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Original Article

Phosphorylation of TOB1 at T172 and S320 is critical for gastric cancer proliferation and progression

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Abstract: We previously revealed that increased phosphorylation of TOB1, a tumor suppressor protein, may promote the progression of gastric cancer. However, the phosphorylated sites on TOB1 and their functional implication in gastric cancer remain to be clarified. Here, we addressed these questions using the gastric mucosal epithelial cell line GES-1 and three gastric cancer cell lines (HGC-27, AGS, and MKN-1). Compared with the control GES-1 cells, the gastric cancer cells showed decreased levels of TOB1 protein and increased levels of phosphorylated TOB1 (p-TOB1) by Western blotting. Then, TOB1 protein was enriched and purified by immunoprecipitation. Two novel phosphorylation sites at threonine 172 (T172) and serine 320 (S320) in TOB1 were identified in gastric cancer MKN-1 cells using LC-MS/MS. Furthermore, treatment with the serine/threonine kinase inhibitor staurosporine (STS; 2 nmol/L, 8 h) significantly decreased the levels of p-TOB1. As a result, the proliferation, migration, and invasion of gastric cancer cells were diminished, accompanied by an increased proportion of cells in G1 phase and a decreased proportion of cells in G2 phase. Taken together, these findings indicate for the first time that TOB1 is phosphorylated at T172 and S320 in gastric cancer cells, which are sensitive to STS. Downregulation of p-TOB1 levels by STS treatment can weaken the malignant phenotype of gastric cancer cells and block their progression through the cell cycle. Moreover, STS may exert its antiproliferative activity in gastric cancers by restoring TOB1 protein activity.

Keywords: Gastric cancer, TOB1, phosphorylation, phosphorylation sites, malignant phenotype

Introduction

The human ErbB2 transcription factor 1 or transducer of ErbB2-1 (TOB1) gene plays an essential role in inhibiting tumor proliferation, invasion, and metastasis. Decreased expression of the TOB1 gene has been reported in lung [1], thyroid [2], breast [3], and skin cancer [4]. In breast cancer, the TOB1 gene has an apoptosis-inducing effect, as shown in MCF-7 cells [5], and TOB1 can increase the sensitivity of MDA-MB-231 breast cancer cells to radiation [6]. TOB1 can inhibit tumor cell proliferation through various pathways, such as negative regulation of c-myc expression [7] and blockade of the PI3K/PTEN signaling pathway [8].

In previous allelotyping for loss of heterozygosity (LOH) using microsatellite markers on chromosomes 17 in 45 patients with gastric cancer, we first described chromosomal LOH regions at 17q21.33 (TOB1 locus), suggesting that the TOB1 gene may be involved in gastric carcinogenesis [9, 10]. Then, we demonstrated that functional inactivation of TOB1 triggered by abnormal expression promotes the development of gastric cancer and that TOB1 exerts an antiproliferative effect in the nucleus [11, 12]. Additionally, four polymorphism sites in the TOB1 gene were revealed to be jointly associated with the risk of gastric cancer in the Han population of northeastern China [13]. Additionally, TOB1 inhibited the proliferation of gastric cancer cells by activating Smad4 and inhibiting the β-catenin signaling pathway [14]. Moreover, miR-25 can inhibit the expression of TOB1 mRNA by binding to the 3'-untranslated region of TOB1, thereby promoting the proliferation, invasion, and metastasis of gastric cancer

cells [15]. Reduced TOB1 expression in the cytoplasm was associated with the clinicopathological characteristics of 90 gastric cancer patients [16]. The TOB1 gene is a tumor suppressor in gastric cancer.

Importantly, the activity of TOB1 is closely related to its phosphorylation status. Suzuki et al. initially revealed that p90rsk1 binds to and phosphorylates TOB1 at serine and threonine sites, indicating that the function of TOB1 is at least in part under control of growth factor-stimulated tyrosine kinases through phosphorylation by p90rsk1 [17]. These authors further demonstrated that TOB1 phosphorylation at certain sites could inactivate its function. TOB1 inhibits the cell cycle by inhibiting the expression of cyclin D1, but this effect can be attenuated by phosphorylation of TOB1 at serines 152, 154, and 164 by phosphorylated extracellular signal-regulated protein kinase 1 (ERK1) and ERK2, as shown in NIH3T3 cells [18]. TOB1 phosphorylation eliminates its antiproliferative effect in thyroid [2] and breast [19] cancers. Western blotting showed that TOB1 protein expression was decreased in 3/4 gastric cancer cell lines, and the ratio of phosphorylated TOB1 (p-TOB1) to total TOB1 protein was increased [11]. Moreover, the level of TOB1 phosphorylation in gastric cancer patient tissue samples is related to its subcellular distribution. Phosphorylation of nuclear TOB1 was higher in gastric cancer tissue than in normal gastric tissue and was associated with poorly differentiated and high TNM stage tumors. Patients with intestinal-type gastric cancer and increased nuclear TOB1 phosphorylation had poor overall survival [12]. Thus, the phosphorylation sites of TOB1 in gastric cancer and their function in the development of gastric cancer remain to be determined.

To address these questions, we purified TOB1 protein by immunoprecipitation (IP) after detecting the expression levels of TOB1 and p-TOB1 in a human gastric epithelial cell line (GES-1) and three gastric cancer cell lines. Then, we identified the phosphorylation sites in TOB1 in MKN-1 cells using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Finally, we determined the effect of inhibiting TOB1 phosphorylation on the aggressive behavior of gastric cancer cells through a series of functional in vitro experiments.

Materials and methods

Cell lines and cell culture

The human gastric mucosal epithelial cell line GES-1 and the human gastric cancer cell lines AGS and HGC-27 were routinely maintained according to a previous report [12]. The human gastric cancer cell line MKN-1 was provided by Zhong Shan Medical University (Guangzhou, China) and grown in DMEM (Gibco BRL, USA) with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. All cell lines were authenticated by Beijing Microread Genetics Co., Ltd. (Beijing, China) using short tandem repeat (STR) analysis.

Western blot analysis

Western blotting was performed as previously described [11]. In brief, total protein was extracted with an appropriate amount of lysis buffer (with protease inhibitor and phosphatase inhibitors at a ratio of 10:1:1, Roche) and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to polyvinylidene difluoride membranes (Sigma-Aldrich, Saint Louis, MO, USA). The membranes were blocked with 5% fat-free milk for 2 h at room temperature and hybridized overnight at 4°C with primary antibodies against TOB1 (1:500, Proteintech Group, USA), p-TOB1 (phospho-S164) (1:500, Abcam, Cambridge, UK), and β-actin (1:500, ZSGB-BIO, Beijing, China), followed by incubation with secondary antibodies (anti-rabbit or anti-mouse, 1:5,000, Rockland, Limerick, PA, USA). Blots were imaged using an Odyssey Infrared Imaging System (Li-COR, Lincoln, NE, USA). ImageJ software was used for band density analysis.

