RESEARCH ARTICLE

Involvement of MAPK and PI3K signaling pathway in sterigmatocystin-induced G_2 phase arrest in human gastric epithelium cells

Xin Xing[1], Juan Wang[1], Ling Xiao Xing[2], Yue Hong Li[1], Xia Yan[2] and Xiang Hong Zhang[1,2]

[1] Department of Pathology, The Second Hospital, Hebei Medical University, Shijiazhuang, P. R. China
[2] Laboratory of Pathology, Hebei Medical University, Shijiazhuang, P. R. China

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Scope: Sterigmatocystin (ST), a mycotoxin commonly found in foodstuff and feedstuff, has been shown to be a carcinogenic mycotoxin in animal models. Many studies showed that the high level of ST contamination in grains might be related to the high incidence of gastric carcinoma in rural areas of China. However, up to now, the potential effects of ST on human gastric epithelium cells remain largely unknown. In this study, we explored the effects of ST on cell-cycle distribution and the regulatory mechanism in immortalized human gastric epithelium cells (GES-1).

Methods and results: The effects of ST on the cell cycle distribution of GES-1 cells were determined with flow cytometric (FCM) analysis, Giemsa staining and immunofluorescence staining, while that on the expression of related gene-Cdc25C, Cdc2, CyclinB1 and the complex of CyclinB1-Cdc2 were studied with Western blot, reverse transcription polymerase chain reaction (RT-PCR) and immunoprecipitation assay respectively. We found that ST induced GES-1 cells arrested at G2 phase by regulating the expression of Cdc25C, Cdc2, CyclinB1 and the formation of CyclinB1-Cdc2 complex. Further study suggested JNK, ERK and PI3K/AKT/mTOR pathways to be involved in the process of G2 arrest induced by ST. The specific inhibitors of JNK and ERK reversed the role of ST, whereas that of PI3K/AKT/mTOR reinforced the effect of ST on cell-cycle distribution.

Conclusion: This study demonstrates that JNK, ERK and PI3K/AKT/mTOR pathways participated in the G2 arrest induced by ST through the deregulation of CyclinB1, Cdc2 and Cdc25C. It may play some roles in the gastric carcinogenesis in ST exposure populations.

Keywords:
ERK / G_2 arrest / JNK / mTOR / Sterigmatocystin

1 Introduction

Sterigmatocystin (ST) is a toxic fungal metabolite mainly produced by *Aspergillus nidulans* and *Aspergillus versicolor*. It is also a precursor involved in the biosynthesis of aflatoxin. The contamination of ST was frequently seen in human food, animal feed and even in indoor environment, such as carpet and building materials [1, 2]. It was reported that ST had the potent mutagenic and carcinogenic effects in animals [3]. ST could enhance the development of intestinal metaplasia of gastric mucosa in *Helicobacter pylori*-infected Mongolian gerbils [4]; induce atypical hyperplasia of glandular stomach in mice [5, 6] and induce p53 mutation and malignant transformation of human fetal gastric mucosa cells *in vitro* [7]. Accordingly, ST has been recognized as a 2B carcinogen (possible human carcinogen) by International Agency for Research on Cancer [8].

Accumulating evidence has indicated that some carcinogenic mycotoxins could affect the process of cell-cycle transition. Deoxynivalenol induced G_2/M phase

Correspondence: Dr. Xiang Hong Zhang, Department of Pathology, The Second Hospital, Hebei Medical University, No. 215, Heping Western Road, Shijiazhuang, P. R. China
E-mail: zhangxianghong2008@163.com
Fax: +86-311-86266229

Abbreviations: ERK, extracellular signal-regulated kinase; **FCM,** flow cytometry; **JNK,** c-Jun-NH2-terminal kinase; **MAPK,** mitogen-activated protein kinase; **mTOR,** mammalian target of rapamycin; **PI3K,** phosphatidylinositol 3-kinase; **ST,** sterigmatocystin

arrest in human intestinal epithelium cells *via* elevated p21 gene expression [9]. Zearalenone could result in the cell-cycle perturbation of human colon cancer cells (Caco-2) *in vitro* [10]. Xie *et al.* found that ST could induce G_2/M phase arrest in murine fibroblasts *via* loss of p53-mediated G_1 checkpoint [11]. It is generally accepted that the imbalance of cell-cycle progression may result in genomic instability, abnormal cell proliferation and even carcinogenesis [12–14].

In eukaryotic cells, the progression of the cell cycle is mainly regulated by the Cdc25C, Cdc2, CyclinB1 as well as the complex of CyclinB1–Cdc2 [15]. Among these proteins, Cdc25C, a tyrosine protein phosphatase, could directly dephosphorylate Cdc2 on Tyr-15 and activate its kinase activity, thereby inducing the formation of CyclinB1–Cdc2 complex, which makes cell-cycle entrance into M phase from G_2 phase [16]. On the other hand, phosphorylation of Cdc25C will lead to the inactivation of Cdc2, and these changes might facilitate cells arrested at G_2 phase [17]. Moreover, recent findings have implicated that c-Jun-NH2-terminal kinases (JNK), extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathways can affect the phosphorylation of targets and may be involved in the process of cell-cycle arrest [18–22].

Epidemiological studies in China suggested that ST may be a putative etiological factor for gastric carcinoma due to the high contaminations in foodstuff in the high incidence areas of gastric carcinoma [23]. Gastric epithelium would be likely exposed directly to ST when contaminated food is ingested. However, up to now, few studies on the direct effects of ST on human gastric epithelial cells have been reported. Therefore, in the present study, we explored the effects of ST on cell-cycle distribution and the regulation mechanism of JNK, ERK and PI3K/AKT/mTOR pathways using an immortalized human gastric epithelium cell line (GES-1).

2 Materials and methods

2.1 Chemicals and reagents

Highly purified ST (>99% purity, benzene free) was purchased from Sigma-Aldrich (S3255, St. Louis, MO, USA). The primary antibodies used for Western blot, immunoprecipitation and immunofluorescence analysis were mouse anti-human CyclinB1 antibody (eBioscience, CA, USA); rabbit anti-human Cdc2, Cdc25C monoclonal antibodies (Epitomics, CA, USA); rabbit anti-human phospho-Cdc2 (Tyr-15), phospho-Cdc25C (Ser-216), phospho-histone H3 (Ser-10), JNK/p-JNK, ERK/p-ERK, mTOR/p-mTOR monoclonal antibodies (Cell Signaling Technology, MA, USA); mouse anti-human PI3K monoclonal antibody (Santa Cruz, CA, USA). SP600125, PD98059 and LY294002 were purchased from Sigma-Aldrich.

2.2 Cell culture and treatment

Human gastric epithelial cell line GES-1 was purchased from Beijing Institute for Cancer Research. The cells were cultured in DMEM (pH 7.0) supplemented with 100 U/mL penicillin, 100 U/mL streptomycin and 10% fetal bovine serum. Cells were randomly divided into control (normal saline), solvent control (DMSO, final solvent concentration is 0.1%) and ST treatment groups. ST was diluted in DMSO and added to the culture flasks to obtain the final concentration of 100, 500, 1000 and 2000 μg/L, respectively. The cells were harvested with 0.25% trypsin 24 h after ST treatment.

