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SLPI promotes the gastric cancer growth and metastasis by regulating the expression of P53, Bcl-2 and Caspase-8

X.-Y. DU[1], X. LIU[1], Z.-J. WANG[1], Y.-Y. WANG[2]

[1]Department of Pathology, Jinzhou Medical University, Jinzhou, China
[2]Department of Oncology, the First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China

Abstract. – OBJECTIVE: The incidence of gastric cancer is very high all over the world, but the mechanism of the occurrence and development of gastric cancer is unclear. Secretory leukocyte protease inhibitor (SLPI) is overexpressed in gastric, lung and ovarian cancers, which accelerates the metastasis of cancer cells. In this research, we mainly explored the expression level and possible mechanism of SLPI in gastric cancer.

PATIENTS AND METHODS: The expression and clinical significance of SLPI in 68 cases of gastric cancer tissues and adjacent tissues were detected by qRT-PCR. Cell Counting Kit-8 (CCK8) assay was used to detect the proliferation ability of gastric cancer cell lines. In addition, we used Western blot to clarify the relationship between SLPI and metastasis.

RESULTS: Compared with the adjacent tissues, we found that SLPI was highly expressed in gastric cancer tissues. We also found that the expression of SLPI was in significant correlation with the survival time, clinical classification and size of the tumor. What's more, SLPI could promote the proliferation and metastasis of gastric cancer by regulating P53, Bcl-2 and Caspase-8 expression through apoptosis signaling pathway.

CONCLUSIONS: We concluded that SLPI was closely related with to invasion and metastasis of gastric cancer. Perhaps we can hopefully find new targets for the treatment of gastric cancer.

Key Words
Gastric cancer, SLPI, Apoptosis pathway, Proliferation, Metastasis.

Introduction

Gastric cancer is the second most common cancer in the world. Maintaining the normal structure of gastric mucosa hugely depends on the dynamic balance of the apoptosis and proliferation of gastric mucosal cells, whose disorder is considered to be an important mechanism of the occurrence and development of gastric cancer[1]. Because of the urgent need of clinical treatment, increasing attention has been paid to tumor-specific gene therapy targeting tumor-specific promoter[2]. In recent years, it was found that secretory leukocyte protease inhibitor (SLPI) gene in various epithelial cancer was overexpressed, such as non-small cell lung cancer, ovarian cancer, and cervical cancer. But in normal tissues such as liver, endocrine, and blood system had no expression or very low expression of SLPI[3,4]. SLPI is a cationic inhibitor of neutrophil elastase and its biological functions include inducing cell proliferation and differentiation, anti-inflammatory function, antiviral function and antibacterial function. The specific expression and killing effect of the gene promoter have been confirmed in ovarian cancer. It has reported that SLPI promoted the metastasis of SNU638 gastric cancer cells by increasing expression of Matrix metalloproteinases-2 (MMP-2) and Matrix metalloproteinases-9 (MMP-9) through Elk-1 signaling[5]. However, there are still many unsolved problems of mechanism in the development of gastric cancer[6]. To further investigate the role of the promoters in gastric cancer, the function of SLPI in apoptosis pathway was carefully studied.

Patients and Methods

Patients Specimens and Clinical Assessments

The data were collected from 132 patients with gastric cancer admitted in the hospital from January 2014 to December 2015 (the Third Affiliated Hospital of Jinzhou Medical University, Jinzhou, China). All the specimens were divided into the same size after the operation and then treated with

Corresponding Author: Yuanyuan Wang, MD; e-mail: yuanyuanwang111@163.com

liquid nitrogen. The clinical data including age, sex, tumor size, lymph node metastasis, stage and pathological grade were recorded. All patients were elucidated about the experiment and the informed consent form was signed by all. This investigation was approved by our Medical Ethics Committee.

Cell Culture

MKN28, MKN45, and GES-1 cell lines were purchased from the Chinese Academy of Sciences (Shanghai, China). MKN28 and MKN45 were gastric cancer cell lines, and normal gastric mucosal cell line GES-1 was set as a control. All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at a 37°C incubator with 5% CO_2.

RNA Extraction and Real-time Quantitative PCR Assays

According to the manufacturer's protocol, total RNA from tissues and cells was extracted using RNA Riso Plus (TaKaRa, Otsu, Shiga, Japan). We used the PrimeScript™ RT reagent Kit to detected the concentration of RNA and cDNA was synthesized using gDNA Eraser (TaKaRa, Otsu, Shiga, Japan). Expression of SLPI in tumor tissue and gastric cancer cell lines were detected by standard fluorescent quantitative PCR assay with SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan).

