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Original Paper

Long Non-Coding RNA XLOC_006753 Promotes the Development of Multidrug Resistance in Gastric Cancer Cells Through the PI3K/AKT/mTOR Signaling Pathway

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Key Words
Xloc_006753 • PI3K/AKT/mTOR • Multidrug resistance • Gastric cancer

Abstract
Background/Aims: The development of multidrug resistance (MDR), which results in disease recurrence and metastasis, is a crucial obstacle to successful chemotherapy for patients with gastric cancer (GC). Long non-coding RNAs (lncRNAs) have been found to play various roles in cancer. This study aimed to investigate the effect of XLOC_006753 on the development of MDR in GC cells. ***Methods:*** The expression levels of XLOC_006753 in GC patients and MDR GC cell lines (SGC-7901/5-FU and SGC-7901/DDP cell line) were assessed by qRT-PCR. Statistical analyses were conducted to determine the relationship between XLOC_006753 expression and clinical features and to assess the prognostic value of XLOC_006753 for overall survival and progression-free survival. Then, a CCK-8 assay was used to detect cell proliferation ability and chemosensitivity. Flow cytometry was used to detect cell cycle and cell apoptosis. A wound-healing assay and transwell assay were used to detect cell migration. The expression of markers for MDR, G1/S transition, epithelial–mesenchymal transition (EMT) and PI3K/AKT/mTOR signaling pathway were examined by western blot. ***Results:*** XLOC_006753 was highly expressed in GC patients and MDR GC cell lines (SGC-7901/5-FU and SGC-7901/DDP

L. Zeng, Q. Liao, Z. Zou and Y. Wen contributed equally to this work.

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cell lines), and its high expression was positively associated with metastasis, TNM stage, tumor size, and poor survival in GC patients. Moreover, XLOC_006753 was an independent prognostic biomarker of overall survival and progression-free survival for gastric cancer patients. Knocking down XLOC_006753 in the two MDR GC cell lines significantly inhibited cell proliferation, cell viability, cell cycle G1/S transition, and migration. XLOC_006753 knockdown also promoted apoptosis. Furthermore, western blots showed that XLOC_006753 knockdown decreased some markers of MDR, G1/S transition, and EMT expression, while increasing caspase9 expression and inhibiting the PI3K/AKT/mTOR signaling pathway in SGC-7901/5-FU and SGC-7901/DDP cells. ***Conclusion:*** High expression of XLOC_006753 promoted the development of MDR, which was activated by the PI3K/AKT/mTOR pathway in GC cells.

Introduction

Gastric cancer (GC) is one of the most common malignancies of human digestive systems, and its high incidence is associated with high mortality rates globally [1-3]. Chemotherapy is usually used in the treatment of gastric cancer both in adjuvant and advanced settings [4]. Although chemotherapy has improved the survival of patients with gastric cancer, the development of multidrug resistance (MDR) is still a crucial problem leading to therapeutic failure and cancer-related death [5]. In addition, the underlying molecular mechanisms of MDR in gastric cancer remain elusive.

Recent studies indicate that long non-coding RNAs (lncRNAs) play essential roles in MDR. For example, lncRNAs, including GHET1 [6], UCA1 [7, 8], ANRIL [9], and PVT1 [10], are related to the development of MDR in GC. Furthermore, recent research has characterized multiple molecular roles of regulatory lncRNAs in the PI3K/Akt/mTOR signaling pathway that are involved in cancer drug resistance. For example, lncRNA HOTALR regulates cisplatin resistance of GC through the PI3K/Akt and Wnt/β-catenin signaling pathways [11, 12]. In addition, Ma et al. found that lncRNA DANCR mediates cisplatin resistance in glioma cells by activating the AXL/PI3K/Akt/NF-κB signaling pathway [13]. Similarly, lncRNA UCA1 enhances drug resistance through the mTOR signaling pathway in breast cancer [14] and EGFR-mutant non-small cell lung cancer [15].

In our study, a novel lncRNA XLOC_006753 was found to be aberrantly expressed in gastric carcinoma, and the expression of XLOC_006753 was associated with metastasis, TNM stage, and tumor size. In addition, XLOC_006753 was up-regulated in SGC-7901/5-FU and SGC-7901/DDP cells. Inhibition of XLOC_006753 using siRNA significantly reduced cell growth and migration and promoted cell apoptosis in SGC-7901/5-FU and SGC-7901/DDP cells. In this study, we further analyzed the effect of XLOC_006753 expression on the development of multidrug resistance in gastric cancer.

Materials and Methods

Patient tissue samples and cell culture

GC tumor tissues and corresponding normal tissues were acquired from 110 cases of patients diagnosed with GC at Sun Yat-Sen University Cancer Center (SYSUCC). This study was approved by the Research Ethics Committees of Sun Yat-Sen University Cancer Center, and written informed consent was obtained from all patients.

Human gastric adenocarcinoma cell line (SGC-7901), maintained in the Surgical Laboratory at Guangzhou Medical University, and the MDR variants SGC-7901/5-FU and SGC-7901/DDP were obtained and maintained in laboratory conditions as described previously [16, 17].

Cell transfection

Well-differentiated GC cells were grown in six-well plates and cultured to 60% confluence. Cells were transfected with a mixture of siRNA-XLOC_006753 (100 nmol/L) and Lipofectamine® 3000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. After a 36-h incubation period, the transfected cells were used for further experiments. The sequences of siRNA-XLOC_006753 were as follows: siRNA-1, sense, 5′-GCUGGAGACAUAUCUUGAUTT-3′, and antisense, 5′-AUCAAGAUAUGUCUCCAGCTT-3′; siRNA-2, sense, 5′-GAGGCCAUUAAAGAAUAAATT-3′, and, antisense:5 ′-UUUAUUCUUUAAUGGCCUCTT-3′ (Gene Pharma, Shanghai, China).

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from GC tumor tissue samples and GC cell lines using TRIzol™ reagent (Invitrogen) according to the manufacturer's instructions. The quality and concentration of the total RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Total RNA (2 μg) was used for reverse transcription with the GoScript Reverse Transcription (RT) System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Real-time qPCR was performed with 2 μL of RT products (cDNA) and 7.5 μL of GoTaq qPCR Master Mix (Promega) in a 15-μL total reaction volume on a CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. Primers for XLOC_006753 amplification were as follows: forward, 5′-ATGACACTGTTTGGAAAGCTGC-3′; reverse, 5′-TAACTGGGGCTGTGTTTGGT-3′. Glyceraldehyde 3-phosphatedehydrogenase (GAPDH) was used as an internal standard with forward primer 5′-CTCCTCCTGTTCGACAGTCAGC-3′ and reverse primer 5′-CCCAATACGACCAAATCCGTT-3′. All experiments were performed in triplicate. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation and cell viability

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). The SGC-7901/5-FU and SGC-7901/DDP cells were transfected with siRNA-XLOC_006753 or negative control siRNA and seeded into 96-well plates (1×10^3 cells/well). At 24, 48, 72, and 96 h, CCK-8 reagent was added, and the cells were cultured for a further 2 h at 37°C. Absorbance at 450 nm was measured using a microplate reader. All experiments were performed in triplicate. For cell viability analysis, after transfection with siRNA-XLOC_006753 or negative control siRNA for 24 h, 5 μg/ml 5-FU or 2 μg/ml DDP was added to the cells for another 48 h. Then, CCK-8 reagent was added, and the cells were cultured for a further 2 h at 37°C, after which absorbance at 450 nm was measured.

