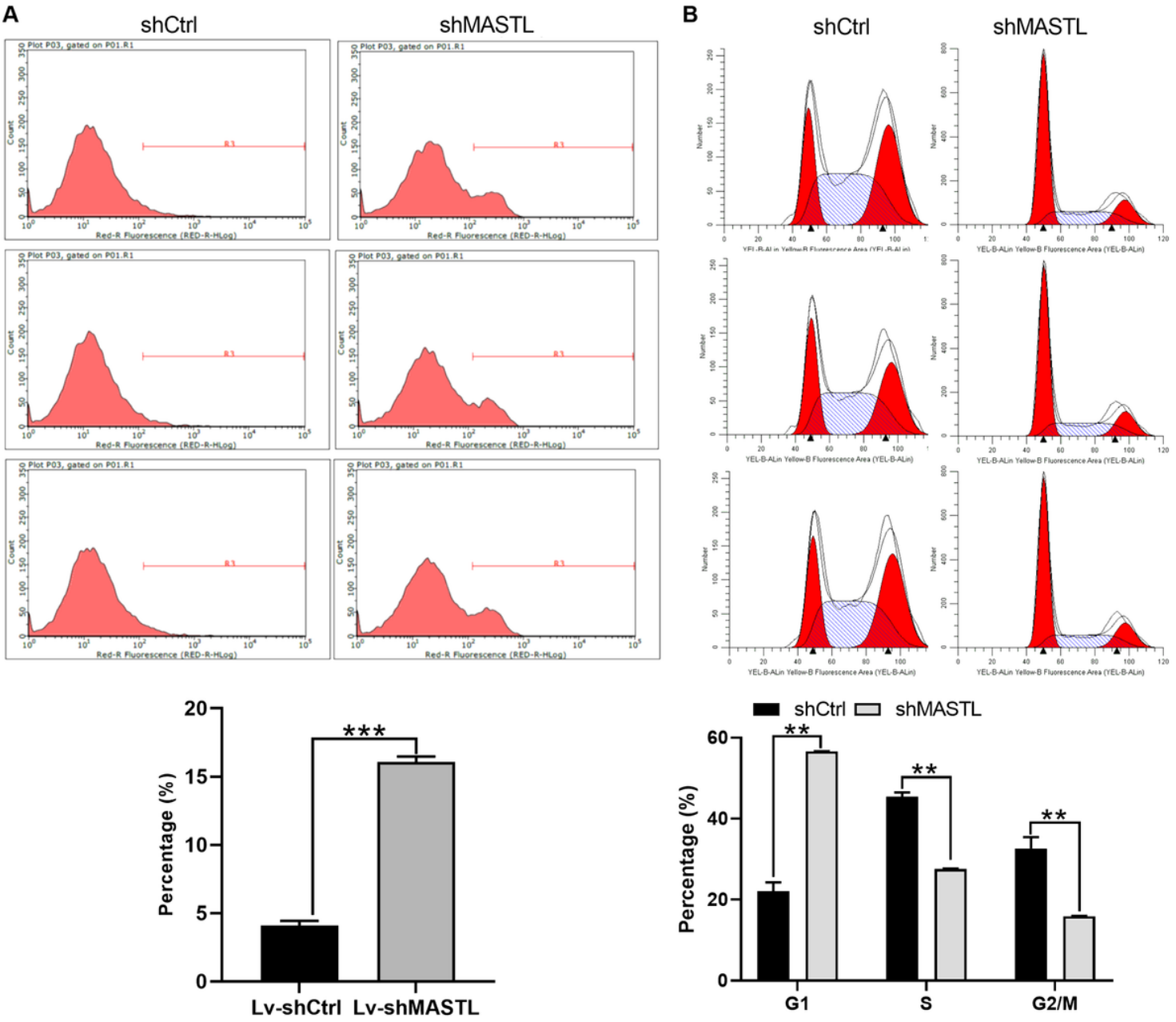

Figure 2

(A, B): Effect of MASTL gene knockdown on apoptosis and cell cycle by FACS detection. After infection with shRNA lentivirus for 5 days, the apoptotic MGC-803 cells in Lv-shMASTL group increased significantly in comparison with that in Lv-shCtrl group ($p < 0.001$), suggesting that MASTL gene was significantly correlated with the apoptosis of MGC-803 cells. The cell cycle results showed that the number of cells in G1 phase of shMASTL group decreased and the number of cells in S and G2 phase of shMASTL group increased following 3 days of culturing compared with the cells in shCtrl group ($P<0.05$, Figure 5(A, B), indicating that silencing MASTL could arrest cell cycle at S and G2 phase.