For JNK, ERK and PI3K signaling pathway activation analysis, GES-1 cells were divided into five groups: control, solvent control, ST 1000 μg/L, pathway-specific blocking agents alone and blocking agent combined with ST 1000 μg/L. The cells in pathway-specific blocking agent groups were pretreated for 30 min with 1 μmol/L SP600125 (JNK inhibitor), 50 μmol/L PD98059 (ERK inhibitor) and 1 μmol/L LY294002 (PI3K inhibitor), respectively. Cells in blocking agent combined with ST 1000 μg/L group were treated with ST 1000 μg/L after the blocking agent treatment. Cells were harvested after exposure for 24 h.

2.3 Cell-cycle analysis

Cells in different groups were trypsinized (Gibco-BRL, USA), washed once with PBS and fixed with 70% ethanol overnight at 4°C. After fixation, cells were washed once with PBS and resuspended in PBS/0.1% Triton X-100 and incubated with 50 U DNase-free RNaseA (Calbiochem, Germany) (30 min, room temperature). After incubation, cells were stained with propidium iodide (Sigma, USA) (20 μg/mL in PBS, 15 min and room temperature). Flow cytometry (FCM) analysis was performed using a FACS Calibur (Becton Dickinson, USA).

2.4 Mitotic indexes count

GES-1 cells were allowed to grow on sterilized glass coverslips in flask and Giemsa staining was used for mitotic index analysis. After exposure to ST at different concentrations for 24 h as described above, the cells were rinsed with PBS three times and fixed with 4% paraformaldehyde for 30 min. Then the coverslips were immersed in freshly prepared 5% v/v Giemsa stain solution for 10 min, subsequently flushed with tap water and left to dry in the air. The cells were observed under a light microscope. At least 1000 cells were observed and ten visual fields were randomly selected for mitosis count analysis. The mitotic cell count was performed independently by three experimenters blind to treatment condition. Mitotic cells were recognized as lack of nuclear membrane and chromosome condensation. The mitotic index was evaluated as the average percent of mitotic cells: the number of cells in mitosis/total number of cells.

2.5 Immunofluorescence staining

GES-1 cells were cultured for 24 h in medium containing different concentrations of ST on glass coverslips, fixed with 4% paraformaldehyde for 30 min and then permeabilized with 0.5% Triton X-100. After blocking with 5% normal goat serum in PBS, the cells were incubated with rabbit monoclonal antibodies against phosphorylated histone H3 (Ser-10) at 4°C overnight. The cells were washed with PBS containing 0.1% Triton X-100 and incubated with FITC-labeled affinity purified anti-rabbit IgG secondary antibody. DNA was counterstained with propidium iodide. The images were captured and observed using TCS-SP2 laser scanning confocal microscope (Leica, Germany).

2.6 Western blot analysis

Whole cell protein from GES-1 cells was extracted using the lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L PMSF, 2 g/L aprotinin and 0.5 mmol/L DTT). Fifty micrograms of protein *per* sample was run on a 15% SDS-PAGE gel, electrotransferred to PVDF membrane, blocked with 5% nonfat milk in TBST and probed or re-probed with the appropriate primary antibodies or secondary antibodies, and then detected by the ECL system. Anti-actin antibody was used as the loading control.

2.7 Immunoprecipitation assay

Precleared cell lysate (120 μg) was incubated with 10 μg anti-CyclinB1 at 4°C with rotation overnight. Then the solution was incubated with 20 μL protein G-Sepharose (Santa Cruz) at 4°C with rotation overnight. The beads were washed five times using lysis buffer and boiled at 95°C for 5 min. Immunocomplexes were resolved by 15% SDS-PAGE and were transferred to PVDF membranes. Association of CyclinB1 with Cdc2 was detected by incubating the blots with anti-Cdc2 antibody.

2.8 The semi-quantitative RT-PCR

Total RNA was extracted from cells using Trizol reagent. One microgram of RNA from each sample was transcribed into cDNA using reverse transcriptase (5 U/μL, 0.5 μL), and the cDNA was used in PCR reaction. The PCR was carried out in a final volume of 50 μL containing cDNA (5 μL), 10 × buffer (5 μL), $MgCl_2$ (25 mmol/L, 3 μL), dNTP (10 mmol/L, 0.5 μL), Taq DNA polymerase (0.3 μL), primers (sense and anti-sense, 10 pmol, respectively) using the following parameters: denaturation at 94°C for 5 min and 35 cycles of reactions of denaturation at 94°C for 50 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s. An aliquot of each PCR product was subjected to 1.5% w/v agarose gel electrophoresis and visualized by staining with ethidium bromide. GAPDH was used as the endogenous control in each test, and the ratio of target gene to GAPDH represented the level of target gene expression. The primers for the amplification of each gene are listed in Table 1.

2.9 Statistical analysis

All results were expressed as mean ± SD from each group; statistical analysis was performed with one-way analysis of variance (ANOVA). The concentration–effect relationship was analyzed with correlation and regression analysis. All statistical analyses were calculated by the statistical software of SPSS 13.0. The *p*-values <0.05 were considered to be significant.

3 Results

3.1 ST induces the GES-1 cell cycle to be arrested at G_2 phase

FCM assay showed that ST could affect the distribution of cell cycle in GES-1 cells. The proportion of cells in G_2/M phase was increased significantly while that of cells in G_0/G_1 and S phase was decreased correspondingly in ST-treated groups compared with the solvent control, especially that in 500, 1000 and 2000 μg/L ST-treated groups (Fig. 1A). To further

Table 1. Primers for RT-PCR amplifications

Gene	Primer	Sequence	Product length (bp)
CyclinB1	Sense	5′-TTG GTT GAT ACT GCC TC-3′	207
	Anti-sense	5′-TGG TCT GAC TGC TTG CT-3′	
Cdc2	Sense	5′-AAA GTG AAG AGG AAG GGG T-3′	441
	Anti-sense	5′-CTG GAG TTG AGT AAC GAG C-3′	
Cdc25C	Sense	5′-TTT ATG TCA TTG ATT GTC GC-3′	286
	Anti-sense	5′-AAG AAG TCT CTG TAG CCG CC-3′	
GADPH	Sense	5′-GGA AGG TGA AGG TCG GAG T-3′	231
	Anti-sense	5′-CCT GGA AGA TGG TGA TGG G-3′	