Flow cytometric analysis of the cell cycle

The SGC-7901/5-FU and SGC-7901/DDP cells were transfected with siRNA-XLOC_006753 or negative control siRNA. After 36 h, the cells were serum-starved for 22 h, replenished with 10% fetal bovine serum-RPMI 1640 medium, and then stained with propidium iodide solution (MULTI SCIENCES, Hangzhou, China). The images of the cell cycle were captured and analyzed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) with FlowJo software (TreeStar Corp., San Carlos, CA, USA).

Flow cytometric analysis of cell apoptosis

SGC-7901/5-FU and SGC-7901/DDP cells were transfected with siRNA-XLOC_006753 or negative control siRNA, followed by double staining with an AnnexinV-FITC/PI apoptosis detection kit (MULTI SCIENCES) according to the manufacturer's instructions. Cell apoptosis rates were assessed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences) and FlowJo software (TreeStar Corp.).

Migration assays

Migration assayed were performed using 24-well Millipore transwell chambers (Millipore Corporation, Billerica, MA, USA). The cells (1×10^5 cells/well), suspended in a serum-free medium, were added to the top of each well, and 600 μL of RPMI-1640 medium containing 10% FBS were added to the lower chamber. After 24 h, the migratory cells were fixed with 4% paraformaldehyde for 20 min and stained with crystal violet solution (Beyotime, Shanghai, China) for 20 min. The migrated cells were photographed and counted. The data are presented as the mean number of cells per field.

Wound healing assay

Cells were seeded into six-well plates and transfected with the negative control siRNA-NC or siRNA-XLOC_006753 for 36 h. Then, the cells were serum-starved for 22 h and replenished with 10% fetal bovine serum RPMI-1640 medium. When the cells reached 100% confluence, an artificial wound was carefully created by scratching the confluent cell monolayer with a 200 μL pipette tip. The wound was imaged immediately at 0 h, and then 12 and 24 h later.

Western blotting

Total proteins extracts were obtained from cells using lysis buffer containing protease inhibitor, and protein concentrations were determined by BCA protein assay kit (KenGEN BioTECH, Nanjing, China). First, 30-μg protein samples were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk for 2 h at room temperature and incubated with anti-antibody at 4°C overnight. After being washed three times with TBST, the membranes were incubated with HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 2 h. β-Tubulin was used as an internal control. Protein expression was visualized using a chemiluminescence system (Tanon 5200, Tanon Science & Technology Co., Ltd., Shanghai, China).

Statistical analysis

SPSS software (version 20.0; IBM Corp., Armonk, NY, USA) was used for statistical analyses. Relative expression of XLOC_006753 in GC tissues and paired normal tissues were evaluated using paired-sample *t*-tests. Chi-squared or Fisher's exact tests were used to examine the association between XLOC_006753 expression and various clinicopathological variables. Survival rates were calculated using a Kaplan–Meier survival analysis. The Cox proportional hazard regression model was used to identify independent prognostic factors. Test results with $P < 0.05$ were considered statistically significant.

Results

XLOC_006753 is upregulated in GC tissues and MDR GC cell lines

The expression of XLOC_006753 was analyzed in 43 cases of GC patients by qRT-PCR, which was normalized using GAPDH as an internal control. XLOC_006753 expression was significantly up-regulated in tumor tissues compared to the paired normal tissues ($P < 0.05$; Fig. 1A). To determine whether XLOC_006753 is involved in tumor progression in MDR gastric

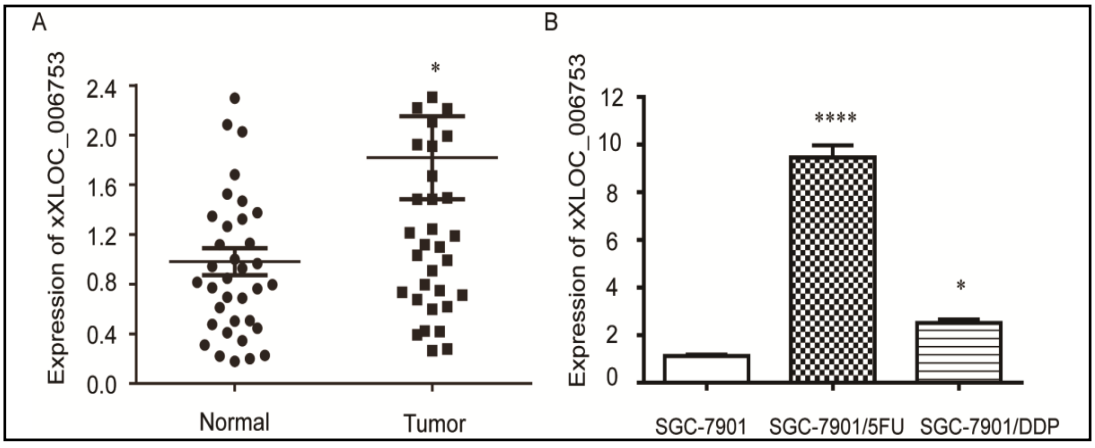

Fig. 1. Expression of XLOC_006753 is upregulated in gastric cancer (GC) tissues and multidrug resistance (MDR) GC cell lines. (A) Real-time qRT-PCR analysis showed that the expression level of XLOC_006753 significantly increased in 36 GC tissues compared to the corresponding normal tissues. *P<0.05 compared to the control group. (B) Expression levels of XLOC_006753 significantly increased in the SGC-7901/5-FU and SGC-7901/DDP cell lines, compared to the SGC-7901 cell line. Expression levels were normalized to GAPDH mRNA. *P<0.05, ****P < 0.0001

cancer cells, its expression levels were determined by qRT-PCR of the MDR lines SGC-7901/5-FU and SGC-7901/DDP as well as in the chemosensitive parental cell line SGC-7901. Expression of XLOC_006753 was up-regulated in both MDR lines compared to the parental line (Fig. 1B).

High XLOC_006753 expression is associated with unfavorable clinical characteristics in GC patients

To investigate the clinical significance of XLOC_006753 overexpression, we analyzed the relationship between XLOC_006753 expression and clinical characteristics in 110 GC patients. GC patients were divided into high and low XLOC_006753 expression groups using the median expression level as the cut-off point (median, 1.0347; range, 0.0313–11.605). The association between XLOC_006753 expression level and clinical characteristics was evaluated by chi-squared and Fisher's exact tests. High XLOC_006753 expression was significantly associated with metastasis, advanced TNM stage, and tumor size in GC patients (Table 1), indicating that XLOC_006753 overexpression is associated with GC progression.