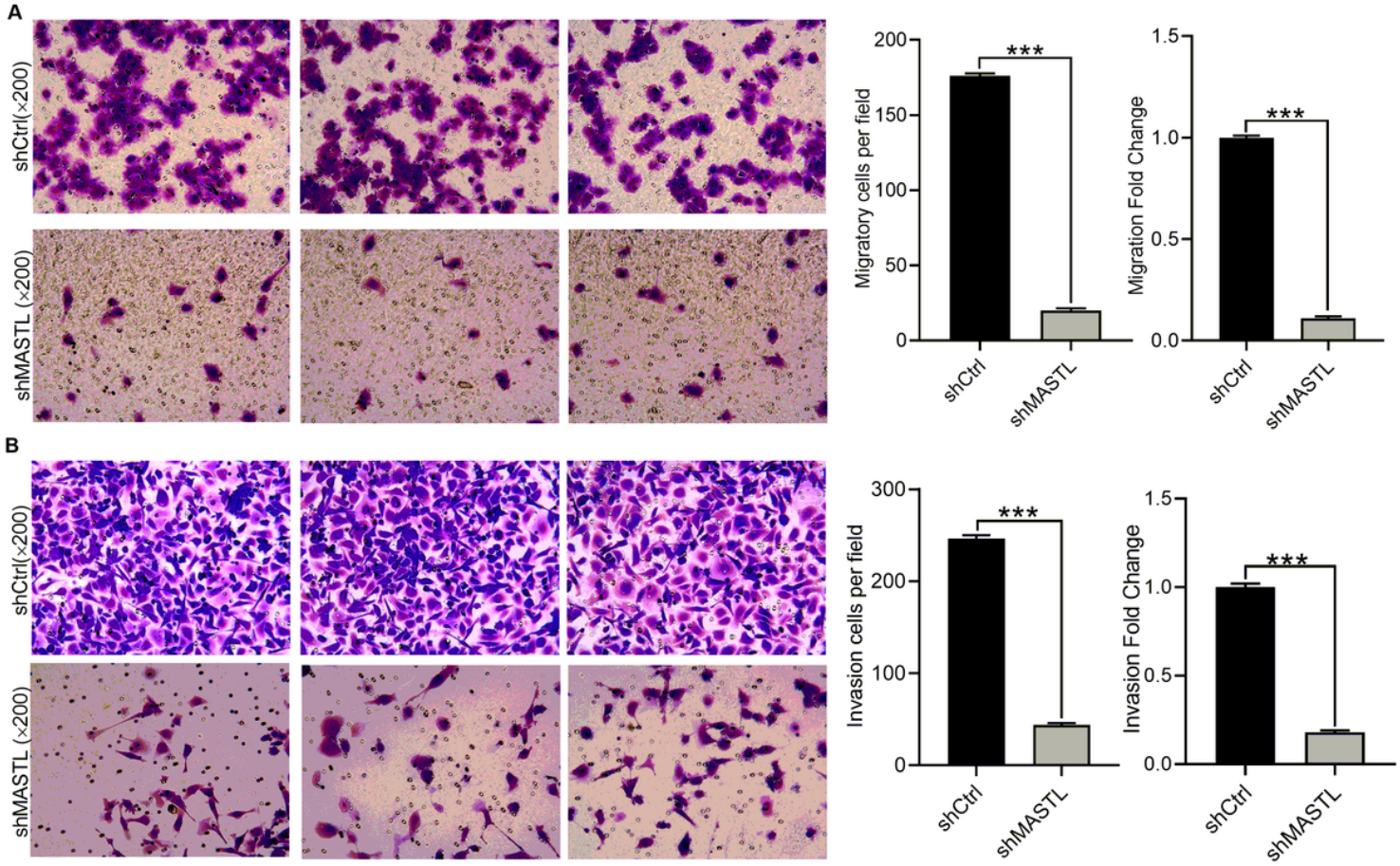

Figure 3

(A, B): MASTL silencing inhibited gastric cancer cell invasion and migration in vitro. The present study determined whether MASTL overexpression inhibits gastric cancer cell migration and invasion. Transwell chamber assays demonstrated that MASTL silencing significantly inhibited the migration and invasion ability of MGC-803 compared with shCtrl group cells ($P<0.05$, Fig 3(A, B)). These results indicate that MASTL silencing could inhibit gastric cancer cell invasion and migration in vitro.