Immunoprecipitation (IP)

To enrich and purify TOB1 protein, IP was performed. Total protein was harvested from cell lysates as described for Western blotting. Protein A/G beads were washed twice with pre-chilled TBS and prepared as a 50% suspension in lysis buffer. To block nonspecific binding, cell lysates containing 5 mg of protein were absorbed with 50 μL of a 50% protein A/G agarose suspension in a 1.5 mL Eppendorf tube at 4°C for 1 h and then centrifuged at

12,000×g for 3 min at 4°C. The supernatant was divided into two fractions, mixed with antibody and protein A/G agarose conjugate prepared by incubation of 5 μg of rabbit-derived IgG (for control) or 5 μg of TOB1 antibody with 50 μL of the 50% protein A/G agarose suspension for 1 h at 4°C and then centrifugation at 12,000×g for 4 min at 4°C. After incubation at 4°C overnight, the reaction precipitate was recovered by centrifugation at 12,000×g for 3 min at 4°C; the precipitate was then added to 500 μL of washing solution, the mixture was placed at 4°C for 2 min and centrifuged at 12,000×g for 3 min at 4°C, and the precipitate was recovered and washed 4 times. Next, the precipitate was added to 100 μL of 2×SDS-PAGE loading buffer, incubated at 95°C for 5 min, and centrifuged at 12,000×g for 5 min; the supernatant was collected to identify the phosphorylation sites.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Purified TOB1 protein (20 μL) was separated by conventional electrophoresis on a 12% SDS-PAGE gel, and protein bands were visualized with Coomassie blue staining solution. The protein bands were cut from the gel and washed with ultrapure water; after centrifugation and removal of the supernatant, the protein/gel mixture was washed with a mixture of NH_4HCO_3 (25 mmol/L, pH 8.0) and 50% acetonitrile. Then, the gel was placed in acetonitrile until it bleached and was dried in a speed-vac. Protein reductive alkylation and enzymatic hydrolysis were performed by the subsequent addition of dithiothreitol (DTT, 10 mmol/L), ammonium iodoacetate (IAM, 55 mmol/L), and 1 μg trypsin. The polypeptides were separated on a C18 column and then dissolved in 15 μL loading buffer. The dissolved peptide was analyzed by LC-MS/MS (ekspert™ nanoLC; AB Sciex TripleTOF 5600-plus). After completion of the experiment, the data were submitted directly to an AB SCIEX TripleTOF 5600-plus mass spectrometer for database retrieval.

After filtering out common contaminating proteins and setting the confidence level to > 95% and unique peptides to > 1, the number of proteins identified in the target protein sample was set to 1, and phosphorylated peptides were screened. In brief, phosphorylation sites on the peptide were identified by the mass-to-charge ratio (m/z) of the fragment ion on the peptide spectrum. If a phosphorylation site was present, the m/z of the corresponding b and y ions should match the theoretical m/z data.

Staurosporine (STS) treatment

Cells were collected, resuspended into a single-cell suspension and seeded in 6-well plates at a density of 2.5×10^5 cells per well. After attaching for 24 h, the cells were incubated with the serine/threonine kinase inhibitor STS at final concentrations of 2 nmol/L, 5 nmol/L, 10 nmol/L, 20 nmol/L, and 50 nmol/L for 24 h. Then, the expression levels of p-TOB1 were detected by Western blotting to determine the optimal drug concentration. In subsequent experiments, the cells were treated with the optimal concentration of inhibitor for 4 h, 8 h, 12 h, and 24 h, and then, the optimal time point of drug action was determined by the expression levels of p-TOB1.

Cell proliferation assay

Cells (1×10^3/well) in 6 replicates were cultured in 96-well plates at 37°C in 5% CO_2. Before each experiment, the original medium was discarded and replaced with 100 μL of medium with 20 μL of CellTiter 96® AQueous One Solution (Promega, Madison, USA) per well; the cells were then incubated for 4 h before the absorbance value of each well was determined at 492 nm using a microplate reader (TECAN Sunrise, Australia). The assay was performed once a day for 6 consecutive days, and the relative absorbance values were calculated to draw the growth curve.

Colony formation assay

Cells were cultured in 6-well plates for 14 days after plating at a density of 1×10^2 cells per well in 3 replicates. The medium was discarded, and the cells were washed with PBS, fixed with methanol for 15 min, washed with PBS and stained with Giemsa solution (Gibco BRL) for 30 min. The cells were visualized under the microscope after being washed.

Wound healing assay

Cells were grown in a six-well plate (5×10^5/well). When the cells reached confluence, a straight line was drawn across the well to create a wound, and the suspended cells were

removed by washing with PBS. The cells were then cultured at 37°C and 5% CO_2, and wound closure was measured at 0 h, 24 h, and 48 h using a microscope (Nikon) at 100× magnification.

Cell invasion assay

Invasion chambers (Corning, NY, USA) containing 500 μL of serum-free medium and 3×10^4 cells were placed in a 24-well plate, 750 μL of medium containing 15% FBS was added to each well, and the cells were cultured at 37°C in 5% CO_2 for 24 h. The cells that did not pass through the membrane in the 24-well plate were gently removed, and the cells that migrated through the membrane were fixed with methanol for 15 min, stained with Giemsa solution for 10 min, and counted using an optical microscope at 100× magnification.

Flow cytometry

For the cell cycle distribution assay, the BD Cycletest™ Plus-DNA Reagent Kit (BD Biosciences, Bedford, MA) was used as described previously [20]. The cells were cultured in serum-free medium for 24 h. After synchronization, the medium was replaced with serum-containing medium for 4 h. The cells were harvested, washed twice with ice-cold PBS and fixed with prechilled 75% alcohol for 24 h at 4°C. The cellular DNA was stained, and the cell cycle distribution was analyzed using flow cytometry analysis (Bio-Rad, Richmond, CA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. All data are expressed as the mean ± standard deviation. Two-way ANOVA was used to identify significant differences in the cell proliferation assay, and Student's t-test was used for the statistical analysis of the experimental and control groups in the colony formation, migration, invasion, and cell cycle distribution assays. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicated a statistically significant difference.