Table 1. Association between XLOC_006753 expression and clinical characteristics in GC: $\chi 2$ or Fisher's exact tests were used for P-value calculation. aPatients were divided into high and low XLOC_006753 expression groups using the median expression level as the cut-off point (1.0347). TNM, tumor-node-metastasis

Characteristics	All cases	XLOC_006753 expression (%) Low	High	P-value
Gender				0.829
Female	29	14(48.3%)	15(51.7%)	
Male	81	41(50.6%)	40(49.4%)	
Age(years)				0.699
≤58	64	33(51.6%)	31(48.4%)	
＞58	46	22(47.8%)	24(52.2%)	
Topography				0.052
T1,T2	15	11(73.3%)	4(26.7%)	
T3,T4	95	44(46.3%)	51(53.7%)	
Lymph Node				0.436
N0,N1,N2	66	35(53%)	31(47%)	
N3	44	20(45.5%)	24(54.5%)	
Metastasis				0.042
M0	96	51(53.1%)	45(46.9%)	
M1	13	3(23.1%)	10(76.9%)	
TNM Stage				0.010
Ⅰ,Ⅱ	30	21(70%)	9(30%)	
ⅢⅣ	80	34(42.5%)	46(57.5%)	
Family Medical History				0.376
Yes	13	8(61.5%)	5(38.5%)	
No	97	47(48.5%)	50(51.5%)	
Smoking				0.076
Yes	41	25(61%)	16(39%)	
No	69	30(43.5%)	39(56.5%)	
Drinking				0.482
Yes	23	13(56.5%)	10(43.5%)	
No	87	42(48.3%)	45(51.7%)	
Tumor Differentiation				0.548
I	24	14(58.3%)	10(41.7%)	
II	37	19(51.4%)	18(48.6%)	
III	49	22(44.9%)	27(55.1%)	
Tumor Size(cm)				0.036
≤5	55	22(40%)	33(60%)	
＞5	55	33(60%)	22(40%)	
General Classification				0.645
Elevated Type	24	10(41.7%)	14(58.3%)	
Ulcerative Type	76	40(52.6%)	36(47.4%)	
Infiltrating Type	10	5(50%)	50(50%)	

XLOC_006753 is associated with poor prognosis and an independent prognostic factor in GC patients

To further explore the prognostic value of XLOC_006753 in GC patients, we analyzed overall survival (OS) and progression-free survival (PFS) of GC patients with high or low XLOC_006753 expression using the Kaplan–Meier method and Log-rank test. The 5-year OS and PFS of patients with high XLOC_006753 expression were only 38.13% and 42.87%, respectively, which were considerably shorter than those with low XLOC_006753 expression (63.71% and 69.28%, respectively; Fig. 2A and B). Thus, a high level of XLOC_006753 is significantly associated with poor prognoses of GC patients.

We further stratified GC patients according to TNM stage. Stage I–II patients with high XLOC_006753 levels have remarkably poorer OS and PFS than those with low XLOC_006753 levels (Fig. 2C and D), and stage III patients with high XLOC_006753 levels had significantly shorter OS than those with low expression levels. Moreover, stage III patients with high XLOC_006753 levels had remarkably poorer PFS than those with low expression levels (Fig. 2E and F). It should be noted that there was a small sample size for stage I–II patients. No significant difference was seen between OS and PFS of patients with high and low

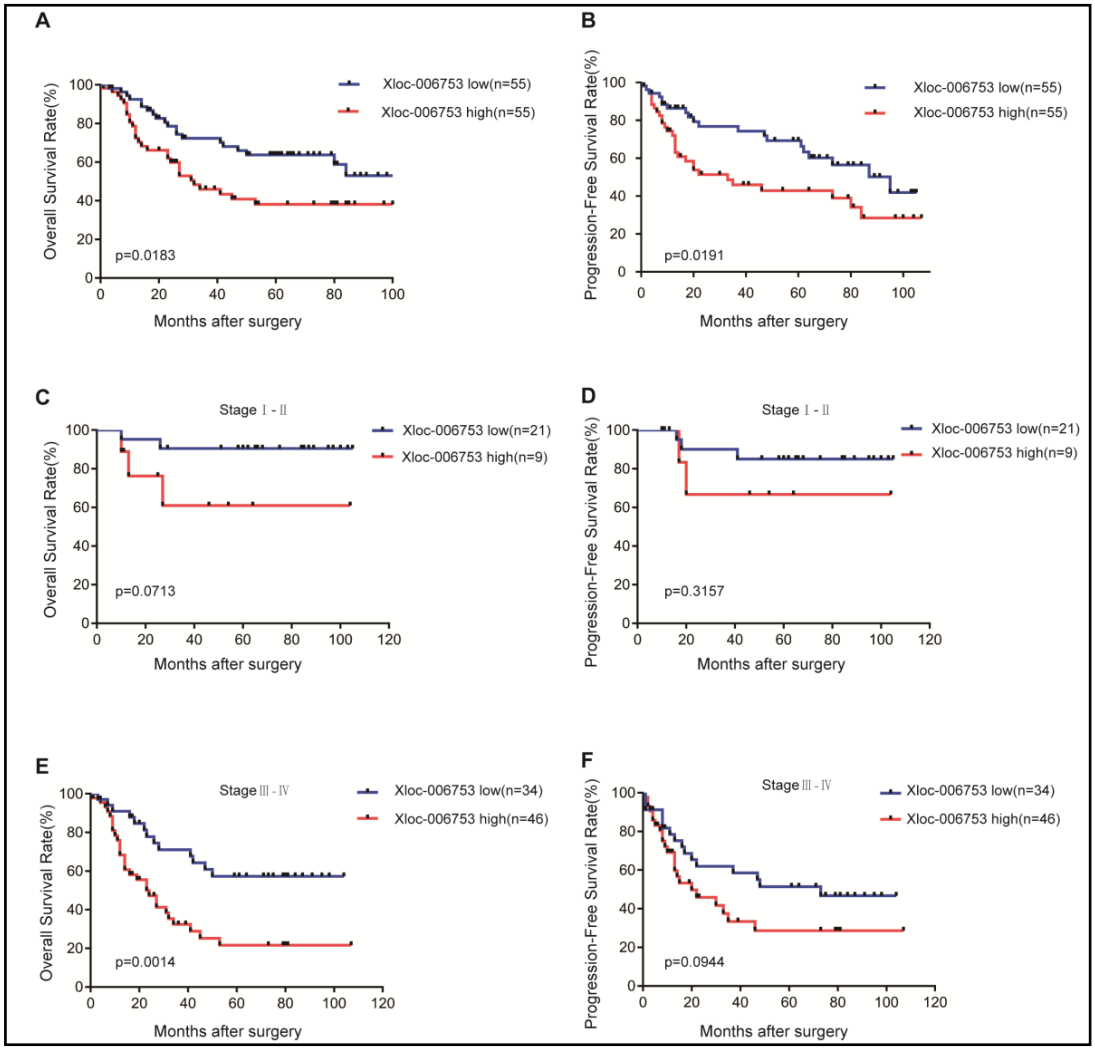

Fig. 2. Higher XLOC_006753 expression was associated with shorter survival times. (A) Overall survival (OS) of 110 gastric cancer (GC) patients with high or low XLOC_006753 expression. (B) Progression-free survival (PFS) of 110 GC patients with high or low XLOC_006753 expression. (C) OS and (D) PFS of stage I–II ESCC patients with high or low XLOC_006753 expression. (E) OS and (F) PFS of stage III ESCC patients with high or low XLOC_006753 expression.