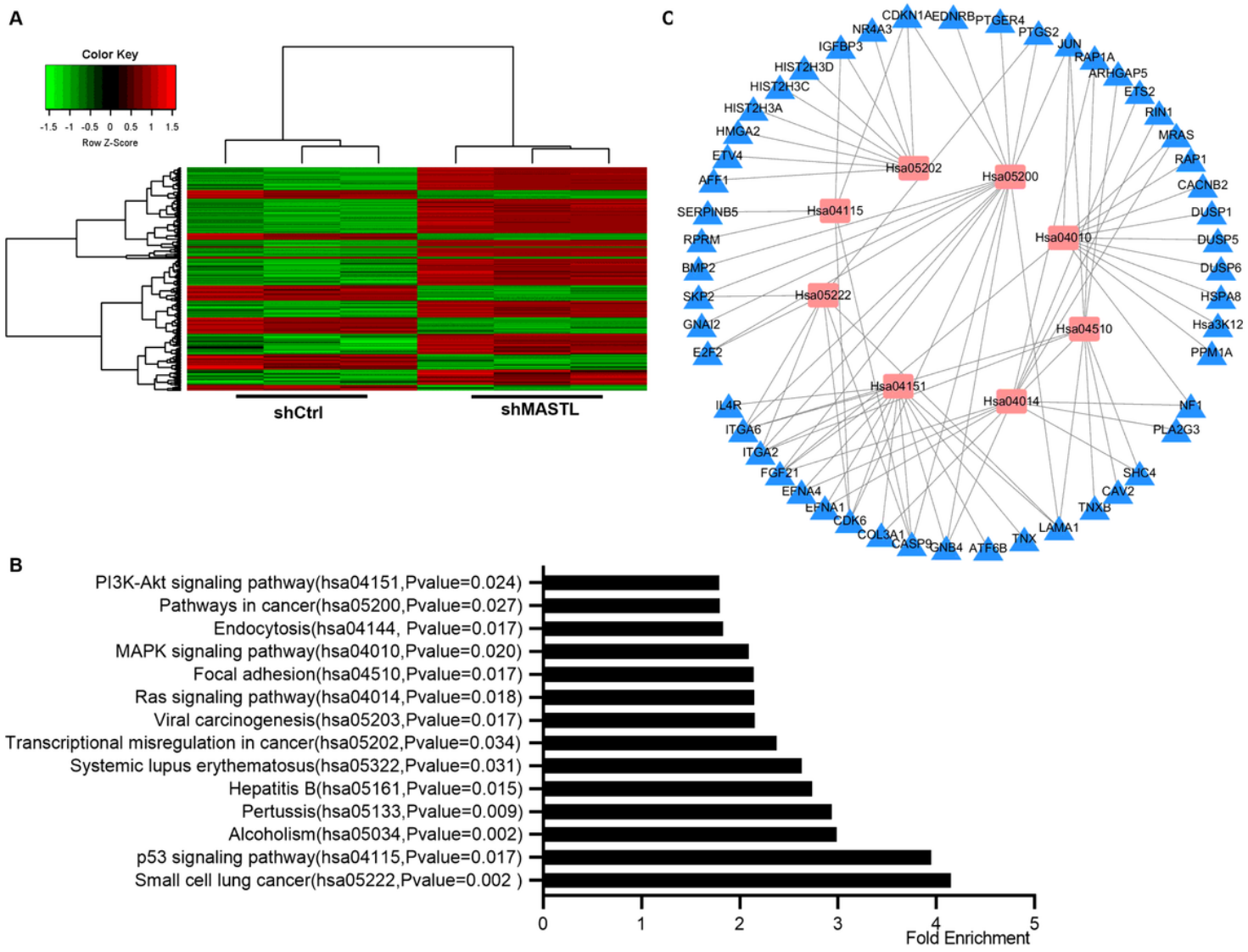

Figure 4

(A, B): Effect of MASTL gene knockdown on tumorigenesis in nude mice and imaging assay in vivo. Lv-shMASTL-infected MGC-803 cells and Lv-shCtrl-infected MGC-803 cells were implanted into the nude mice. Compared with Lv-shCtrl group, the fluorescence expression of Lv-shMASTL group was decreased ($P < 0.05$, Fig 4A). After 6 weeks, fluorescence imaging assay showed that there were significantly more the total radiant efficiency of region of interest (ROI) in xenografts of mice group developed from Lv-shMASTL-infected MGC-803 cells than that in the corresponding Lv-shCtrl-infected group ($P < 0.05$, , Fig 4B).