Results

TOB1 phosphorylation in gastric cancer cell lines

Our previous study revealed that the p-TOB1/total TOB1 ratio was increased in gastric cancer cell lines. To examine the phosphorylation status of TOB1, we first predicted the phosphorylation sites of TOB1 using PhosphoSitePlus (https://www.phosphosite.org/simpleSearchSubmitAction) and NetPhos (http://www.cbs.dtu.dk/services/NetPhos/). PhosphoSitePlus was used to search for the phosphorylation status of TOB1 (**Figure 1**), in which the known phosphorylation sites were mainly derived from reported data analyses of low-throughput (LTP) and high-throughput (HTP) assays such as MS. We found that a total of 7 of the 345 amino acid residues of human TOB1 protein are phosphorylated, i.e., Y40, S152, S154, S164, T204, S205, and S268 (**Figure 1A**). Among them, the S152, S154, and S164 phosphorylation sites were identified by LTP methods, and the Y40, T204, S205, and S268 phosphorylation sites were identified by HTP MS (**Figure 1B**). In addition, the NetPhos server also predicted that TOB1 might be phosphorylated at other sites (e.g., S14, Y15, Y17, Y40, S152, S154, S164, T172, S320, etc.). **Table 1** shows that TOB1 could be phosphorylated at many serine, threonine, and tyrosine residues, including the reported phosphorylation sites retrieved from PhosphoSitePlus.

Since phosphorylation of TOB1 could occur at more than one known site, we subsequently investigated the phosphorylation sites of TOB1 in the gastric mucosal epithelial cell line GES-1 and the gastric cancer cell lines HGC-27, AGS, and MKN-1. We detected the protein levels of TOB1 and p-TOB1 using Western blotting, which showed that p-TOB1 levels (**Figure 2B**) were higher in three malignant cell lines than in normal gastric mucosa cells, although total TOB1 protein levels were lower in the gastric cancer cells (**Figure 2A**). Thus, these gastric cancer cell lines are suitable for further determination of TOB1 phosphorylation sites.

Identification of TOB1 phosphorylation sites

To determine the phosphorylation sites in cell lines expressing p-TOB1, we first enriched TOB1 protein by IP (**Figure 3A**); TOB1 protein expression was identified by Western blotting in all four cell lines using an antibody specific for IP (**Figure 3B**) and was present in the IP blots (**Figure 3C**).

The protein in the post-IP elute was sequentially alkylated, enzymatically digested, desalted, drained, dissolved, and analyzed by LC-MS/

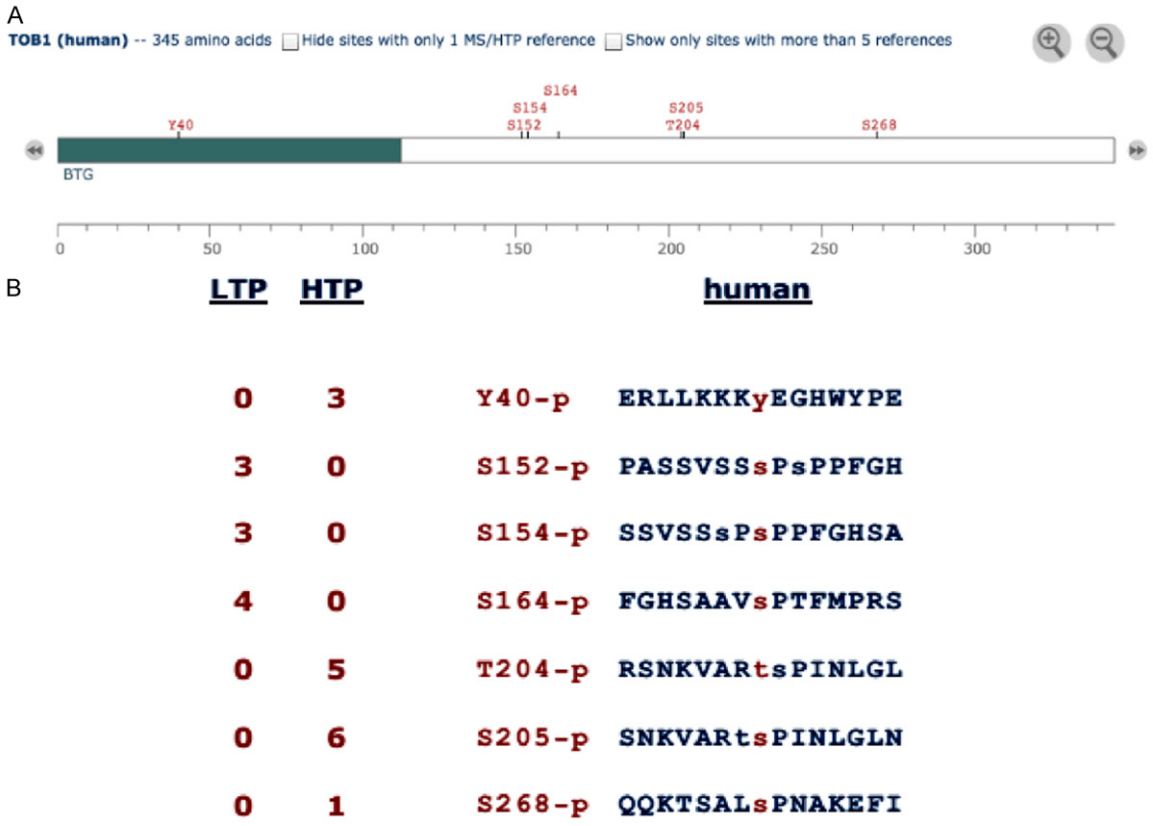


Figure 1. Phosphorylation sites in TOB1 reported in PhosphoSitePlus. A. Phosphorylated sites in TOB1. B. Phosphorylation sites in TOB1 identified by high-throughput (HTP) and low-throughput (LTP) methods. The numbers under LTP and HTP indicate the number of references for the phosphorylation site.

Table 1. Phosphorylation sites in TOB1 (345 amino acids) retrieved from the NetPhos database

Sequence	# x	Context	Score	Kinase	Answer
Sequence	14 S	NFIISYLYN	0.654	PKC	YES
Sequence	15 Y	FIISYLYNK	0.438	INSR	.
Sequence	17 Y	ISYLYNKLP	0.411	INSR	.
Sequence	40 Y	LKKKYEGHW	0.505	INSR	YES
Sequence	45 Y	EGHWYPEKP	0.437	INSR	.
Sequence	50 Y	PEKPYKGSG	0.884	unsp	YES
Sequence	53 S	PYKGSGFRC	0.659	PKC	YES
Sequence	72 S	IEQASKESG	0.950	unsp	YES
Sequence	75 S	ASKESGLDI	0.982	unsp	YES
Sequence	91 S	PQDLSVWID	0.456	CaM-II	.
Sequence	100 S	PFEVSYQTG	0.474	CkII	.
Sequence	101 Y	PEVSYQIGE	0.847	unsp	YES
Sequence	113 Y	VKVLYVDDN	0.914	unsp	YES
Sequence	131 S	EIKNSFNPE	0.573	PKA	YES
Sequence	143 S	FMPISDPAS	0.509	cdc2	YES
Sequence	147 S	SDPASSVSS	0.960	unsp	YES
Sequence	148 S	DPASSVSSS	0.989	unsp	YES
Sequence	150 S	ASSVSSSPS	0.924	unsp	YES

MS. The TOB1 protein sequence was inputted into the mass spectrometer according to the search parameters shown in **Table 2**, the protein was randomly degraded into peptides of different molecular weights, and the recognition mode for peptide modification was set to phosphorylation recognition (**Figure 4**). The results after analyzing the relevant information of the target protein spectrum showed that the phosphorylated amino acids were threonine 172 (T172, **Figure 4A**) and serine 320 (S320, **Figure 4B**).