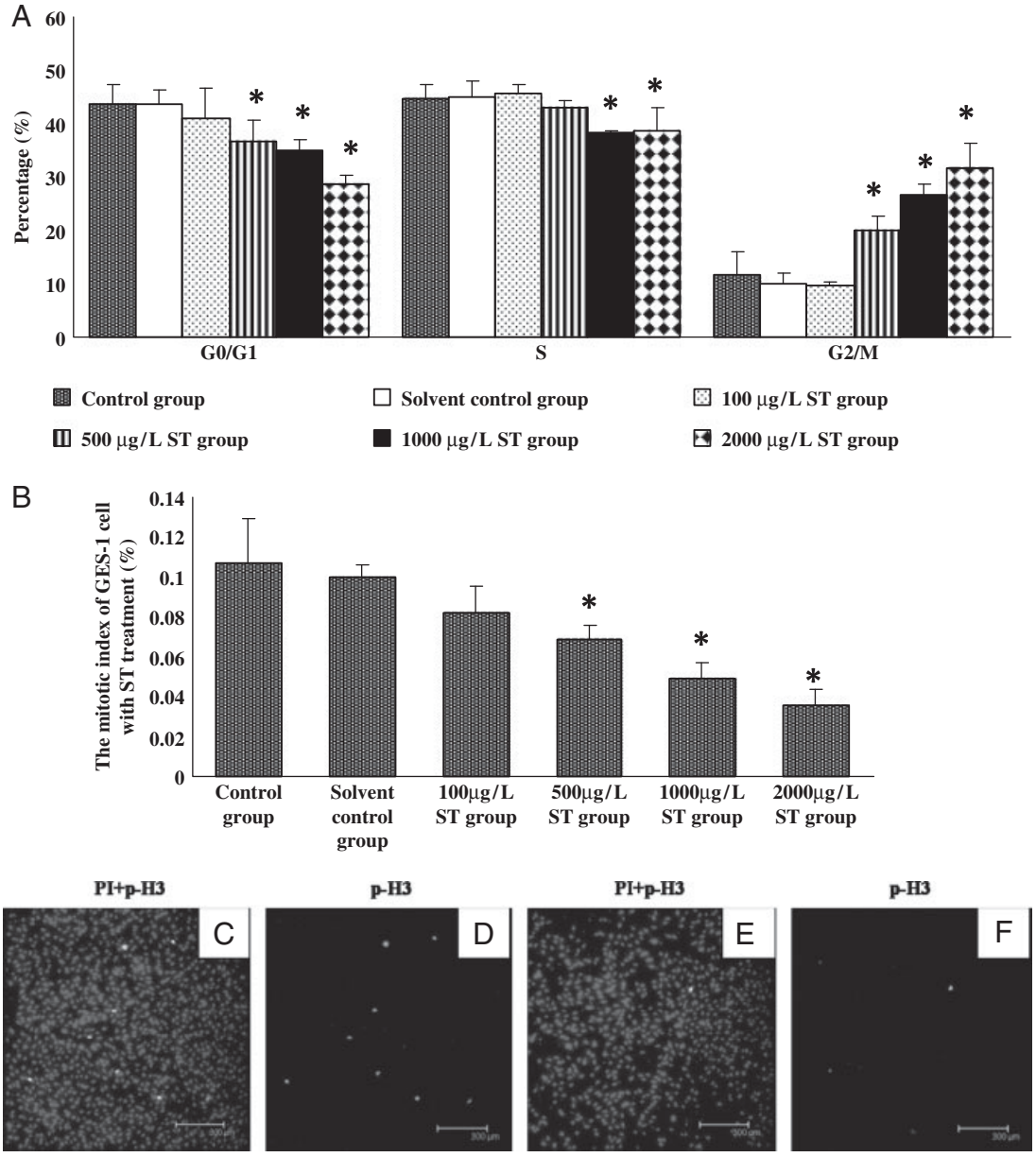

Figure 1. The changes of cell-cycle distribution, mitotic index and phospho-histone H3 level in GES-1 cells induced by ST within the concentration range from 0 to 2000 μg/L *in vitro*. (A) FCM assay for cell-cycle distribution. (B) The down-regulation effect of ST on the mitosis index of GES-1 cells detected by Giemsa. (C–F) GES-1 cells were seeded onto glass coverslips and, after 2000 μg/L ST treatment for 24 h, cells were fixed, permeabilized and subjected to staining with PI to visualize nuclei and with anti-phosphorylate histone H3 (Ser-10) antibody as a mitosis marker (D and F). Images were analyzed using a confocal microscope (C and E). (C and D) Solvent control group; (E and F) 2000 μg/L ST group. $^{*}p<0.05$, compared with solvent control group.

clarify the ratio of cells in M phase exactly, two assays including Giemsa staining (mitosis index) and immunofluorescence with phospho-histone H3 were then performed. The mitosis index was significantly decreased ($p<0.05$) in 500, 1000 and 2000 μg/L ST-treated groups (Fig. 1B), and phospho-histone H3 expression in ST treatments was also noticeably diminished compared with the other treatment cells (Fig. 1C–F). Thus, from these data we concluded that ST could cause the cell cycle arrested at G_2 phase.

3.2 CyclinB1, Cdc2 and Cdc25C are involved in the G_2 phase arrest induced by ST

CyclinB1, Cdc2 and Cdc25C proteins are all critical in controlling transition of cell cycle from G_2 phase to M phase. We hypothesized that these proteins might be involved in the G_2 phase arrest induced by ST. Therefore, the expression of Cdc2, Cdc25C and CyclinB1 was detected by RT-PCR and Western blot assay, respectively. The results showed that

Cdc25C mRNA was reduced in ST-treated cells in a concentration-dependent manner ($r = -0.881$, $n = 3$, $p < 0.01$), whereas the expression of CyclinB1 and Cdc2 mRNA was both increased in ST-treated cells relative to the control (Fig. 2A). At protein level, the results revealed that the total Cdc25C protein was decreased by ST treatment, whereas the level of p-Cdc25C (Ser-216) was increased correspondingly in ST-treated groups compared with the solvent control (Fig. 2B). Consistent with the change at mRNA level, the expression of CyclinB1 protein was also upregulated by ST treatment compared with the solvent control group ($r = 0.743$, $n = 3$, $p < 0.01$, Fig. 2B). As for the change of Cdc2 protein expression, we found that the level of p-Cdc2 (Tyr-15) was increased correspondingly in ST-treated cells, though the total Cdc2 protein was decreased in ST-treated cells compared with control one (Fig. 2B).

The complex of CyclinB1–Cdc2 is defined as the maturation-promoting factor, which promotes cell entrance into mitosis from the G_2 phase. Therefore, we determined the effects of ST on CyclinB1–Cdc2 protein interactions by immunoprecipitation. As shown in Fig. 2C, the complex CyclinB1–Cdc2 was decreased noticeably in all the ST treatment groups within the concentration ranging from 100 to 2000 μg/L. Although the expression of CyclinB1 protein was increased by ST treatment, its ability to bind with Cdc2 was decreased markedly. Taken together, these observations indicated that the total Cdc2 and Cdc25C protein were both decreased while p-Cdc2 (Tyr-15) and p-Cdc25C (Ser-216) were increased correspondingly by ST treatment within the concentration ranging from 100 to 2000 μg/L, and the complex of CyclinB1/Cdc2 in ST-treated cells was also decreased significantly, suggesting that CyclinB1, Cdc2 and Cdc25C were involved in the G_2 phase arrest induced by ST.