CCK8 Assays

The target cells were seeded into 96 well plates at a density of 2000 cells per well, and three replicates were set for each group. 10 ml of Cell Counting Kit 8 (Dojundo Laboratories, Kumamoto, Japan) was added into 100 ml of DMEM in per well, which were co-cultured at 37°C incubator for 2 hours in darkness. The absorbance at 450 nm was detected for each 96 well plate. Data were collected for 5 days. All experiments were repeated three times.

Plasmid Transfection

The target cells were cultured in DMEM culture medium containing 15% fetal bovine serum (FBS) respectively. Logarithmic growth phase cells were collected $5*10^4$ cells per well and incubated in 24 well plates. When the cells grow to 60%-70% density, we transfected two cells according to Lipofectamine manual (Invitrogen, Carlsbad, CA, USA). Then, the plasmid lipid complex was added to the cells.

Western Blot Assays

Whole cell lysates were prepared by lysis buffer (50 mM Tris-HCl, 150 mMNaCl, 1% Triton-X100, 1 mM each $MgCl_2$, $MnCl_2$ and $CaCl_2$, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 10 mM sodium fluoride). Proteins were separated by SDS-PAGE and were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Quantitative determination of protein concentration was performed in standard bovine serum albumin (BSA). 100 mg of samples were added to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% denaturing gel. The protein was transferred to polyvinylidenedifluoride (PVDF) membrane (Millipore, Billerica, MA, USA) after electrophoresis, which was blocked in the 5% skimmed milk for 1 hour at room temperature. Then phosphate buffered saline (PBS) was used to wash the membranes. The membrane was incubated with respective secondary antibody. Enhanced chemiluminescent (ECL) detection system was applied to test the immunoblots. GraphPad Prism software was used to analyzed the protein bands (GraphPad, GraphPad Software, Inc., La Jolla, CA, USA).

Statistical Analysis

All data were presented as the average of SD. At least three independent replicates were conducted for each experiment. A *t*-test was used to analyze the differences between groups, and the log-rank test was applied to do survival analysis. The result was treated as significant only when the p-value<0.05. We used the Graphpad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) for all statistic analysis.

Results

SLPI was Highly Expressed in the Gastric Cancer Tissue

To investigate the effect of SLPI in gastric cancer, we detected the expression of SLPI in 68 cases of gastric cancer tissues and adjacent normal tissues using real-time fluorescence quantitative PCR. Compared with the adjacent tissues, SLPI was highly expressed in gastric cancer tissues. What's more, we also found that the expression of SLPI was even higher in diffuse-type gastric cancer compared with the intestinal-type gastric cancer (according to Lauren criteria). These results indicated that SLPI is associated with the

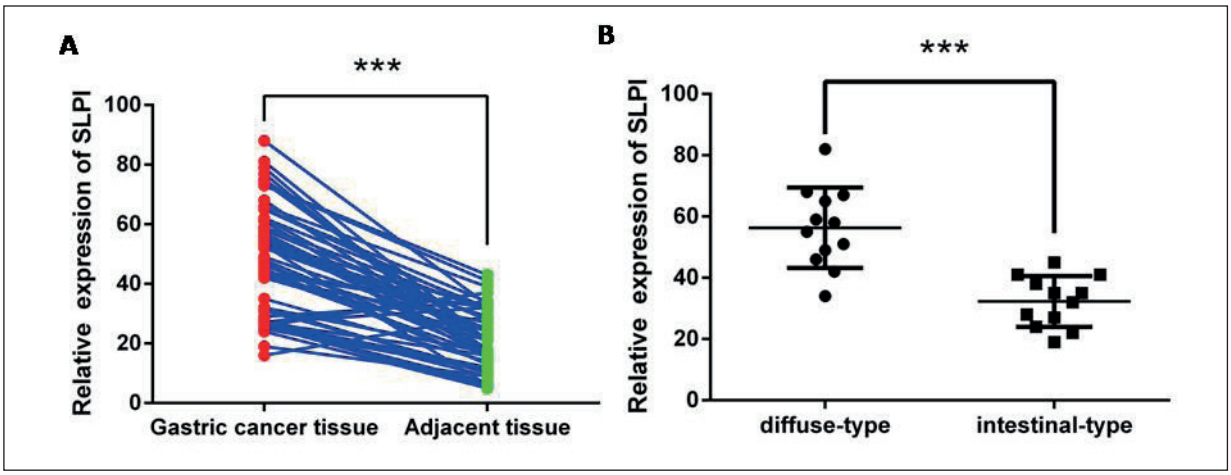

Figure 1. SLPI was highly expressed in the gastric cancer tissue. **A**, The expression of SLPI in the gastric cancer tissue and adjacent tissue was investigated by the qRT-PCR assay. ***p<0.001; **B**, The expression of SLPI in gastric cancer tissue was analyzed according to clinical subtype, ***p <0.001.