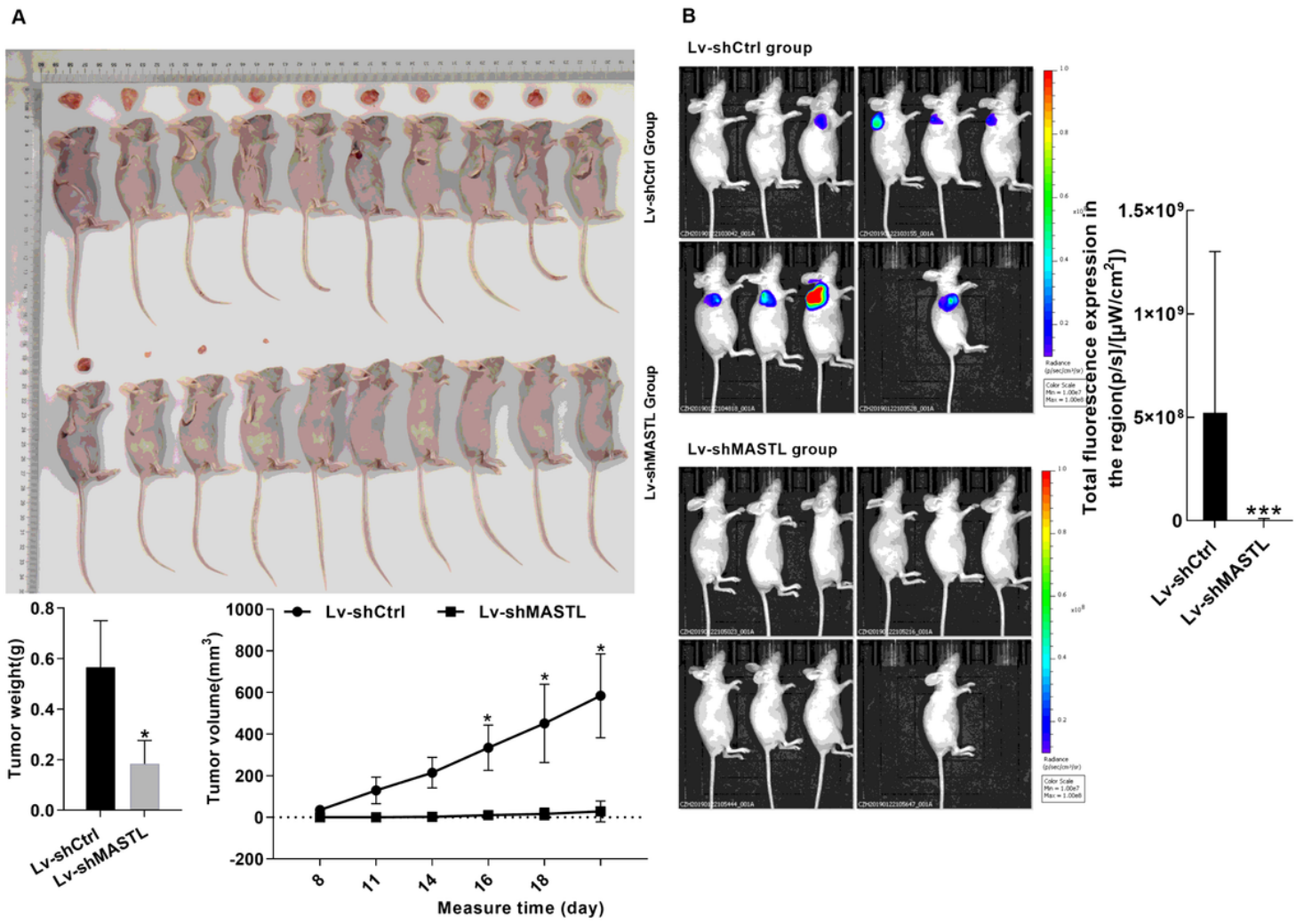

Figure 5

(A, B, C): Affymetrix mRNA microarray analysis of aberrantly expressed genes and pathway analysis with bioinformatical method. Hierarchical clustering of 391 genes were aberrantly expressed including 124 up-regulated genes, and 267 down-regulated genes screened by Affymetrix mRNA microarray between MGC-803 cells of Lv-shMASTL group and MGC-803 cells of Lv-shCtrl group (cut-off ≥1.5, ($P < 0.05$, , Fig 5A). KEGG pathway analysis showed that 291 aberrantly expressed genes were enriched in 14 pathways, among which, 9 pathways were closely related to tumorigenesis were closely related to tumorigenesis. 9 pathways were closely related to tumorigenesis were closely related to tumorigenesis including Small cell lung cancer, p53 signaling pathway, MAPK signaling pathway, Transcriptional misregulation in cancer, Ras signaling pathway, Pathways in cancer, Viral carcinogenesis, Focal adhesion, PI3K-Akt signaling pathway. In summary, this kind of bioinformatical analysis facility subsequent molecular mechanisms to discover the effects of silencing MASTL in gastric cancer cells ($P < 0.05$, Fig 5(B.C)).