The coverage of the target protein peptide sequence in the sequence coverage pane of ProteinPilot software is shown in **Figure 4C**. MS detected a total of five peptides with confidence > 95%, and these peptides contained two phosphorylated peptides, i.e., T172 and S320, which have not been reported in PhosphoSitePlus but were predicted (NetPhos server) (**Table 1**). Thus, the

Sequence	151 S	SSVSSSPSP	0.812	unsp	YES
Sequence	152 S	SVSSSPSPP	0.994	unsp	YES
Sequence	154 S	SSSPSPPFG	0.930	unsp	YES
Sequence	160 S	PFGHSAAVS	0.502	PKG	YES
Sequence	164 S	SAAVSPTFM	0.759	unsp	YES
Sequence	166 T	AVSPTFMPR	0.448	GSK3	.
Sequence	171 S	FMPRSTQPL	0.543	PKA	YES
Sequence	**172 T**	**MPRSTQPLT**	**0.799**	**unsp**	**YES**
Sequence	176 T	TQPLTFTTA	0.436	CaM-II	.
Sequence	178 T	PLTFTTATF	0.495	cdc2	.
Sequence	179 T	LTFTTATFA	0.559	PKG	YES
Sequence	181 T	FTTATFAAT	0.887	PKC	YES
Sequence	185 T	TFAATKFGS	0.795	PKC	YES
Sequence	189 S	TKFGSTKMK	0.872	PKC	YES
Sequence	190 T	KFGSTKMKN	0.890	PKC	YES
Sequence	195 S	KMKNSGRSN	0.991	unsp	YES
Sequence	198 S	NSGRSNKVA	0.993	unsp	YES
Sequence	204 T	KVARTSPIN	0.517	PKC	YES
Sequence	205 S	VARTSPINL	0.996	unsp	YES
Sequence	223 S	QKAISSSMH	0.986	unsp	YES
Sequence	224 S	KAISSSMHS	0.454	GSK3	.
Sequence	225 S	AISSSMHSL	0.505	cdc2	YES
Sequence	228 S	SSMHSLYGL	0.930	unsp	YES
Sequence	230 Y	MHSLYGLGL	0.506	INSR	YES
Sequence	236 S	LGLGSQQQP	0.617	ATM	YES
Sequence	264 T	QQQKTSALS	0.463	GSK3	.
Sequence	265 S	QQKTSALSP	0.548	cdc2	YES
Sequence	268 S	TSALSPNAK	0.988	unsp	YES
Sequence	284 S	QGQGSSTNG	0.839	unsp	YES
Sequence	285 S	GQGSSTNGM	0.465	cdc2	.
Sequence	286 T	QGSSTNGMF	0.507	cdc2	YES
Sequence	294 S	FPGDSPLNL	0.644	unsp	YES
Sequence	299 S	PLNLSPLQY	0.546	cdk5	YES
Sequence	303 Y	SPLQYSNAF	0.415	INSR	.
Sequence	304 S	PLQYSNAFD	0.525	cdc2	YES
Sequence	313 Y	VFAAYGGLN	0.471	EGFR	.
Sequence	**320 S**	**LNEKSFVDG**	**0.915**	**unsp**	**YES**
Sequence	328 S	GLNFSLNNM	0.525	cdc2	YES
Sequence	334 Y	NNMQYSNQQ	0.580	EGFR	YES
Sequence	335 S	NMQYSNQQF	0.475	CaM-II	.

#: position of phosphorylated amino acids. x: category of phosphorylated amino acids. Context: the sequence of phosphorylated amino acid; each sequence contains 9 amino acids, the phosphorylated amino acid and the 4 amino acids before and after. Score: the phosphorylation site prediction score. Kinase: kinase that phosphorylates the amino acid. Answer: indicates whether the prediction is meaningful.

T172 and S320 sites of TOB1 are phosphorylated in MKN-1 gastric cancer cells.

Downregulation of p-TOB1 levels by a serine/threonine kinase inhibitor

Proteins are phosphorylated by protein kinases, and the antiproliferative activity of TOB1 decreases after phosphorylation, as stated in the introduction. To establish the link between p-TOB1 levels and the antiproliferative function of TOB1 in gastric cancer, we treated MKN-1 cells with the serine/threonine kinase inhibitor STS. As revealed in the Western blotting analysis (**Figure 5**), STS inhibited the phosphorylation of TOB1, resulting in decreased levels of p-TOB1 in gastric cancer cells.

In the next step, we determined the optimal working concentration and optimal duration of STS treatment. STS was dissolved in DMSO and prepared to final working concentrations of 2, 5, 10, 20, and 50 nmol/L with RPMI-1640 medium; 1% DMSO was used as a control. MKN-1 cells were plated in six-well plates and cultured in a 5% CO_2 incubator at 37°C; after the cells attached to the plates, the medium was changed to medium containing 1% DMSO or different concentrations of STS. After 24 h of culture, proteins were extracted, and p-TOB1 levels were detected by Western blotting (**Figure 5A**). The results showed that the levels of p-TOB1 were significantly decreased in MKN-1 cells treated with 2 nmol/L STS, which was determined to be the optimal concentration of STS.

To determine the optimal duration of STS treatment of MKN-1 cells, the cells were inoculated in a six-well plate. After the cells adhered to the plate, the medium was replaced with medium containing 2 nmol/L STS or 1% DMSO (control). After 4 h, 8 h, 12 h, and 24 h, the cells were collected, total protein was extracted, and the p-TOB1 level was detected by Western blotting. As shown in **Figure 5B**, the level of p-TOB1 in MKN-1 cells was significantly decreased after treatment with 2 nmol/L STS for 8 h, which was determined to be the optimal duration of STS treatment. These results demonstrated that STS could inhibit the phosphorylation of TOB1 and decrease the levels of p-TOB1. The inhibitory effect was optimal at 8 h of treatment with 2 nmol/L STS.