occurrence and progression of gastric cancer, but the mechanism remains unclear.

The Clinical Characteristic of SLPI

Based on the above results, we wanted to examine the clinical significance of SLPI. At first, we analyzed the correlation between the expression of SLPI and the survival time of patients with gastric cancer. It was found that the expression level of SLPI was negatively correlated with the survival time of patients with gastric cancer. We also found that expression level of SLPI showed a positive correlation with tumor size. These results suggested that the expression of SLPI might be significantly related to the proliferation of gastric cancer.

Suppression of SLPI Would Lead to Decreased Proliferation in Gastric Cancer Cell Lines

Based on results above, we found that SLPI was positively correlated with the size of gastric cancer so that we wanted to check whether SLPI could regulate the proliferation of gastric cancer cells. We detected the expression of SLPI in two gastric cell lines: MKN28, MKN45 and normal gastric mucosal cell line GES-1. Surprisingly, compared with GES-1, we found that SLPI was highly expressed in gastric cancer cell lines MKN28 and MKN45. Then, we inhibited the expression of SLPI in MKN28 and MKN45, and it was found that the suppression of SLPI expression could lead to the decrease of the proliferation of MKN28 and MKN45.

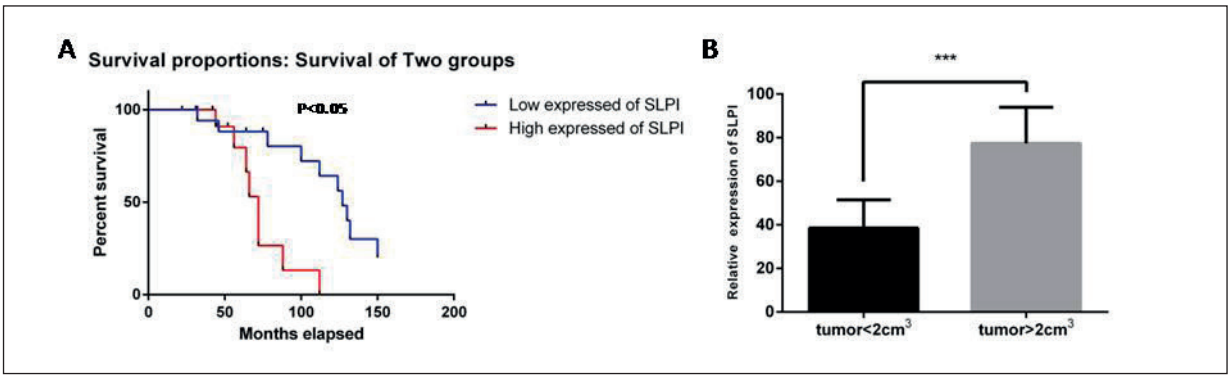

Figure 2. The clinical characteristic of SLPI. **A**, Association between patient survival time (log-rank test) and expression of SLPI. **B**, The expression of SLPI in gastric cancer tissue was detected according to the tumor size. ***p <0.001.