XLOC_006753 expression in stage I–II subgroups. Therefore, XLOC_006753 expression level predicts the survival of GC patients independently of clinical stage, particularly for OS, indicating that XLOC_006753 can provide additional prognostic information for the clinical staging system.

To determine whether XLOC_006753 is an independent prognostic factor in GC patients, we analyzed the relationship of XLOC_006753 expression and various clinicopathological parameters with patient survival using univariate and multivariate Cox proportional hazard models. Univariate analysis indicated that T stage, node status, metastasis, TNM stage, and XLOC_006753 expression are significant predictors for OS of GC patients (Table 2). Moreover, T stages, lymph node status, metastasis, and TNM stage are also significant predictors for PFS in GC patients, and XLOC_006753 is a significant predictor for OS and PFS in GC patients. Multivariate Cox regression analysis demonstrated that XLOC_006753 is an independent risk predictor for OS (HR, 2.333; 95% CI, 1.229–4.428, $P = 0.010$) in GC patients, but not for PFS (Table 2). These results suggest that XLOC_006753 is a potential biomarker for predicting the overall survival of GC patients.

Table 2. Univariate and multivariate Cox regression analysis of XLOC_006753 and survival in patients with gastric cancer (GC)

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Overall Survival						
Gender (Female vs male)	1.568	0.847-2.904	0.153			
Age (>58y vs≤58y)	0.901	0.500-1.622	0.728			
Topography	2.211	1.209-4.041	0.010	3.771	0.411-4.563	0.240
Lymph Node	1.615	1.225-2.128	<0.001	1.487	1.119-1.975	0.006
Metastasis	2.824	1.557-5.119	0.002	2.037	1.074-3.861	0.029
TNM Stage	4.661	1.840-11.809	<0.001	2.681	0.835-8.604	0.097
Family Medical History	1.760	0.822-3.770	0.146			
Smoking(yes vs no)	0.900	0.496-1.633	0.730			
Drinking(yes vs no)	0.916	0.443-1.896	0.814			
Tumour Differentiation	1.397	0.747-2.613	0.295			
Tumour Size (≥5cm vs <5cm)	1.256	0.708-2.228	0.436			
General Classification	1.479	0.847-2.583	0.169			
XLOC_006753 expression(high vs low)	2.172	1.203-3.922	0.010	2.333	1.229-4.428	0.010
Progression-Free Survival						
Gender (Female vs male)	1.557	0.842-2.880	0.158			
Age (>58y vs≤58y)	0.821	0.456-1.479	0.521			
Topography	2.277	1.238-4.187	0.008	1.934	1.009-3.707	0.047
Lymph Node	1.625	1.234-2.139	<0.001	1.410	1.063-1.870	0.017
Metastasis	2.667	1.120-6.348	0.027	2.200	1.185-4.084	0.012
TNM Stage	4.885	1.929-12.372	<0.001	2.592	0.795-8.449	0.114
Family Medical History	1.906	0.890-4.085	0.097			
Smoking(yes vs no)	0.869	0.478-1.573	0.639			
Drinking(yes vs no)	0.922	0.446-1.908	0.828			
Tumour Differentiation	1.352	0.723-2.527	0.345			
Tumour Size (≥5cm vs <5cm)	1.245	0.702-2.209	0.453			
General Classification	1.542	0.895-2.658	0.119			
XLOC_006753 expression (high vs low)	2.042	1.131-3.686	0.018	1.911	0.576-6.347	0.290

XLOC_006753 promotes cell proliferation and viability in MDR GC cells

To assess the biological significance of XLOC_006753 in MDR GC cells, we transfected SGC-7901/5-FU and SGC-7901/DDP cells with two XLOC_006753 siRNAs and one control siRNA-NC. The efficiency of siRNA-mediated XLOC_006753 knockdown was evaluated by qRT-PCR, achieving a significant reduction of XLOC_006753 (Fig. 3A and B). A CCK-8 cell proliferation assay was also performed. XLOC_006753 siRNAs, but not negative control siRNA, significantly reduced proliferation of SGC-7901/5-FU and SGC-7901/DDP (Fig. 3C and D). These results indicate that XLOC_006753 is important to MDR GC cell proliferation. Then, after the SGC-7901/5-FU and SGC-7901/DDP cell lines were transfected with siRNA-XLOC_006753 or siRNA-NC for 24 h, these cells and SGC-7901 cells were treated with 5 μg/ml 5-FU or 2 μg/ml DDP for another 48 h, followed by a CCK-8 cell viability assay. SGC-7901/5-FU and SGC-7901/DDP cells transfected with siRNA-XLOC_006753 had a low viability that was similar to that of SGC7901, which suggested that XLOC_006753 knockdown reduced cell viability of SGC-7901/5-FU and SGC-7901/DDP cells (Fig. 3E).

To investigate the mechanism by which XLOC_006753 enhances the drug resistance of GC cells, we examined the protein levels of several MDR-related proteins (MDR1, LRP1 and BCRP) by western blot. We found that the protein levels of MDR1, LRP1, and BCRP decreased in SGC-7901/5-FU and SGC-7901/DDP cells transfected with siRNA-XLOC_006753 compared to the control (Fig. 3F).

XLOC_006753 promotes G1/S cell cycle progression in MDR GC cells through the G1/S pathway

Because XLOC_006753 knockdown inhibits MDR of GC cells, we further investigated whether the cell cycle is affected by XLOC_006753 knockdown. Flow cytometry analysis shows that XLOC_006753 downregulation significantly increases G1 cell population while decreasing S cell population in SGC-7901/5-FU cells (Fig. 4A and B). A similar result was