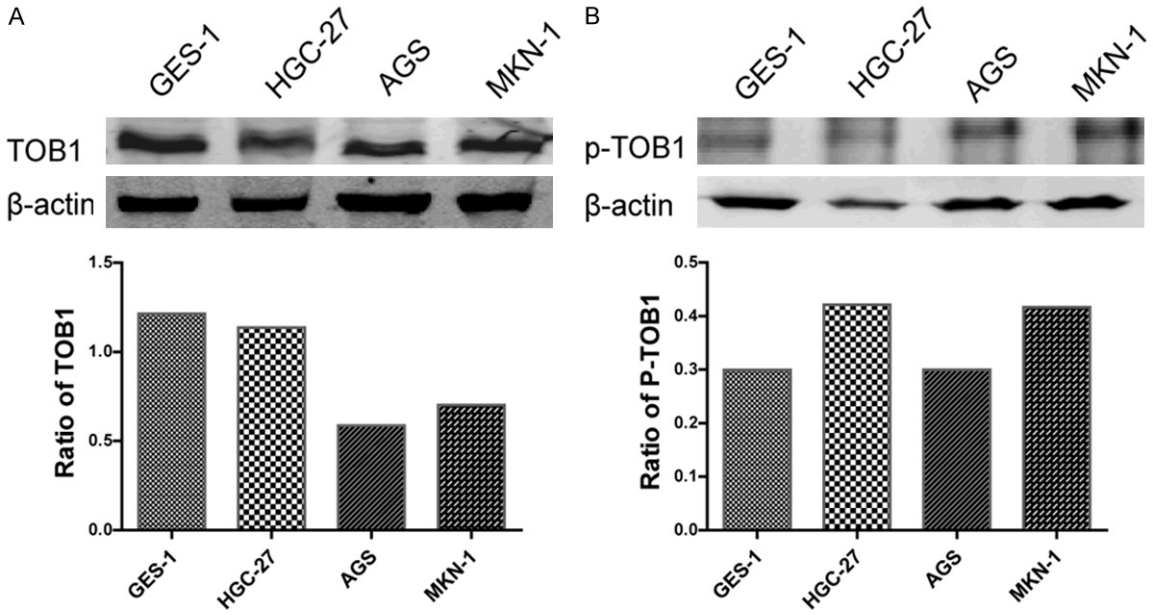


Figure 2. Total TOB1 and p-TOB1 levels in normal gastric mucosa cells and gastric cancer cells. (A) Western blot analysis revealed lower expression of TOB1 protein and (B) higher expression of p-TOB1 in the malignant cell lines HGC-27, AGS, and MKN-1 compared to the normal gastric mucosa cell line GES-1.

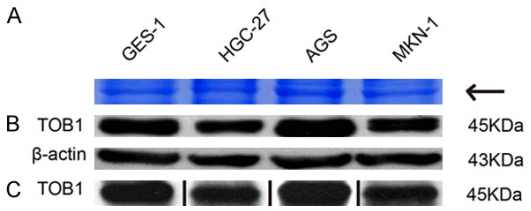


Figure 3. TOB1 protein was enriched by immunoprecipitation in normal gastric mucosa cells and gastric cancer cells. (A) Total protein was extracted from GES-1, HGC-27, AGS, and MKN-1 cells with a high extraction efficiency. (B) Western blotting was performed to detect the efficacy of the IP-grade antibody and (C) IP-enriched TOB1 protein.

Effect of downregulating p-TOB1 levels on the biological behavior of gastric cancer cells

Attenuating the proliferation of gastric cancer cells following the inhibition of TOB1 phosphorylation: To ascertain the effect of downregulating TOB1 phosphorylation on the growth of gastric cancer cells, we analyzed the growth curves of MKN-1 cells treated with or without STS; the cell growth curve was plotted with the relative absorbance as the ordinate and the number of days (1~6 days) as the abscissa. As shown in **Figure 6A**, the growth of MKN-1 cells treated with STS was significantly slower than that of cells without STS treatment ($P < 0.001$), indicating that STS inhibited the phosphorylation of TOB1 and decreased the growth rate of gastric cancer cells. We then performed colony formation experiments to verify this effect of STS on proliferation. As shown in **Figure 6B**, colony formation by MKN-1 cells was significantly reduced after STS treatment ($P < 0.001$), indicating that the inhibition of TOB1 phosphorylation by STS inhibited the proliferation of gastric cancer cells.

Attenuating the migration and invasion of gastric cancer cells following a decrease in p-TOB1 levels: To determine the effect of downregulating p-TOB1 levels on gastric cancer cell migration, we analyzed the migration rate of MKN-1 cells using wound healing assays after reducing p-TOB1 levels. The results showed that the migration rate of MKN-1 cells treated with 2 nmol/L STS was significantly slower than that of MKN-1 cells not exposed to STS (**Figure 6C**, $P < 0.001$), indicating that reducing p-TOB1 levels also decreased the migration of gastric cancer cells. The STS-treated cells were then subjected to an invasion chamber experiment. As shown in **Figure 6D**, STS treatment significantly reduced the number of invading MKN-1 cells compared with control treatment ($P < 0.01$), suggesting a decrease in cell invasion ability and the functional association between the

Table 2. LC-MS/MS detection parameters

Item	Value
Type of search	Identification
Enzyme	Trypsin
Cys Alkylation	Iodoacetamide
Special Factors	Phosphorylation emphasis
Instrument	Triple TOF 5600-plus
Bias Correction	TRUE
Background Correction	TRUE
ID focus	Biological modifications
Search Effort	Thorough ID
Protein Mass	Unrestricted
Database	TOB1.fasta (a total of 1 target sequence)

inhibition of TOB1 phosphorylation and the inhibition of gastric cancer cell invasion.

Increasing the proportion of gastric cancer cells in G1 phase after decreasing p-TOB1 levels

To investigate the effect of downregulating p-TOB1 levels on the cell cycle distribution of gastric cancer cells, we performed cell cycle analysis of MKN-1 cells after decreasing p-TOB1 levels. The results (**Figure 6E**) showed that STS treatment significantly increased the proportion of MKN-1 cells in G1 phase ($P < 0.05$) but decreased the proportion of cells in G2 phase ($P < 0.05$), indicating that STS-mediated inhibition of TOB1 phosphorylation can block the cell cycle progression of gastric cancer cells.