3.3 ST activates JNK, ERK and PI3K/AKT/mTOR signaling pathways in GES-1 cells

Studies have shown that the activation of JNK, ERK and PI3K/AKT/mTOR signaling pathways was involved in the

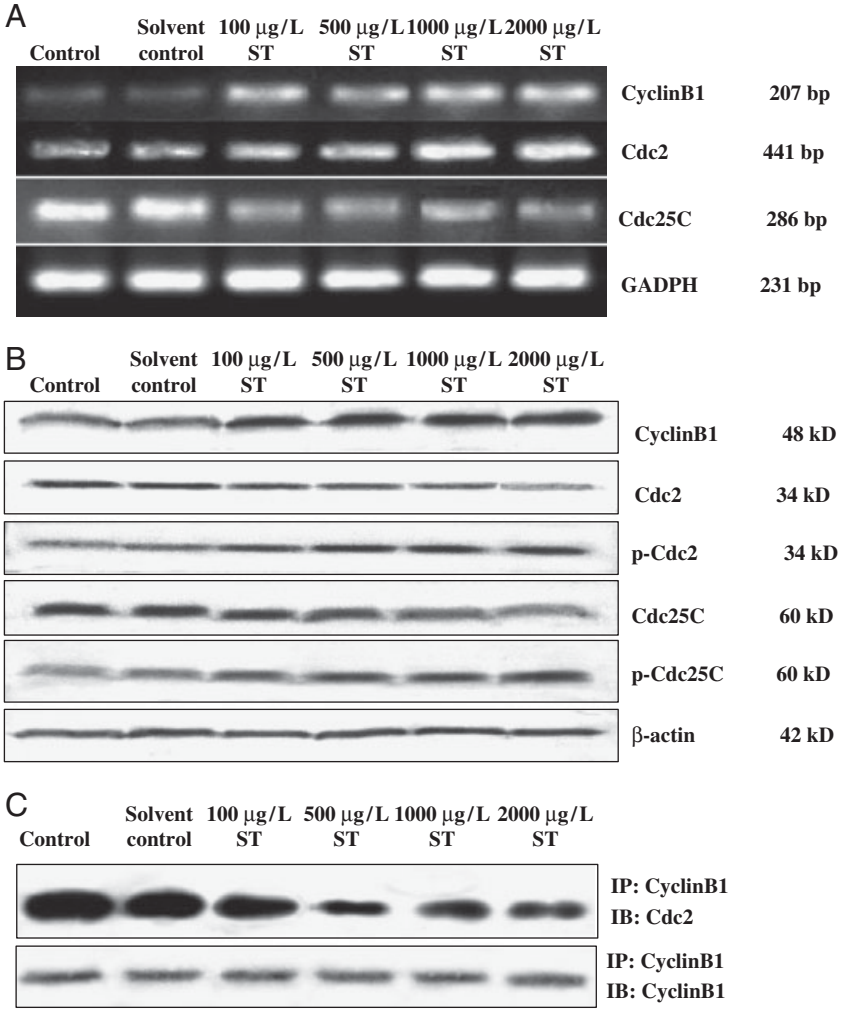

Figure 2. The changes of CyclinB1, Cdc2 and Cdc25C expression and the CyclinB1–Cdc2 complex in GES-1 cells induced by ST within the concentration range from 0 to 2000 μg/L *in vitro*. (A) CyclinB1, Cdc2 and Cdc25C mRNA were detected by RT-PCR after cells had been treated for 24 h. (B) CyclinB1; Cdc2 and p-Cdc2 (Tyr-15); Cdc25C and p-Cdc25C (Ser-216) were detected by Western blot after cells had been treated for 24 h. (C) The formation of CyclinB1–Cdc2 complex in GES-1 cells treatment with ST by immunoprecipitation.

cell-cycle arrest [21, 24, 25]. To examine whether these pathways were involved in ST-induced G_2 phase arrest, we evaluated the activity of these pathways in GES-1 cells after 1000 μg/L ST treatment. Results showed that there were no effects of ST on the expression of pan-JNK, pan-ERK and pan-PI3K proteins. But the levels of p-JNK, p-ERK and p-mTOR were all increased significantly after ST treatment. To further demonstrate whether ST could enhance JNK, ERK and PI3K/AKT/mTOR pathway activation, GES-1 cells were untreated or treated with ST 1000 μg/L in the absence or presence of JNK-specific inhibitor SP600125, ERK-specific inhibitor PD98059 or PI3K-specific inhibitor LY294002, respectively. Incubation of the cells with SP600125 alone or with SP600125 combined with ST led to a reduction of p-JNK when compared with that in the ST treatment alone (Fig. 3). Incubation of cells with ERK-specific inhibitor PD98059 showed the similar change of p-ERK as that of p-JNK. Simultaneously, PI3K inhibitor LY294002 markedly decreased the level of p-mTOR both in LY294002 alone and in LY294002 with ST treatment groups as compared with that in ST treatment group (Fig. 3). From these results we can see that ST treatment could activate JNK, ERK and PI3K/AKT/mTOR signaling pathways in GES-1 cells.

3.4 Activation of JNK, ERK and PI3K signaling pathways participates in the G_2 arrest induced by ST in GES-1 cells

To demonstrate that the activation of JNK, ERK and PI3K/AKT/mTOR signaling pathways was involved in cell-cycle arrest, we next examined the cell-cycle distribution and CyclinB1, Cdc2 and Cdc25C protein expression using FCM and Western blot after pretreatment with the specific inhibitors of JNK, ERK and PI3K/AKT/mTOR, respectively. From three independent tests, we demonstrated that 1000 μg/L ST treatment could increase the proportion of cells in G_2/M phase, decrease the expression of Cdc2 and Cdc25C protein and up-regulate the levels of p-Cdc2 and p-Cdc25C when compared with the control group (Figs. 4–6); all these changes could result in the G_2 phase arrest of GES-1 cells. As compared with the cells in ST-treated alone, the number of cells in G_2/M was decreased significantly in the SP600125 combined with 1000 μg/L ST group detected by FCM (Fig. 4A), and the expression of Cdc2/p-Cdc2 and Cdc25C/p-Cdc25C at protein levels was all reversed by SP600125 pretreatment (Fig. 4B and C). SP600125 also could counteract the role of ST on the expression of CyclinB1 mRNA but had no effects on Cdc2 and Cdc25C mRNA expression (Fig. 4D and E). It indicated