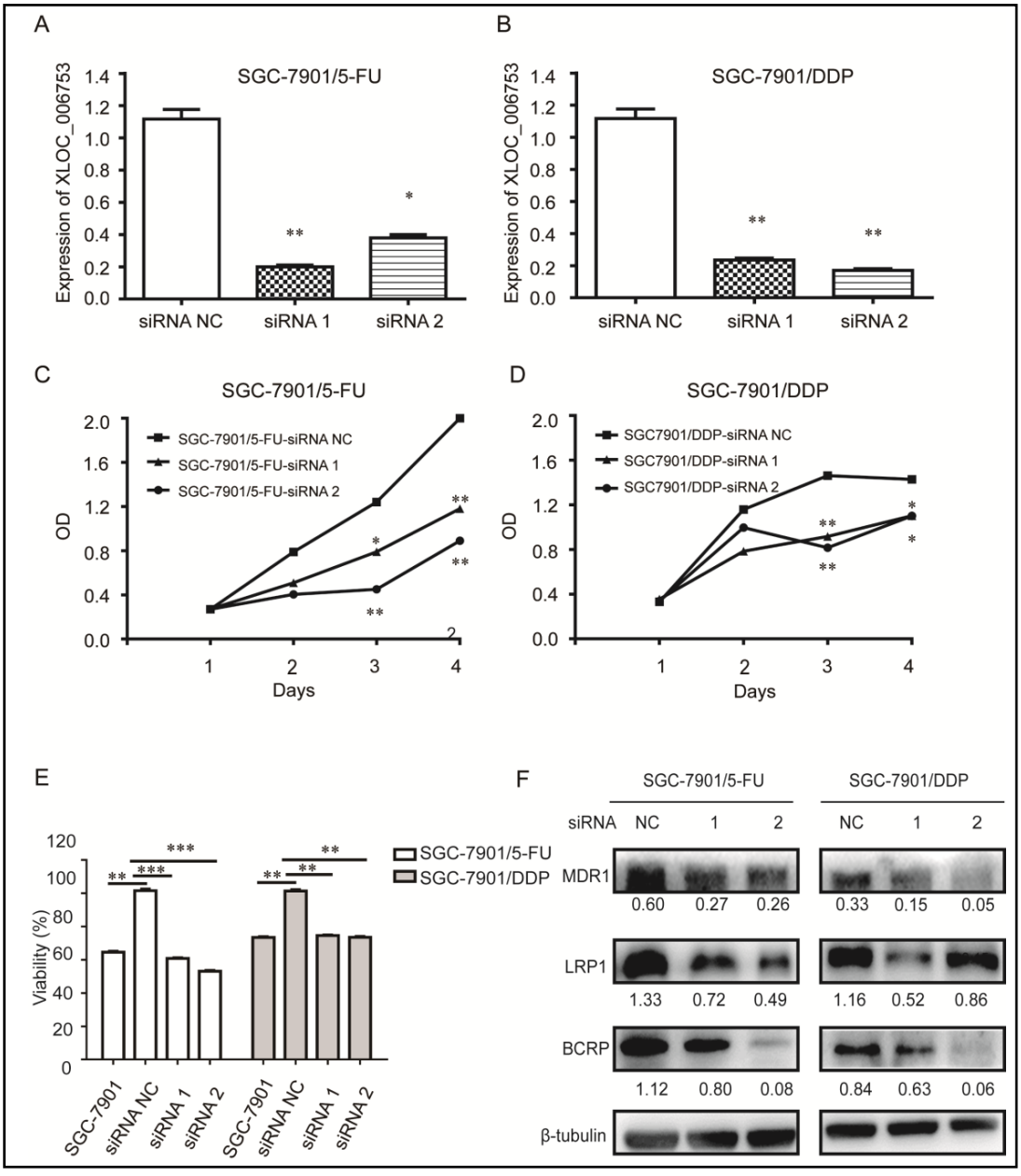

Fig. 3. XLOC_006753 knockdown inhibited cell proliferation and cell viability. Real-time qRT-PCR analysis of XLOC_006753 expression was conducted for (A) SGC-7901/5-FU and (B) SGC-7901/DDP cell lines transfected with two specific siRNAs or one negative control (NC) sequence, respectively. A CCK-8 assay was used to evaluate cell growth in (C) SGC-7901/5-FU and (D) SGC-7901/DDP cells transfected with two siRNAs or one NC sequence, respectively. Each value represents the mean (± S.D.) of three independent experiments (*P<0.05; **P<0.01). The y-axis indicates optical density (OD). (E) The viability of SGC-7901/5-FU and SGC-7901/DDP cells transfected with siRNA-XLOC_006753 and SGC-7901 cells treated with 5-FU (5 μg/ml) or DDP (2 μg/ml) was determined using CCK-8 assays. (F) Western blot analyses indicated that XLOC_006753 knockdown down-regulates the expression of MDR1, LRP1, and BCRP proteins in SGC-7901/5-FU and SGC-7901/DDP cells.

obtained in SGC-7901/DDP cells (Fig. 4C and D). These results indicate that XLOC_006753 knockdown causes G1 cell cycle arrest in both MDR GC cell lines.

We examined whether XLOC_006753 knockdown affects the G1/S pathway. Indeed, XLOC_006753 knockdown in SGC-7901/5-FU and SGC-7901/DDP cells causes a decrease in cyclin D1, cyclin D3, and CDK2/6 (Fig. 4E). Taken together, our results demonstrate that XLOC_006753 is important for cell cycle progression through cyclin D1, cylin D3, and CDK2/6.