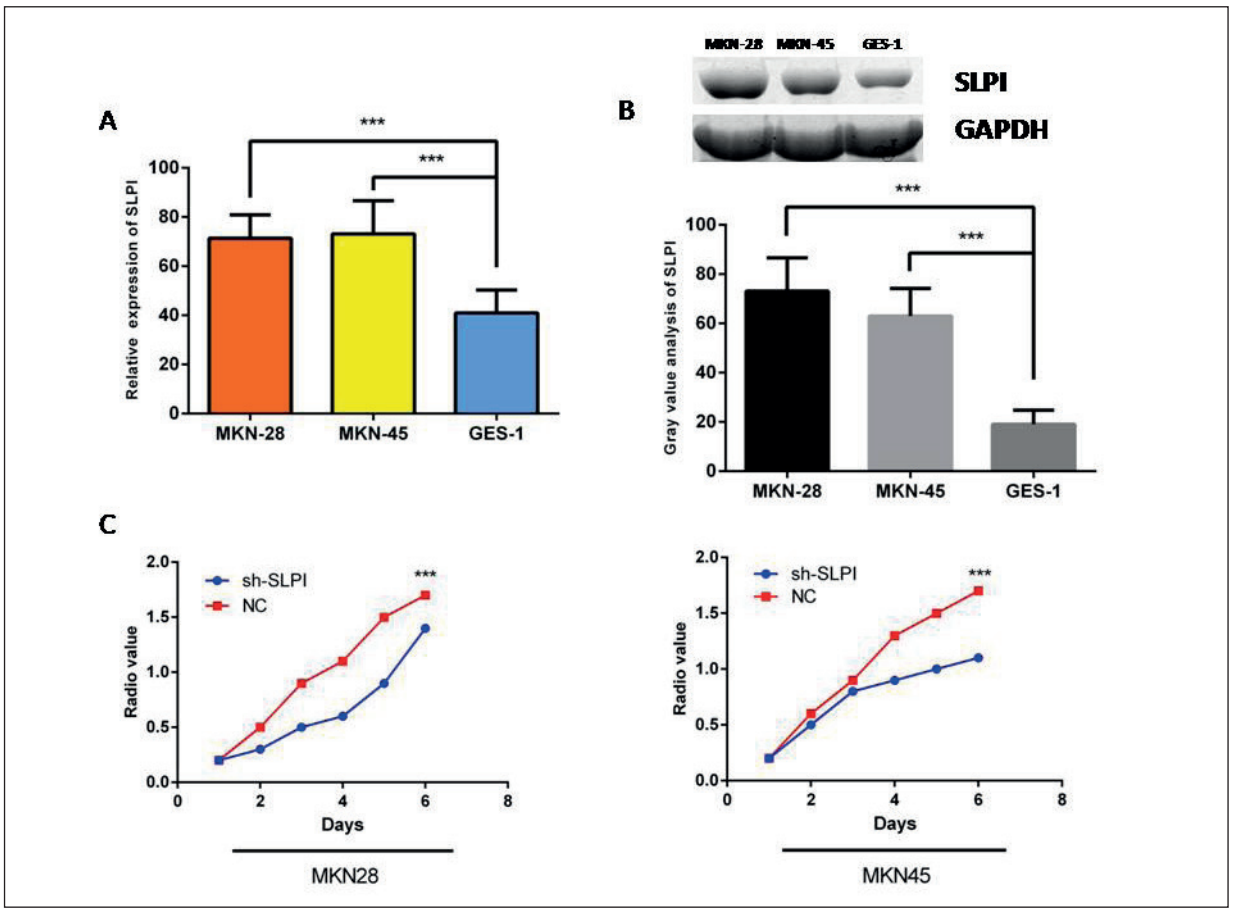

Figure 3. Suppression of SLPI would lead to the reduction of proliferation in gastric cancer cell lines. **A**, The expression of SLPI in the gastric cancer cell lines and GES-1 was detected by the qRT-PCR assay. ***p <0.001. **B**, The expression of SLPI in the gastric cancer cell lines and GES-1 was detected by Western blot. ***p <0.001. (C) CCK8 assays were used to detect the proliferation ability of gastric cancer cell after SLPI was suppressed. ***p <0.001.

SLPI Promotes the Proliferation and Invasion Ability of Gastric Cancer Cell Line Via Regulating the Expression of P53, Bcl-2, and Caspase-8.

As it was shown, SLPI was closely correlated with the proliferation of gastric cancer, and we would like to check whether SLPI could regulate the invasion ability of gastric cancer. So we detected the expression of SLPI and E-cadherin (E-cad) using Western blot, and we found that suppression of SLPI decreased the expression of E-cad in MKN28 and MKN45.Then, we wanted to clarify the mechanism of proliferation and invasion of gastric cancer. It has been reported that SLPI could affect the secretion of TNF-α in DCs, so we wanted to investigate whether SLPI was related to the apoptosis pathway. As is known, P53, Bcl-2, and Caspase-8 are key proteins in apoptosis pathway, so we detected whether SPLI could promote the ability of proliferation and invasion via regulating these three proteins. Interestingly, we found that the expression of P53 and Caspase-8 were increased and the expression of Bcl-2 and E-cad were decreased when the expression of SLPI was suppressed in MKN-28 and MKN-45. These results revealed that SLPI could change the biological characteristics of the tumor by regulating the apoptosis pathway of the cell.