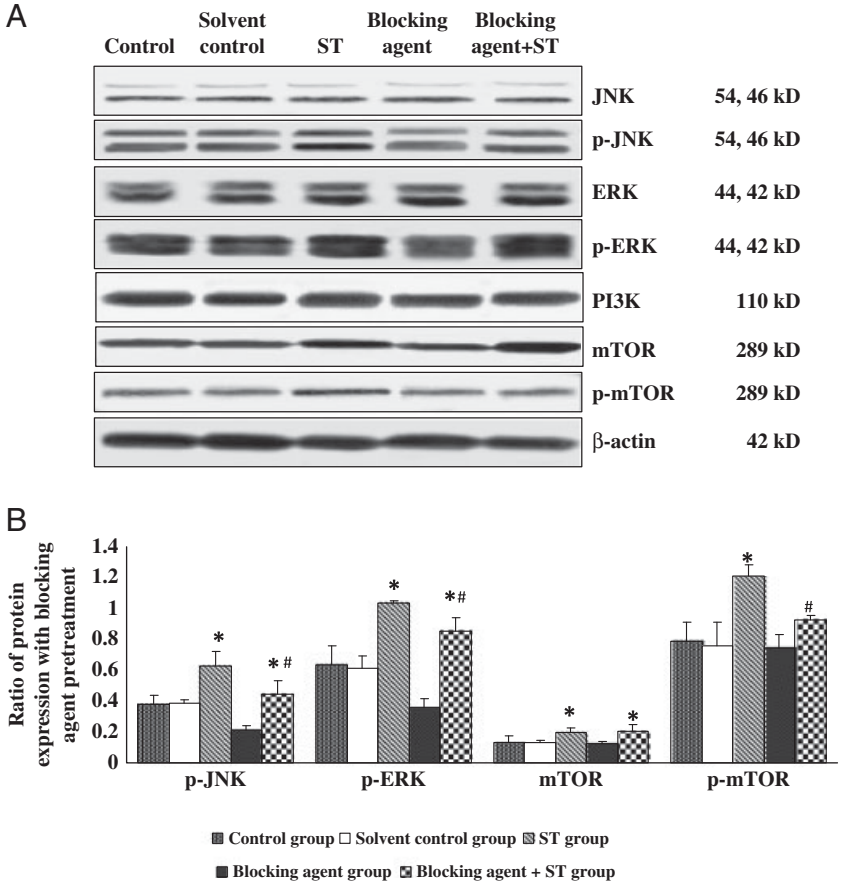

Figure 3. Western blot detected the expression of JNK, ERK and PI3K/AKT/mTOR in GES-1 cells. (A) Representative immunoblots showed that pretreatment with SP600126, PD98059 and LY294002 could block or partly block the activation of JNK, ERK and PI3K/AKT/mTOR signaling pathways induced by ST, which confirmed that ST could activate these pathways. (B) Intensities of the immunoreactive bands were quantified by densitometric scanning. Values are means ± SD. $^{*}p < 0.05$, compared with solvent control group; $^{\#}p < 0.05$, compared with ST group.

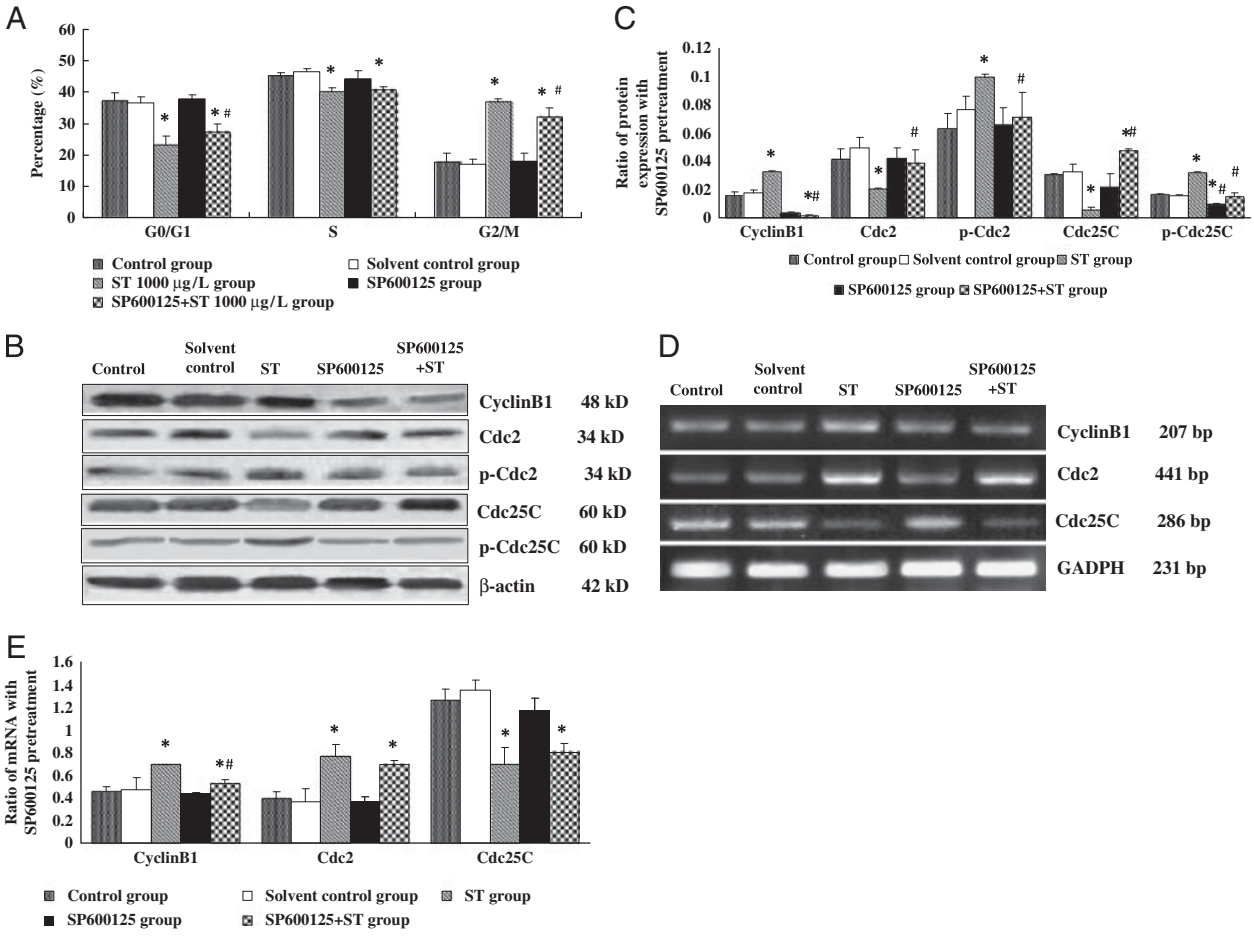

Figure 4. The activation of JNK signaling pathway participated in the G_2 arrest of GES-1 cell cycle induced by ST. (A) Flow cytometry results showed that JNK signaling pathway inhibitor SP600125 pretreatment relieved the G_2 arrest; (B) Representative immunoblots showed that SP600125 pretreatment reversed the expression of Cdc25C, Cdc2, CyclinB1 and the phosphorylation of Cdc25C (Ser-216) and Cdc2 (Tyr-15) induced by ST. (D) The expression of CyclinB1 mRNA was reversed by SP600125, which was analyzed by RT-PCR. (C and E) Intensities of the immunoreactive bands and the electrophoresis bands were quantified by densitometric scanning. Values are means ± SD. $^{*}p<0.05$, compared with solvent control group; $^{\#}p<0.05$, compared with ST group.

that SP600125 pretreatment could partly block the effect of ST on cell-cycle distribution. Treatment of cells with ERK-specific inhibitor PD98059 could also counteract the role of ST on the expression of Cyclin B1 and the level of p-Cdc2 and p-Cdc25C in GES-1 cells, but the expression of Cdc2 and Cdc25C proteins was not affected. Pretreatment with PD98059 could reverse the increasing of Cdc2 mRNA induced by ST, while did not affect CyclinB1 and Cdc25C mRNA (Fig. 5). These results showed that the activation of JNK and ERK signaling pathway participated in the G_2 phase arrest of cell cycle induced by ST.