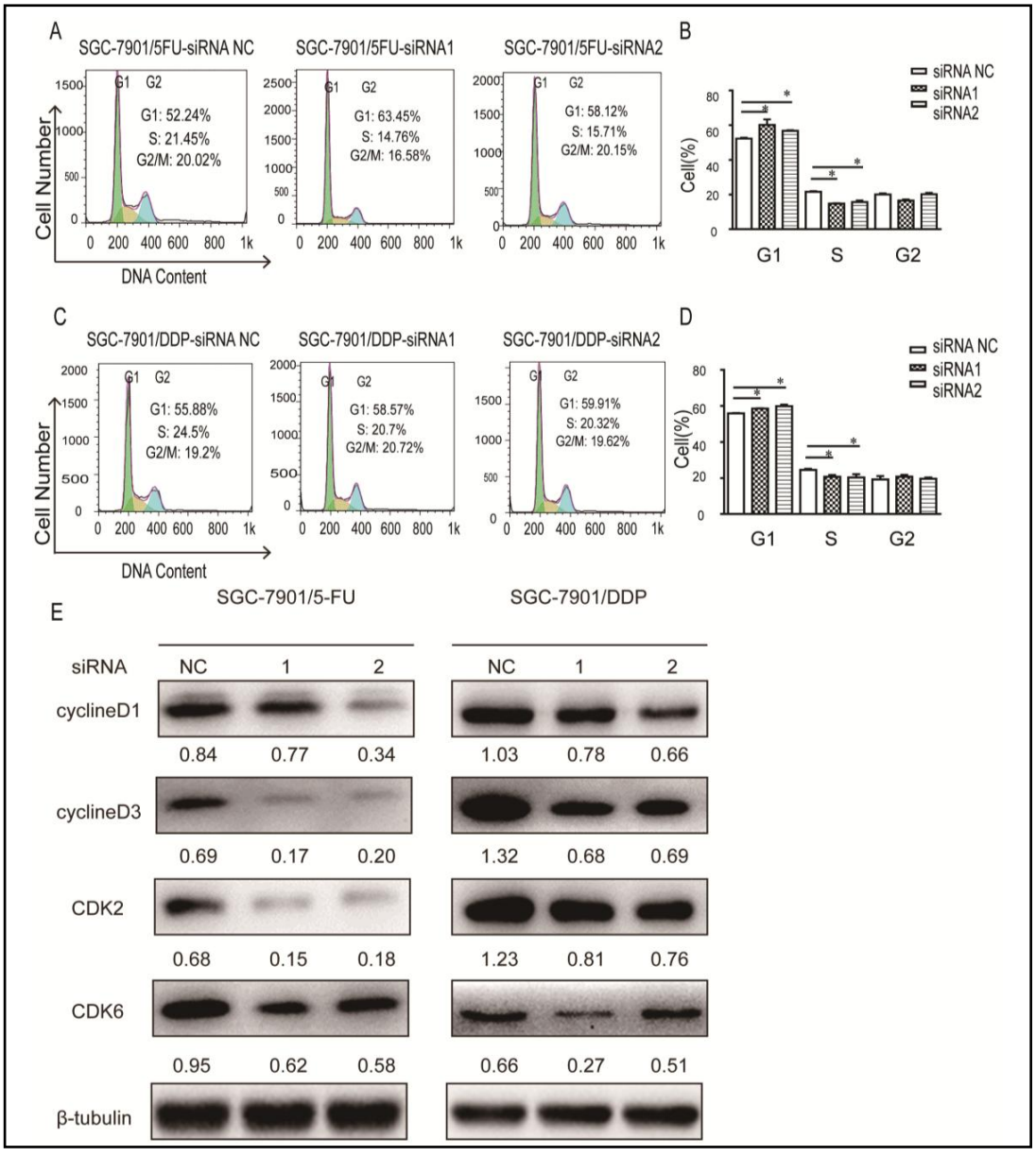

Fig. 4. XLOC_006753 knockdown induces G1/S arrest of multidrug resistance (MDR) gastric cancer (GC) cells. (A and C) Flow cytometry analysis of cell cycles of the indicated MDR GC cells transfected with siRNAs and negative control (NC) sequences. The proportion of G1-phase cells significantly increased and the proportion of S-phase cells clearly decreased in XLOC_00675 knockdown cell lines compared with the control cells in both (B) SGC-7901/5-FU and (D) SGC-7901/DDP cell lines (*P<0.05). (E) Western blot analysis indicated that XLOC_006753 knockdown downregulated the expression of cyclin D1, cyclin D3, CDK2, and CDK6 proteins in SGC-7901/5-FU and SGC-7901/DDP cells.

XLOC_006753 inhibits apoptosis in MDR GC cells by altering expression of apoptosis-associated proteins

The effect of XLOC_006753 knockdown on the apoptosis of SGC-7901/5-FU and SGC-7901/DDP cells was determined by flow cytometric analysis with Annexin V-FITC and PI double staining. Compared with SGC-7901/5-FU-siRNA-NC (2.34%) and SGC-7901/DDP-siRNA-NC cells (8.83%), the number of apoptotic cells was significantly increased in the SGC-7901/5-FU-siRNA 1 (11.97%), SGC-7901/5-FU-siRNA 2 (7.02%), SGC-7901/DDP-siRNA 1 (10.3%), and SGC-7901/DDP-siRNA 2 (18.8%) (Fig. 5A–D). Next, we used immunoblotting analysis to monitor expression changes in apoptosis-associated proteins in SGC-7901/5-FU and SGC-7901/DDP cells with ablation of XLOC_006753. When compared with control cells, caspase9 protein levels were increased (Fig. 5E). These results revealed that XLOC_006753 overexpression promoted apoptosis in SGC-7901/5-FU and SGC-7901/DDP cells by inhibiting expression of caspase9.

XLOC_006753 promotes migration in MDR GC cells through enhancing epithelial–mesenchymal transition (EMT)

We also investigated the potential role of XLOC_006753 in cell migration using transwell and wound healing assays. In the transwell assay, down-regulation of XLOC_006753 significantly reduced the number of migrated cells for SGC-7901/5-FU and SGC-7901/DDP cells by approximately 60% and 45%, respectively (Fig. 6A). In the wound healing assay, cell

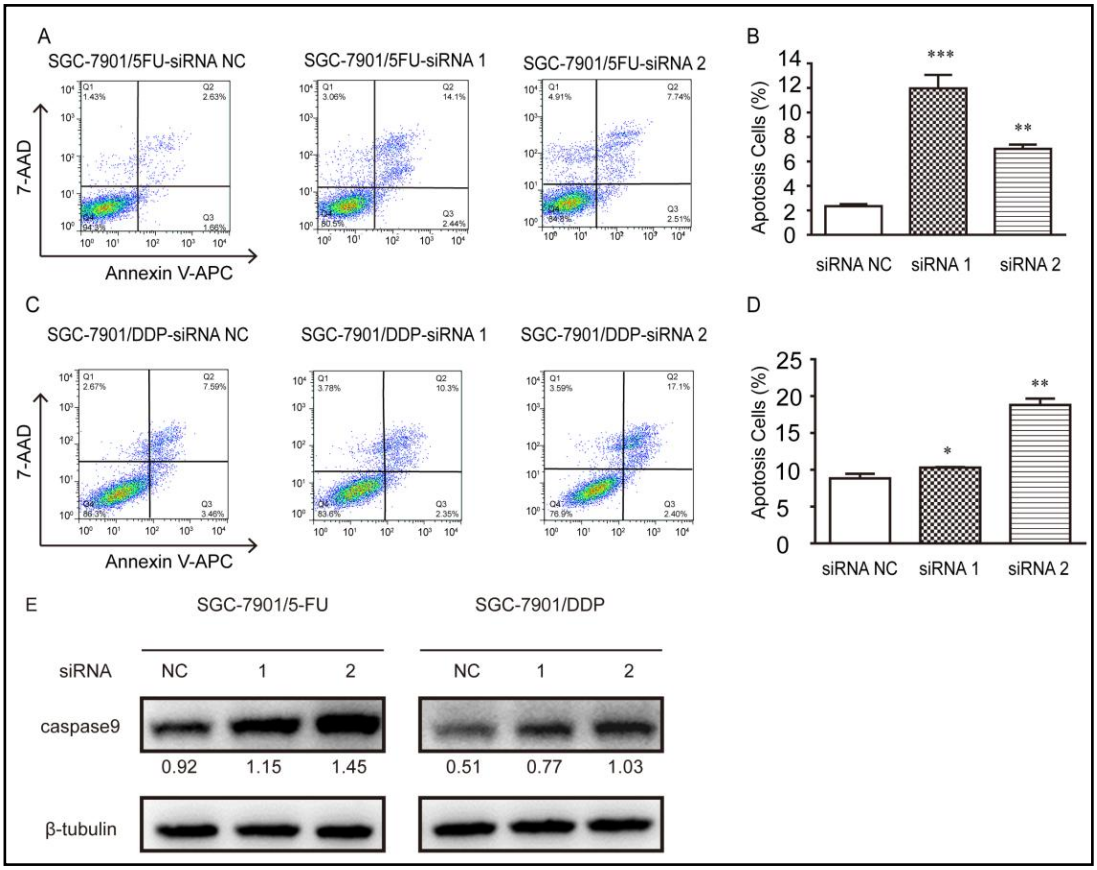

Fig. 5. Depletion of XLOC_006753 promoted cell apoptosis of MDR GC cells. (A and C) Flow cytometry analysis of cell apoptosis of the indicated MDR GC cells transfected with siRNAs and negative control (NC) sequences. Compared to the control group, the cell apoptosis rate increased significantly in (B) SGC-7901/5-FU and (D) SGC-7901/DDP cells with XLOC_006753 knockdown (*P<0.05, **P<0.01). (E) Western blot analysis indicated that XLOC_006753 knockdown upregulates the expression of caspase9 proteins in SGC-7901/5-FU and SGC-7901/DDP cells.