Discussion

The TOB1 gene was first discovered by the Japanese scholar Matsuda in 1996 in a study of the interaction of ErbB-2 receptor-type protein tyrosine kinases with proteins. A recent study indicated that TOB1 proteins and other peptide enzymes derived from degradable and unstable proteins may have proteolytic activity [21]. The TOB1 protein encoded by this gene belongs to the BTG/TOB antiproliferative protein family and plays a tumor suppressor role in many types of cancer, as shown in later studies. Our group reported that TOB1 is also a gastric cancer-associated tumor suppressor gene. We previously identified the 17q21.33 LOH region of TOB1 on chromosome 17 in 45 primary gastric cancer tissue samples using low-density and high-density microsatellite markers [9, 10]. Subsequently, we found decreased TOB1 protein expression in 75% of gastric cancer tissues and an increased p-TOB1/total TOB1 ratio in 3/4 gastric cancer cell lines, which was associated with functional inactivation of the gene [11]. Thus, the reduction of TOB1 protein and the elevation of p-TOB1 levels in the nucleus of gastric cancer tissues contribute to the development of gastric cancer, and TOB1 plays an antiproliferative role in the cell nucleus [12]. In addition, polymorphisms (rs12601477, rs4626, rs34700818, and rs61482741) in the TOB1 gene were also found to be closely associated with the risk of gastric cancer in the Han population of northeastern China [13].

Studies have shown that TOB1 phosphorylation occurs at serine and threonine sites [17]. Using deletion analysis and phosphopeptide mapping, Suzuki et al. found that ERK1/2 can phosphorylate TOB1 in NIH3T3 cells at serines 152, 154, and 164 upon growth factor stimulation [18]. To determine whether TOB1 is phosphorylated at these known sites or at other sites in gastric cancer, we first used two databases of phosphorylation sites, PhosphoSitePlus and NetPhos, to screen and predict phosphorylation sites on TOB1. We found that a total of 7 of the 345 amino acid residues of human TOB1 protein are phosphorylated, i.e., Y40, S152, S154, S164, T204, S205, and S268, based on PhosphoSitePlus. We also found using NetPhos that TOB1 protein might be phosphorylated at other sites, e.g., S14, Y15, Y17, Y40, S152, S154, S164, T172, S320, etc.

Thus, we speculated that TOB1 may be phosphorylated at many sites in gastric cancer. Therefore, we used LC-MS/MS to detect and analyze the phosphorylation sites in TOB1 in three gastric cancer cell lines, which had higher levels of p-TOB1 than the normal GES-1 cell line. We detected two novel phosphorylation sites, T172 and S320, which are consistent with the predictions in NetPhos and have not been reported thus far. According to the predicted results, TOB1 may be phosphorylated at many amino acids; however, only two phosphorylation sites were detected by MS in MKN-1

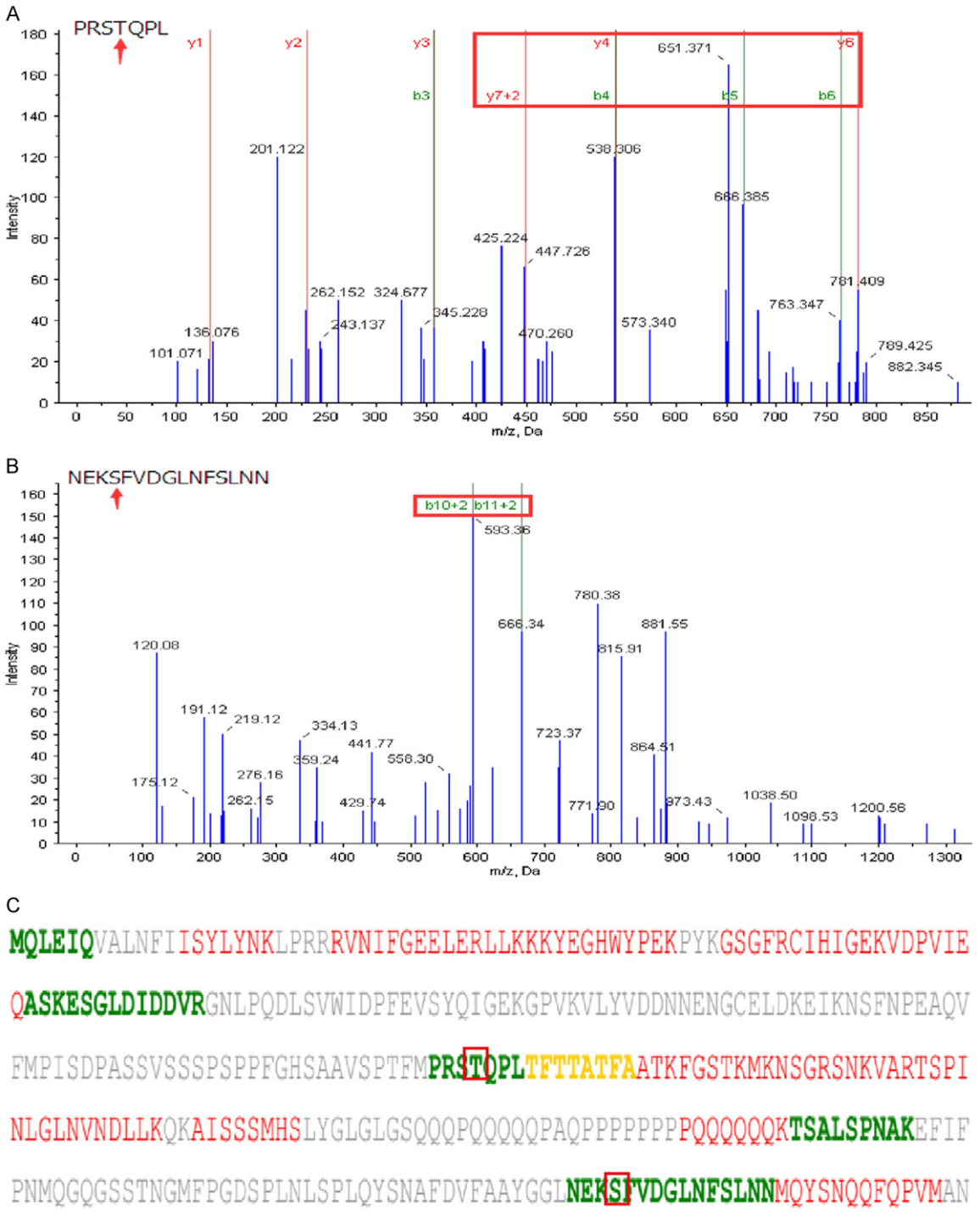


Figure 4. Phosphorylated peptide mass spectra of TOB1 protein in the MKN-1 sample. A. Threonine 172 (T172) was identified in the phosphopeptides of the MKN-1 sample. B. Serine 320 (S320) was identified in the phosphopeptides of the MKN-1 sample. The red box indicates a match. The M/S is of the b and y ions, the upper left panel shows the amino acid sequence, and the red arrow below the amino acid indicates a phosphorylated residue. C. TOB1 phosphorylation sequence information in the MKN-1 sample. The confidence levels of the peptide sequence are presented in different colors: green (confidence > 95%, trustworthy), yellow (confidence between 50~95%, possible), red (confidence between 0~50%, untrustworthy), and gray (completely untrustworthy/ignored). The letters T and S in the red box represent phosphorylated threonine (Thr) and serine (Ser).