On the contrary, the PI3K/AKT/mTOR signaling pathway played a different role in ST-induced G_2 arrest. The G_2/M fraction was even higher in LY294002 combined with ST treatment cells than that in ST treatment cells (Fig. 6A). Furthermore, LY294002 pretreatment could further potentiate the effects of ST on the expression of Cdc2, Cdc25C and p-Cdc2, p-Cdc25C levels (Fig. 6B and C). LY294002 could also further potentiate the effects of ST on the expression of Cdc2 mRNA (Fig. 6D and E). These results suggest that PI3K/AKT/mTOR signaling pathway activation might represent a novel negative regulation on the G_2 phase arrest of cell cycle induced by ST in GES-1 cells.

4 Discussion

All epidemiological *in vivo* and *in vitro* studies have demonstrated that ST has carcinogenic potency. A large-scale study conducted in the 1990s in China revealed the prevalence of ST contamination in wheat, corn and rice to be high at 98, 89 and 72%, and at concentration of up to 68.9, 32.2 and 13.9 μg/kg, respectively [26]. Further study showed that both the contamination rate and the content of ST in grains from high incidence area of gastric cancer were significantly higher than that from low incidence area in

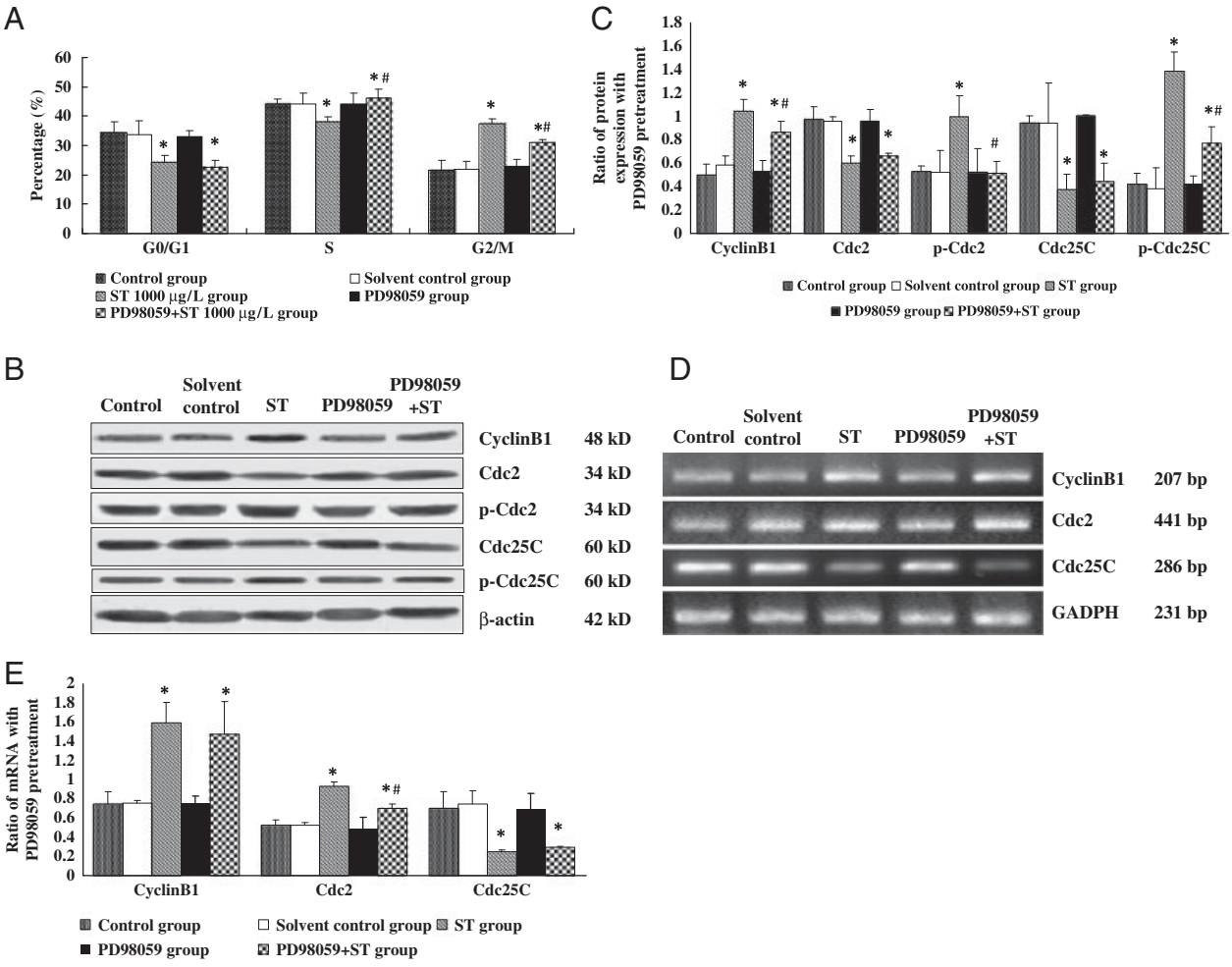

Figure 5. The activation of ERK signaling pathway participated in the G_2 arrest of GES-1 cell cycle induced by ST. (A) Flow cytometry results showed that ERK signaling pathway inhibitor PD98059 pretreatment relieved the G_2 arrest; (B) Representative immunoblots showed that PD98059 pretreatment reversed the expression of CyclinB1 and the phosphorylation of Cdc25C (Ser-216) and Cdc2 (Tyr-15) induced by ST, but had no effects on the expression of Cdc25C and Cdc2 protein. (D) The expression of Cdc2 mRNA was reversed by PD98059, which was analyzed by RT-PCR. (C and E) Intensities of the immunoreactive bands and the electrophoresis bands were quantified by densitometric scanning. Values are means ± SD. $^{*}p<0.05$, compared with solvent control group; $^{\sharp}p<0.05$, compared with ST group.