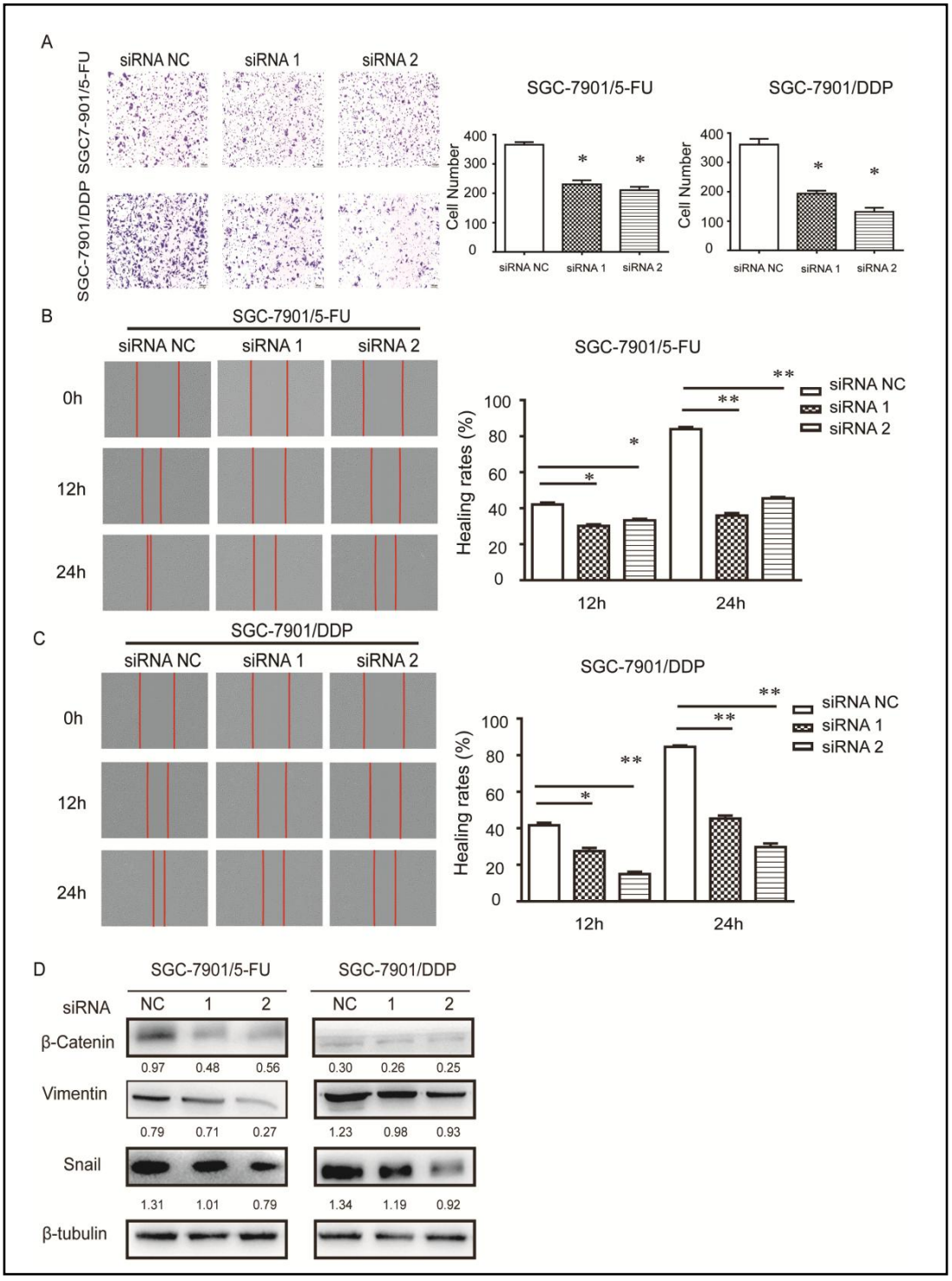

Fig. 6. Downregulation of XLOC_006753 inhibited the migration of multidrug resistance (MDR) gastric cancer (GC) cells. (A) Knockdown of XLOC_006753 reduced the migration of SGC-7901/5-FU and SGC-7901/DDP cells. The cells were transiently transfected with XLOC_006753 siRNA(s) and negative control (NC) sequences, and the number of migrated cells decreased when cells (observed under ×100 magnification) were transfected with XLOC_006753 siRNA(s) compared to those transfected with NC sequences. Wound healing assay showed that downregulation of XLOC_006753 inhibited cell migration over 12 h and 24 h in (B) SGC-7901/5-FU and (C) SGC-7901/DDP cell lines (×100 magnification). (D) Western blot analysis indicated that XLOC_006753 knockdown decreased β-catenin, Vimentin, and Snail expression.

migration rate was markedly decreased in SGC-7901/5-FU-siRNA 1 (29% and 37%), SGC-7901/5-FU-siRNA 2 (34% and 45%), SGC-7901/DDP-siRNA 1 (27% and 45%), and SGC-7901/DDP-siRNA 2 (15% and 30%) compared with SGC-7901/5-FU-siRNA-NC (43% and 84%) and SGC-7901/DDP-siRNA-NC (42% and 84%) at 12 h and 24 h, respectively (Fig. 6B and C).

The EMT was also investigated in the migration inhibition in MDR caused by XLOC_006753 knock down. Western blot analysis was performed to determine the expression of EMT markers. We found that the expression of β-catenin, Vimentin, and Snail were reduced in SGC-7901/5-FU and SGC-7901/DDP cells in association with XLOC_006753 knockdown (Fig. 6D). These results demonstrate that XLOC_006753 down-regulation inhibits the EMT.

XLOC_006753 promotes MDR by activation of the PI3K/AKT/mTOR signaling pathway

To determine whether the PI3K/AKT/mTOR signaling pathway was affected by XLOC_006753, the expression levels of PI3K, Akt, p-Akt, mTOR, p-mTOR, P70 S6 kinase, p-P70 S6 kinase, S6, p-S6, 4E-BP1, and p-4E-BP1 were analyzed by western blot. As shown in Fig. 7A, the expression levels of PI3K, p-AKT (Thr308), p-AKT (Ser473), p-mTOR (Ser2448), P70 S6 kinase/p-P70 S6 kinase (Thr389), p-S6 (Ser235/236), and p-4E-BP1 (Thr37/46) were reduced by siRNA-XLOC_006753 transfection.