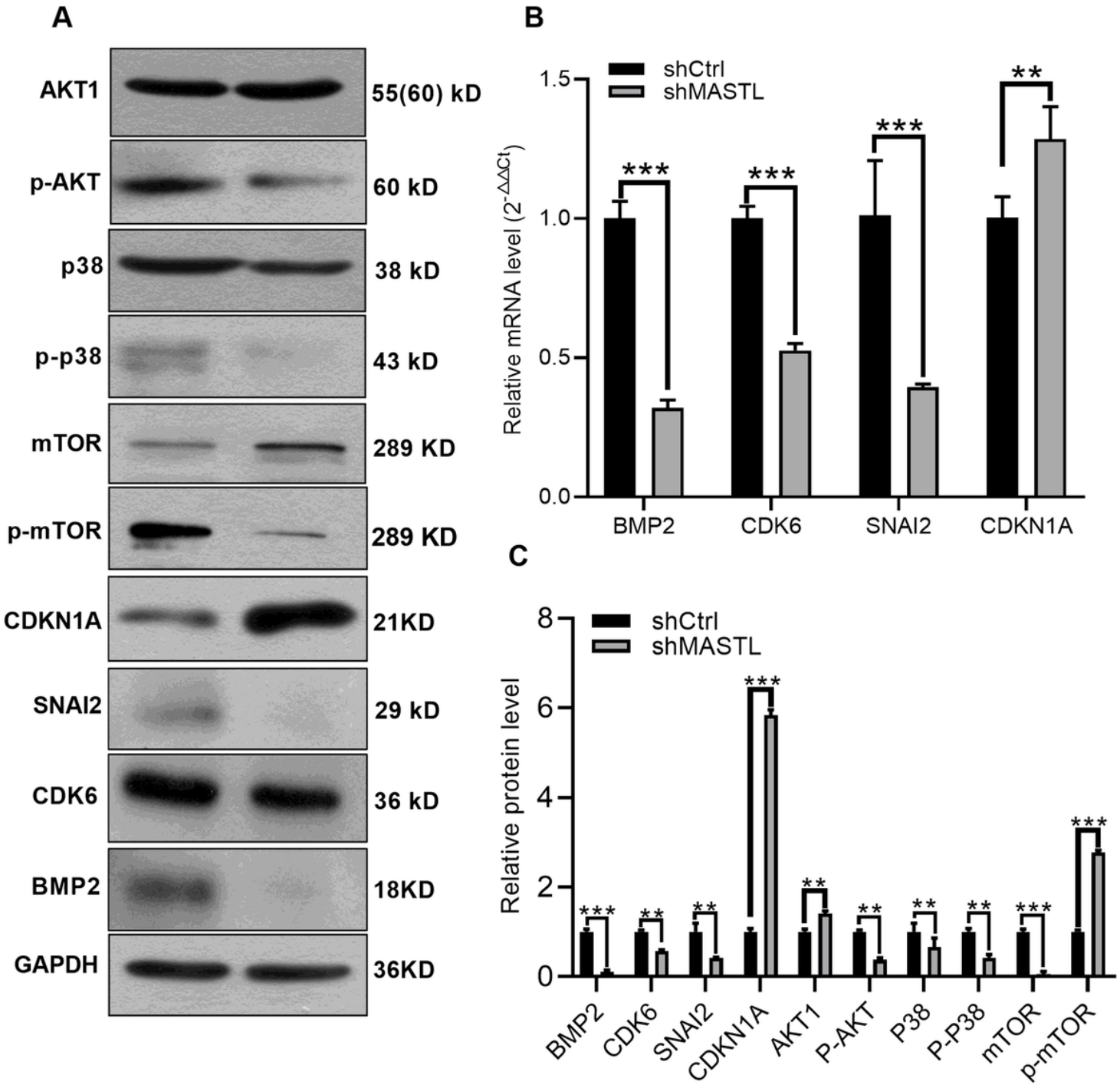

Figure 6

Effects of MASTL gene knockdown on several aberrantly expressed genes validation by RT-qPCR and western blotting. RT-qPCR and western blotting validation discovered that CDK6, BMP2 and SNAI2 are downregulated, and CDKN1A are upregulated (Figure. 6(A, B, C)). In addition, Western blotting analysis showed that phosphorylation form of p-mTOR, p-AKT and p-P38 proteins were downregulated significantly, (Figure. 6(A, B)). suggesting that CDK6, BMP2, SNAI2, CDKN1A, p-mTOR, p-AKT and p-P38 which play important roles in the cell growth, migration and arresting cell cycle of MGC-803 cells

following MASTL silencing. Furthermore, insightful studies are needed to clarify the mechanisms of MASTL in gastric cancer progression.