China [23]. *In vivo* studies have shown that ST exposure can induce hepatocellular carcinoma [27], lung adenocarcinoma [5] and squamous skin cell carcinoma [28] in experimental animals. Additional *in vivo* experiments using NIH mice [6] have shown that long-term (24 wk) ST oral exposure could induce atypical hyperplasia of mucosa epithelial cells of glandular stomach and enhance the development of gastric mucosal metaplasia in *H. pylori*-infected Mongolian gerbils [4]. For consistency, *in vitro* experiments had demonstrated that ST could induce p53 mutation and malignant transformation of human fetal gastric mucosa cells *in vitro* [29].

The present study demonstrated the direct toxic effects of ST on the human gastric epithelium (GES-1) cell cycle *in vitro*. It is generally accepted that induction of cell-cycle arrest is the important bio-effect of many carcinogenic mycotoxins [29, 30]. Several mycotoxins have been identified to play roles in G_2/M phase arrest. OTA was shown to inhibit cell-cycle progression by arresting cells at G_2/M phase in V79 Chinese hamster lung fibroblasts [31] and GES-1 cells at G_2 phase *in vitro* [32]. Deoxynivalenol could induce G_2/M arrest in human intestinal epithelium cells [9]; and as for ST, preliminary results by Xie *et al.* had shown that ST could induce G_2/M phase arrest in murine fibroblasts *via* loss of p53-mediated G_1 checkpoint [11]. However, whether ST could affect the cell cycle of human gastric cells is unknown. Therefore, we evaluated the effects of ST on the cell-cycle distribution of human gastric GES-1 cells *in vitro* in this study. The FCM results revealed that the ST treatment could increase the proportion of G_2/M phase cells significantly. Further analysis confirmed that cells in M phase were decreased significantly after ST treatment by using Giemsa staining and immunofluorescence assay of

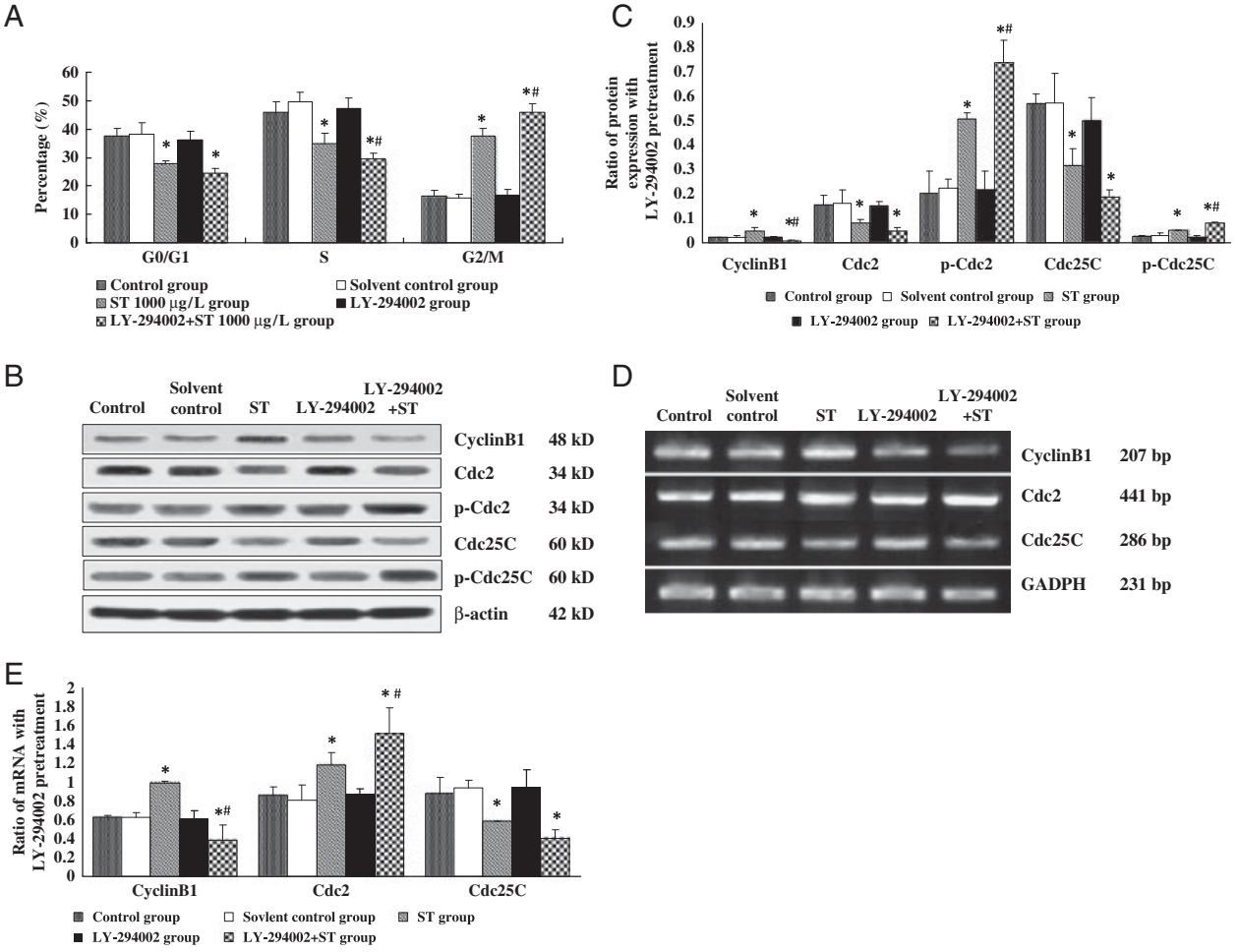

Figure 6. The activation of PI3K/AKT/mTOR signaling pathway participated in the G_2 arrest of GES-1 cell cycle induced by ST. (A) Flow cytometry results showed that PI3K/AKT/mTOR signaling pathway inhibitor LY294002 pretreatment promoted the G_2 arrest; (B) Representative immunoblots showed that LY294002 pretreatment promoted the phosphorylation of Cdc25C (Ser-216) and Cdc2 (Tyr-15) induced by ST. (D) The expression of Cdc2 mRNA was promoted by LY294002, which was analyzed by RT-PCR. (C and E) Intensities of the immunoreactive bands and the electrophoresis bands were quantified by densitometric scanning. Values are means ± SD. $^{*}p < 0.05$, compared with solvent control group; $^{\#}p < 0.05$, compared with ST group.

phospho-histone H3. Thus, it is clear that ST could induce GES-1 cell-cycle arrest at G_2 phase. It was generally accepted that aberrant cell cycle may lead to the chromosomal instability and may result in carcinogenesis. Our results suggest that ST-induced G_2 arrest could play some roles in gastric carcinogenesis of high incidence area in China.