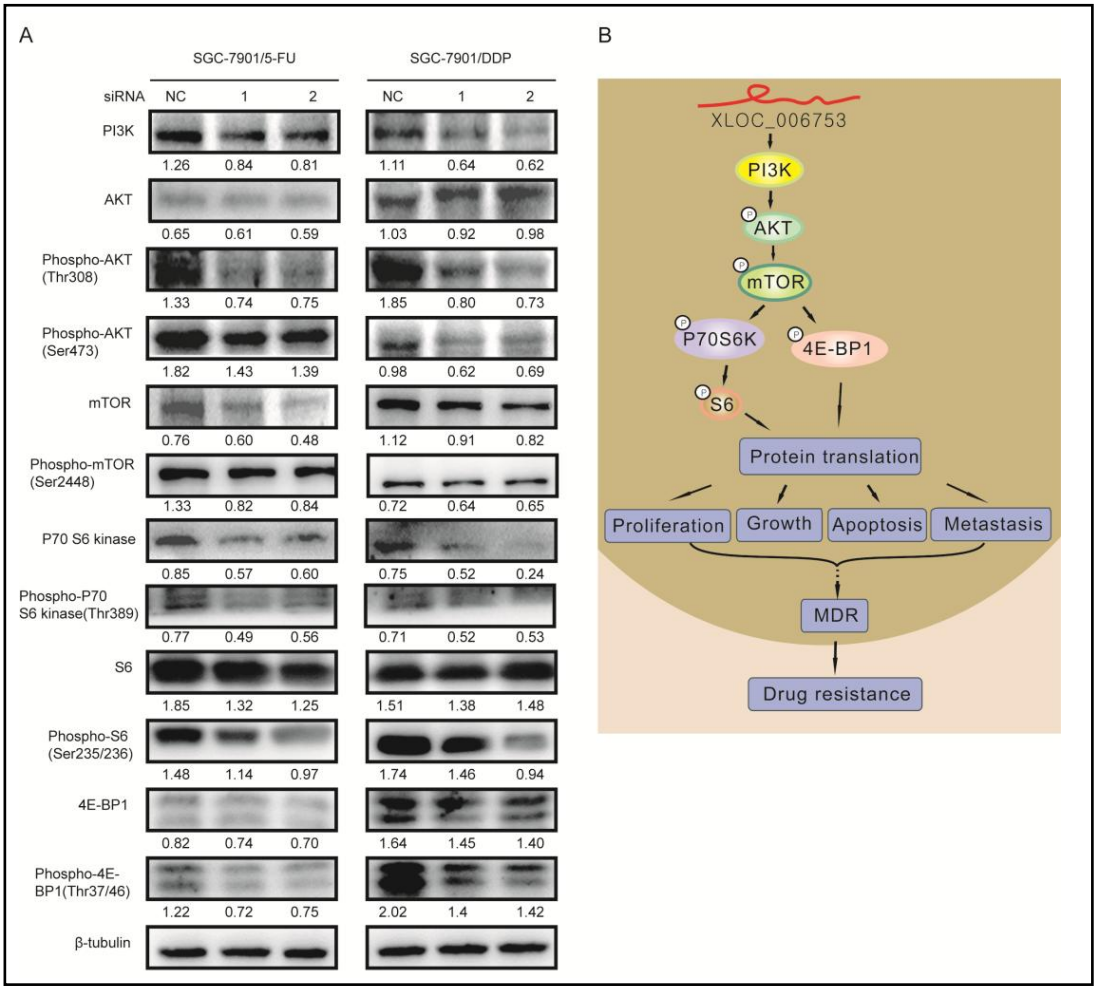

Fig. 7. Diagram summarizing the role of XLOC_006753 in multidrug resistance (MDR) regulation in gastric cancer cells. (A) Western blot analysis was conducted to assay the expression of elements in the PI3K/AKT/mTOR pathway. (B) Schematic of the model showing that XLOC_006753 induces cell proliferation and metastasis and inhibits apoptosis by activating the PI3K/AKT/mTOR signaling pathway to promote MDR.

Based on these reduced expression levels, XLOC_006753 may regulate acquired MDR in GC cells by increasing proliferation and growth, inhibiting apoptosis, and promoting metastasis. The abnormal expression of XLOC_006753 increased activation of the PI3K/Akt/mTOR signaling pathway to promote drug resistance (Fig. 7B).

Discussion

Growing evidence has implicated lncRNAs in a wide range of biological functions, and more and more lncRNAs have been identified as new prognostic markers of GC with potential uses in clinical diagnosis and prognostic evaluation [18-21]. In the present study, we have detected the expression of XLOC_006753 in 110 cases of GC patients and found that the expression of XLOC_006753 in 36 GC tissues was significantly higher than that in paracancerous tissues. The expression of XLOC_006753 had no association with the sex, age, topography, lymph node status, family medical history, smoking, drinking, or tumor differentiation of patients. However, expression level was significantly associated with metastasis, TNM stage, and tumor size. Furthermore, XLOC_006753 is an independent predictive factor for OS, suggesting that XLOC_006753 is a potential biomarker for GC patient prognoses.

Recent studies have suggested that lncRNAs are associated with the regulation of important characteristics of cancer, including proliferation, apoptosis, metastasis, and drug resistance [22-26]. For example, lncRNA AKHE promotes tumor progression in hepatocellular carcinoma (HCC) via activation of NOTCH2 signaling [27]. Overexpression of lncRNA IGFBP4-1 reprograms energy metabolism to promote lung cancer (LC) cell proliferation and metastasis and inhibits apoptosis [28]. Similarly, lncRNA LINC01296 overexpression upregulates the expression of MYCN and promotes cell viability, migration, and invasion in human cholangiocarcinoma (CCA) cells [29]. Furthermore, Liang et al. reported that lncRNA LINP1 is upregulated in doxorubicin- and 5-fluorouracil-resistant cells and induced chemoresistance, indicating LINP1 is a potential therapeutic target and may be key to reducing chemoresistance in breast cancer (BC) [30]. Wen et al. showed that lncRNA GAS5 could influence cisplatin resistance in cervical cancer (CC) by regulating the phosphorylation of Akt [31]. Xue et al. found that lncRNA HOTAIR is upregulated in tamoxifen-resistant BC tissues; its overexpression increased BC cell proliferation and conferred tamoxifen resistance in BC [32]. In this study, we found that XLOC_006753 was upregulated in MDR GC cell lines; its knockdown significantly inhibited cell proliferation, cell cycle, cell viability, and metastasis and induced apoptosis in MDR GC cells. Therefore, we conclude that overexpression of XLOC_006753 promotes tumor progression in MDR GC cells.

To our knowledge, the phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of the rapamycin (PI3K/AKT/mTOR) signaling pathway is one of the main growth regulatory pathways in both normal and cancer cells [33]. The PI3K/AKT/mTOR pathway regulates cell proliferation, differentiation, cellular metabolism, and cytoskeletal reorganization leading to apoptosis and cancer cell survival. Activation of the PI3K/AKT/mTOR pathway mediated through molecular aberrations is instrumental in promoting tumor development as well as resistance to anticancer therapies [34, 35]. In our study, we investigated whether the expression level of XLOC_006753 was associated with tumor progression or MDR via the PI3K/AKT/mTOR pathway in GC cells. SGC-7901/5-FU and SGC-7901/DDP cells transfected with siRNA- XLOC_006753 exhibited decreased p-AKT, mTOR/p-mTOR, P70 S6K/p-P70 S6K, p-S6, and p-4E-BP1 expression, which suggests that chemotherapy resistance changes induced by XLOC_006753 might be mediated by the PI3K/AKT/mTOR pathway.

Conclusion

To date, lncRNA XLOC_006753 expression in cancers and its significance has not been reported. Our study shows that XLOC_006753 is up-regulated in GC and MDR GC cell lines. Furthermore, we demonstrate that overexpression of XLOC_006753 is able to promote multidrug resistance in gastric cancer by induction of cell proliferation, the cell cycle, and metastasis and reduction of apoptosis through the PI3K/AKT/mTOR pathway. This result indicates that XLOC_006753 knockdown may be a promising therapeutic strategy for GC MDR reversal.

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Disclosure Statement

There are no conflicts of interest to disclose for any authors.

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