gastric cancer cells. It is possible that the experimental results may be not complete because MS is a shotgun proteomics method. Moreover, the expression of the TOB1 gene was

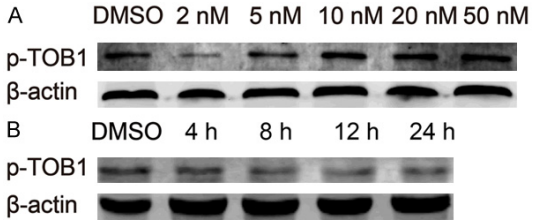


Figure 5. The optimal concentration and treatment duration of the serine/threonine kinase inhibitor STS to inhibit TOB1 phosphorylation in MKN-1 cells. A. Cells were incubated with STS at different concentrations for 24 h; Western blotting revealed a significant decrease in p-TOB1 levels at 2 nmol/L. B. Cells were treated with 2 nmol/L STS for different durations, and the level of p-TOB1 began to decrease significantly at 8 h.

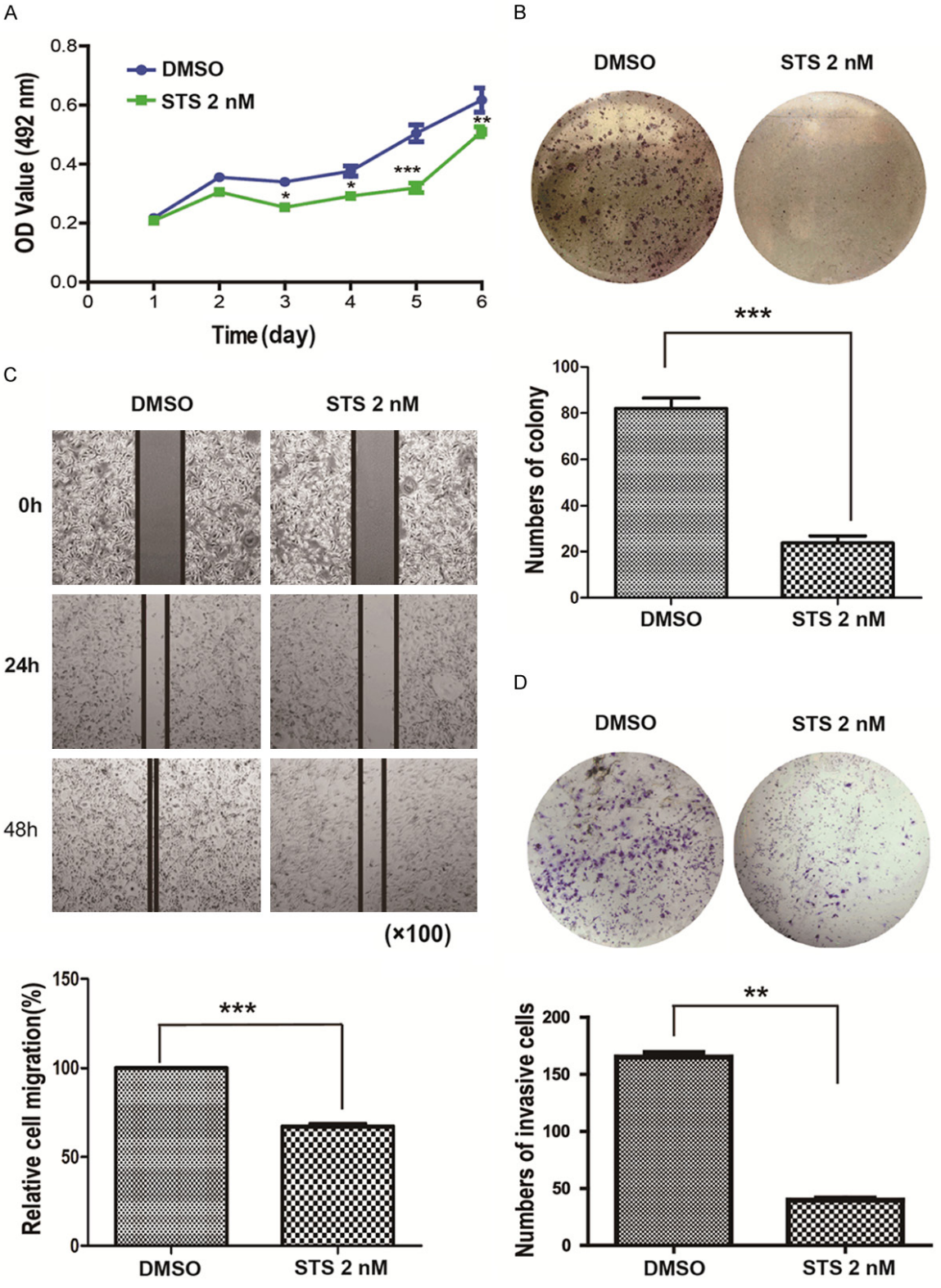
decreased in gastric cancer cells (characteristic inhibition of tumor suppressor genes), which resulted in the low target TOB1 protein abundance and a low p-TOB1 peak on the mass spectrum that might be masked by other peaks. In addition, protein phosphorylation and dephosphorylation are dynamic and reversible processes, and the physiological state of cells affects phosphorylation at different timepoints. These factors could have led to the identification of fewer phosphorylation sites in this study. However, what interests us is that the two phosphorylation sites detected in this study were previously unknown. As they differ from the previously reported phosphorylation sites, we will continue to analyze and verify these two phosphorylation sites in gastric cancer samples. Since TOB1 is a member of an antiproliferative protein family, the non-phosphorylated state is the active form of TOB1; thus, the phosphorylation of TOB1 at specific sites by some kinases could significantly reduce or eliminate its antiproliferative function, thereby promoting tumor formation and development [17].