Discussion

With the development of genetic engineering technology and molecular biology, treatment of cancer has advanced to the level of the gene. To meet the urgent need of clinical treatment, tumor-specific gene therapy with tumor-specific promoter are under the spotlight. Looking for a specific therapeutic target is a hot spot in

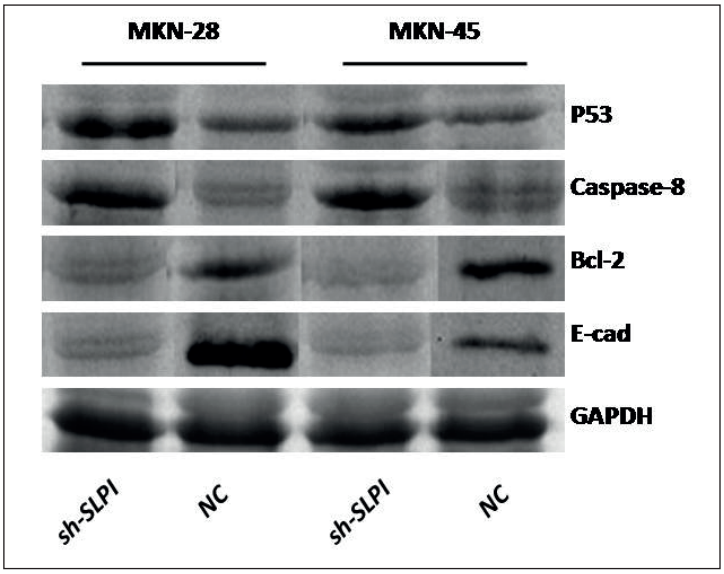

Figure 4. SLPI promotes the proliferation and invasion of the ability of gastric cancer cell line via regulating the expression of P53, Bcl-2, and caspase-8. The expression of P53 and Caspase-8 were increased and the expression of Bcl-2 and E-cad were decreased when the expression of SLPI was suppressed in MKN-28 and MKN-45.

cancer research at present. Secretory leukocyte protease inhibitor (SLPI) is in Kazal type serine protease inhibitor family, which has the ability to regulate cell differentiation and proliferation. Whether SLPI plays an important role in the occurrence and development of gastric cancer is the topic that our experiments wanted to clarify[7-10].

This research indicated that SLPI was highly expressed in gastric cancer compared with normal tissue, and its expression was even higher in the diffuse-type gastric cancer compared with the intestinal-type gastric cancer. Subsequently, we found that patients with higher SLPI expression had shorter survival time and larger size of the tumor. DNA level of SLPI in gastric cancer cells was obviously higher than normal gastric mucosal cells. SLPI played a key role in the development of gastric cancer. To verify whether SLPI was related to cell proliferation, we performed CCK8 experiments. The result showed that suppression of SLPI could lead to decreased proliferation of gastric cancer cells.

What's more, cell proliferation and apoptosis signaling pathways were closely related, so we wanted to know whether the expression of SLPI was related to tumor invasion and metastasis via regulating apoptosis signaling pathway. Using Western Blot, we found that the expression of P53 and Caspase-8 were increased and the expression of Bcl-2 and E-cad were decreased when expression of SLPI was suppressed in MKN-28 and MKN-45. These results showed that SLPI was a key factor which promoted tumor development and its expression was significantly related with cell apoptosis. As is known, the apoptosis signal pathway including extrinsic pathway, which initiates apoptosis mainly by promoter caspase-8, and intrinsic pathway, where mitochondria plays an important role to initiate apoptosis via activation of proapoptotic protein Bax, and anti-apoptotic protein Bcl-2 can inhibit the expression of Bax[11-14]. Furthermore, P53 is a tumor suppressor gene, and its biological function includes monitoring the integrity of DNA in the G period. In apoptosis pathway involving protein P53, P53 can specifically inhibit the expression of Bcl-2, thus significantly promote the expression of Bax. The accumulation and activation of protein P53 induce cell apoptosis[15]. As it was shown, suppression of SLPI could induce the changes of three key proteins, which increased the Bcl-2 level, but decreased P53 and Caspase-8 level.

These results showed that SLPI was a tumor promoting factor and provided a new target for treatment of gastric cancer. Moreover, because of the regulatory effect of SLPI on several key proteins in the apoptotic pathway, it has great potential to be fully explored.