A variety of stimuli including chemicals, irradiation and the transforming factor β1 have been shown to impact detrimentally on signal factors involved in animal cell mitosis, Cdc25C, Cdc2, CyclinB1 and CyclinB1–Cdc2 complex [33–36]. Moreover, abnormal expression and/or phosphorylation of these factors have been reported in many human cancers and cell lines suggesting their importance in carcinogenesis [37–40]. To explore the putative effects of these proteins in the G_2 phase arrest induced by ST, we further evaluated the changes in Cdc25C, Cdc2 and CyclinB1 expression at mRNA and protein level, the phosphorylation levels of Cdc25C and Cdc2 and the formation of CyclinB1–Cdc2 complex after ST treatment. Our findings showed that 24 h after ST treatment at different concentrations from 100 to 2000 μg/L, the expression of Cdc2 and Cdc25C protein was both down-regulated significantly, whereas the level of p-Cdc25C and p-Cdc2 was both up-regulated. In addition, the complex CyclinB1–Cdc2 was decreased significantly in all ST-treated cells. All these changes may be involved in the cell-cycle arrest at G_2 phase induced by ST. The expression of these regulation factors at mRNA level was consistent with the changes in the protein with the exception of Cdc2. We supposed that there might be modifications of mRNA stability and/or translational efficiency, or alternatively, some regulation at the post-translational level may exist.

Down-regulation of Cyclin B1 is an important mechanism used by p53 to stabilize G_2 arrest. Lack of this protection

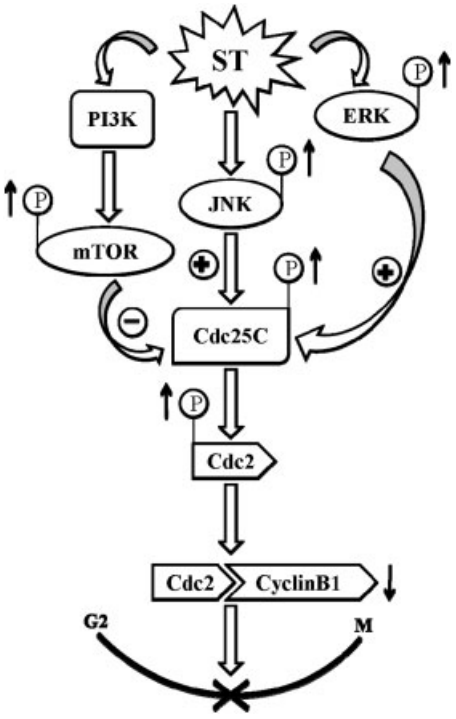

Figure 7. Proposed diagrams of ST-induced G_2 arrest in GES-1 cells.

could cause the cells to enter mitosis with damaged DNA, which contributes to genomic instability and can trigger cell death. Unexpectedly, it showed an increase in CyclinB1 expression after ST exposure both at mRNA and protein level in our study. Some reports suggested that CyclinB1 is considered to be an oncogene, and its overexpression could be found in many kinds of tumor cells [41], as well as in HPV-infected cell [42]. Hence, we presumed that the high expression of CyclinB1 induced by ST might have relationship with its carcinogenesis. Further study concerning the mechanisms need to be carried out. Our results at least demonstrated that the complex CyclinB1–Cdc2 was decreased noticeably after ST exposure, which might favor the cells to be arrested at G_2 phase.

Regulation of Cdc25C and Cdc2 occurs through a variety of mechanisms including phosphorylation, and the signal transduction pathways, especially mitogen-activated protein kinase (MAPK) (JNK, ERK and P38) and PI3K/AKT/mTOR pathways, play important roles on the phosphorylation of these factors [43–46]. The activation of JNK could not only phosphorylate and stabilize p53 to effect the phosphorylation of Cdc25C [47] but also activate Chk2 to phosphorylate Cdc25C on Ser216 [48] to regulate cell cycle. And the activated p-ERK1/2 could bind and phosphorylate Cdc25C on its ser216 residue [49]. The inhibition of AKT/mTOR could induce G_2/M arrest in cells by increasing the association of p21/WAF1/Cdc2 complex and the levels of inactivated phospho-Cdc2 and phospho-Cdc25C [50]. Studies have shown that cell-cycle arrest induced by melatonin and cardiotoxin III was related with the activation of MAPK and PI3K signaling pathways, which was accompanied with the corresponding inactivation of Cdc25C phosphatase and the CyclinB1–Cdc2 complex [18, 51]. Meanwhile, many mycotoxins, such as Patulin [52], deoxynivalenol [53] and OTA [54], could play their biological effects by influencing MAPKs and/or PI3K signaling pathways. Yet there is no evidence about whether these pathways are involved in ST-induced G_2 phase arrest, so we further evaluated the role of these pathways on the cell-cycle distribution in GES-1 cells. The results showed that the levels of the p-JNK, p-ERK and p-mTOR were all up-regulated significantly after 1000 μg/L ST treatment. Pretreatment of the cells with the specific inhibitor (SP600125, PD98059 or LY294002) could specifically inhibit the phosphorylation of JNK, ERK and mTOR induced by ST, respectively. Thus, the data confirmed that ST could activate JNK, ERK and PI3K/AKT/mTOR signaling pathways in GES-1 cells. To our knowledge, ST is a carcinogenic mycotoxin, and it could induce DNA damage [55]; hence, the possible mechanisms that ST can induce the activation of ERK, JNK and PI3K/AKT/mTOR pathways might depend on the role of DNA damage.

Next, we demonstrated that after ST treatment, the activation of these pathways was accompanied by the change in the phosphorylation level of Cdc25C and Cdc2, and the proportion of cells in G_2/M phase. JNK- and ERK-specific inhibitor (SP600125 and PD98059) pretreatment could significantly reduce the proportion of cells in G_2/M phase induced by ST and partly restore the activity of Cdc25C phosphatase and Cdc2 kinase in GES-1 cells *in vitro*. On the contrary, PI3K-specific inhibitor (LY294002) pretreatment reinforced the role of ST on cell-cycle distribution. The proportion of cells in G_2/M phase and the Cdc25C and Cdc2 activity were all even higher by LY294002 pretreatment when compared with ST treatment alone. Based on these data, we indicated that JNK, ERK and PI3K/AKT/mTOR signaling pathways were all involved in the G_2 arrest induced by ST. The activation of JNK and ERK pathways may play positive roles, while PI3K/AKT/mTOR signaling pathway play a negative role in the ST-induced G_2 arrest. It should be noted that the results in our study are different from that in the previous studies which suggested that MAPK and PI3K signaling pathways play a similar role in the disruption of cell cycle induced by external stimuli [56–58]. Obviously, regulation of cell cycle is a complex process, more extensive investigation of related proteins/signaling pathways should be observed further to get a more comprehensive insight on the effects of ST on the cell cycle.

In conclusion, our data suggested that ST exposure activated the JNK, ERK and PI3K/AKT/mTOR signaling pathways in GES-1 cells, accompanied with the changes on cdc25–Cdc2–CyclinB1/CyclinB1–Cdc2 complex, which may contribute to ST-mediated cell-cycle arrest at G_2 phase (Fig. 7). Thus, notwithstanding its limitations, the results suggest that G_2 arrest induced by ST might be involved in the carcinogenesis of ST.

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