It has been reported that TOB1 phosphorylation can be inhibited by the serine/threonine kinase inhibitor STS in NIH3T3 cells [17]. To determine whether STS can reduce the levels of p-TOB1 in gastric cancer cells, we selected MKN-1 gastric cancer cells, which harbor phosphorylated TOB1, and treated them with STS. Western blotting analysis showed that STS treatment (2 nmol/L for 8 h) significantly decreased p-TOB1 levels in MKN-1 cells. STS can inhibit the phosphorylation of TOB1 in gastric cancer cells, which is consistent with the report in NIH3T3 cells [17].

Studies have shown that TOB1 phosphorylation is closely related to tumorigenesis and development, and TOB1 phosphorylation can eliminate its antiproliferative effect. By immunohistochemical analysis, Iwanaga et al. found that TOB1 phosphorylation occurred in 76% of lung adenocarcinoma tissues and that TOB1 expression and phosphorylation decreased in the early clinical stages, such as bronchial dysplasia, indicating that the antiproliferative effect of TOB1 plays an important role in the early stages of lung cancer formation [1]. Helms et al. analyzed tissue microarrays containing 725 cases of node-negative breast cancer and found that TOB1 phosphorylation was positively correlated with the proliferation marker Ki67, suggesting that high levels of p-TOB1 can eliminate its antiproliferative effect and that lymph node-negative breast cancer has a poor prognosis [19]. The uncontrolled proliferation of tumor cells and their migration and invasion are biological characteristics of malignant tumors. Exploring the mechanisms that control the malignant biological behavior of gastric cancer cells and objectively evaluating the value of TOB1 phosphorylation as a clinical tumor marker were also goals of our research. Our series of functional analyses revealed that inhibition of TOB1 phosphorylation by STS resulted in significant decreases in the growth and proliferation of MKN-1 cells, a marked decrease in migration and invasion, an increase in the proportion of gastric cancer cells in G1 phase, and a decrease in the proportion of gastric cancer cells in G2 phase. In support of our finding, the replacement of serines in TOB1 with glutamic acids to mimic phosphorylation led to a significantly weaker ability to inhibit cell cycle progression to S phase from G0/G1 phase in NIH3T3 cells [22]. These results indicate that inhibition of TOB1 phosphorylation can inhibit the malignant phenotype of gastric cancer cells and that the TOB1 gene inhibits the proliferation of gastric cancer cells by regulating cell cycle progression.

Conclusion

In conclusion, this study revealed the presence of TOB1 phosphorylation at T172 and S320, two unreported phosphorylation sites, in MKN-1 gastric cancer cells; serine/threonine kinase inhibition by STS can inhibit the phosphorylation of TOB1, and reducing the phosphorylation of TOB1 can significantly attenuate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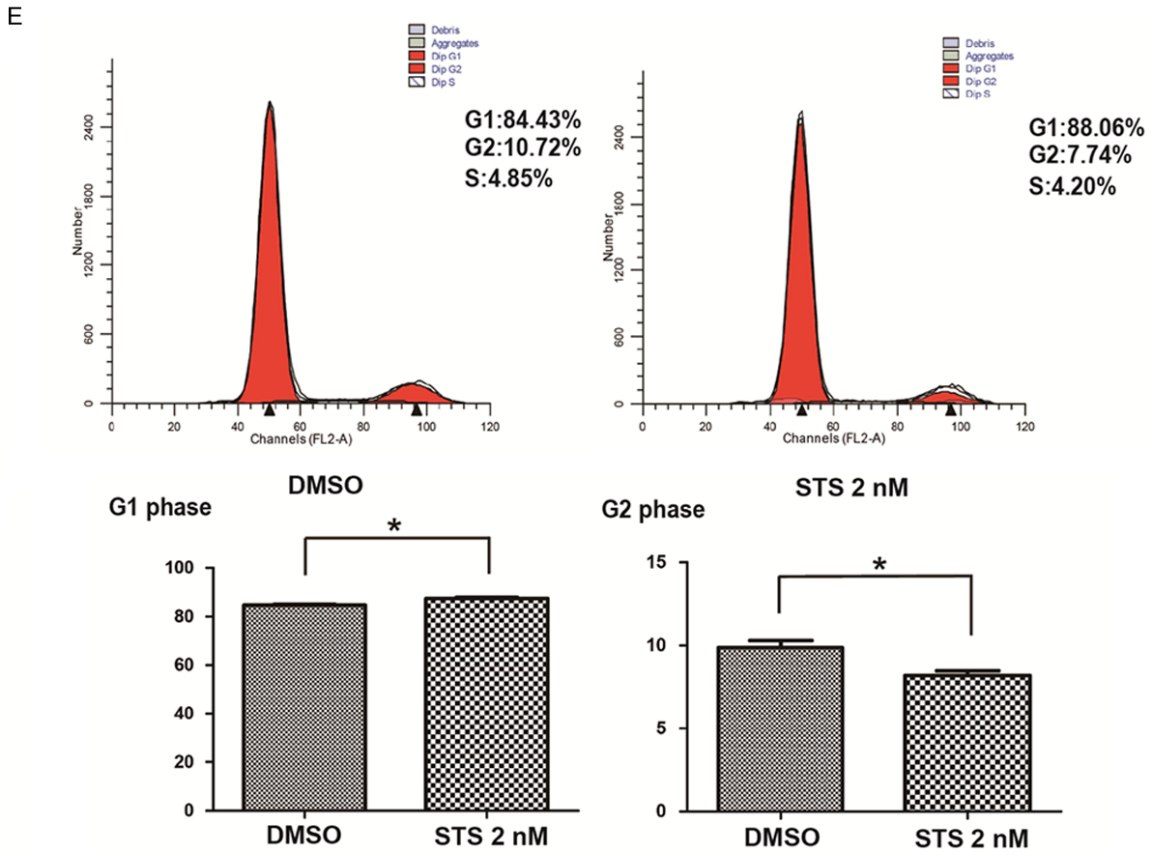

Figure 6. Analysis of the malignant potential of MKN-1 cells after STS treatment. A. Growth curve of gastric cancer cells. B. Colony formation by gastric cancer cells. C. Migration of gastric cancer cells. D. Invasion of gastric cancer cells through Matrigel-coated membranes. E. The cell cycle distribution of gastric cancer cells. ANOVA and Student's t-tests were performed; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with DMSO treatment.

the malignant phenotypes of gastric cancer cells. The results of this study preliminarily confirm that decreasing TOB1 phosphorylation could restore its antiproliferative activity in gastric cancer cells. In future studies, we will use a more specific phosphokinase inhibitor to observe the changes in TOB1 phosphorylation levels and to explore the signaling pathway regulated by TOB1 phosphorylation; with this approach, we can more accurately inhibit TOB1 phosphorylation and restore the tumor suppressor function of TOB1 in gastric cancer.

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Disclosure of conflict of interest

None.

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