Conclusions

In this research, we found that SLPI was highly expressed in the gastric cancer tissue. SLPI could regulate the expression of P53, Bcl-2 and Caspase-8 in apoptosis pathway to promote the proliferation and invasion ability of gastric cancer. In the future, SLPI may be a potential target for the treatment of gastric cancer.

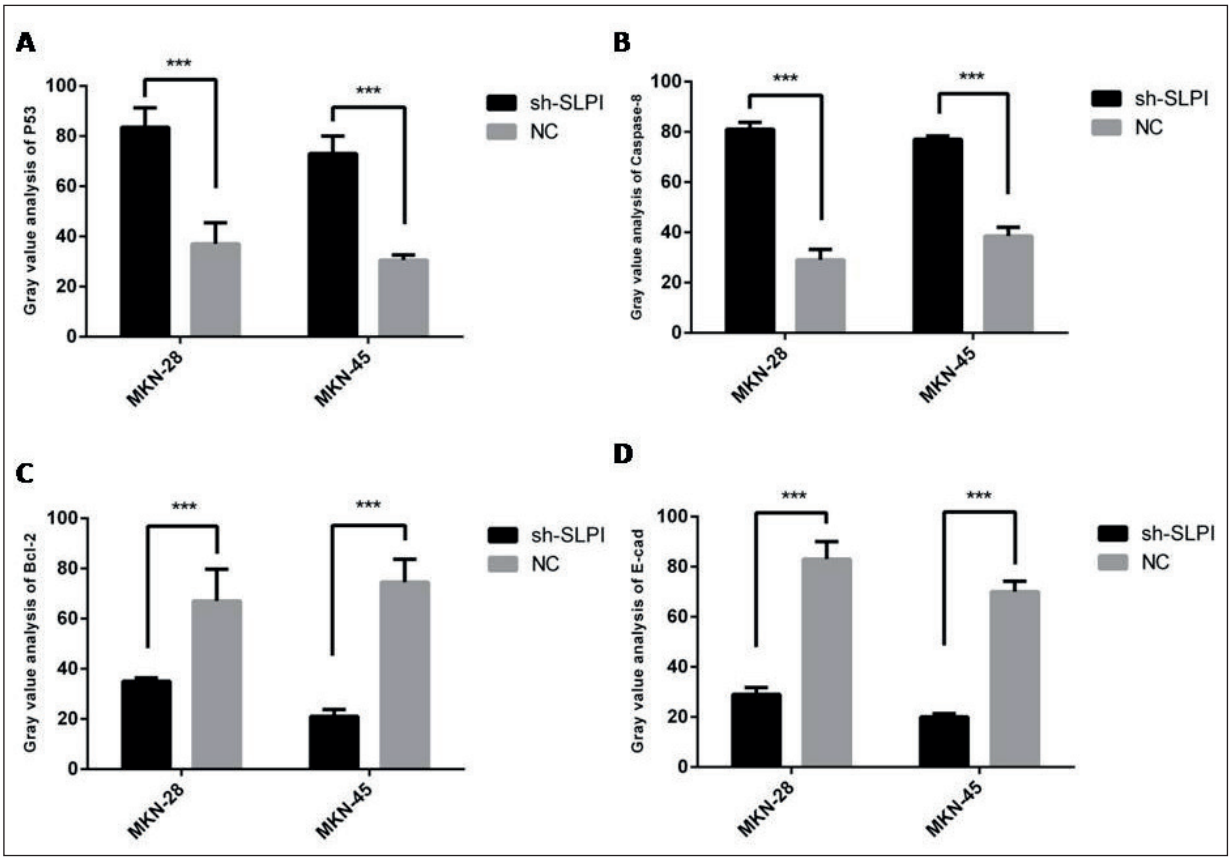

Figure 5. The expression of P53, Bcl-2, Caspase-8, and E-cad in gastric cancer cells was detected by Western blot, after the suppression of SLPI. ***$p < 0.001$; **A**, The gray value assay of P53 via Western blot, after the suppression of SLPI. ***$p < 0.001$; **B**, The gray value assay of Caspase-8 via Western blot, after the suppression of SLPI. ***$p < 0.001$; **C**, The gray value assay of Bcl-2 via Western blot, after the suppression of SLPI. ***$p < 0.001$; **D**, The gray value assay of E-cad via Western blot, after the suppression of SLPI. ***$p < 0.001$.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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