The role and significance of SVIL protein in the occurrence and development of gastric cancer

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Abstract

Aim: To discuss role and significance of SVIL protein in the occurrence and development of gastric cancer. Materials and methods: Using GSE84437 as research object and analysis by bioinformatics. In vitro study, after knockdown SVIL in MGC-803 and SGC-7901, observing cell biological activities including proliferation, apoptosis, invasion and migration by MTT, flow cytometry, transwell and wound healing assay. Results: By bioinformatics analysis, SVIL was closely correlation with gastric cancer, low expression group was significantly better compared with high expression groups in survival rate (P¡0.001); in cell experiment, with SVIL knockdown, MGC-803 and SGC-7901 cell proliferation were significantly depressed with apoptosis significantly increasing (P¡0.001), meanwhile, cells invasion and migration abilities were significantly inhibited with SVIL knockdown in MGC-803 and SGC-7901 cell lines (P¡0.001). Conclusion: SVIL overexpression might be closely correlated with gastric cancer development.

The role and significance of SVIL protein in gastric cancer occurrence and development

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Key words: SVIL; Gastric Cancer; MGC-803; SGC-7901

Running Title: SVIL and gastric cancer

Introduction

Gastric cancer is one of the common tumors of the digestive tract. Its incidence and mortality remain constantly high, ranking fifth and third among all tumors, respectively, with a gradual increasing trend in China as well [1]. Parthenogenesis of gastric cancer is a multifactorial process that can be attributed by dietary habits, *Helicobacter pylori* (HP) infection, genetics, etc.

Patients with early gastric cancer may have no obvious clinical symptoms, or even are asymptomatic. About >50% of patients experience metastasis upon diagnosis. Current therapeutic options for gastric cancer include chemotherapy, radiotherapy, targeted therapy, immunotherapy, etc. There is still an unsatisfactory 5-year survival rate of patients with local advanced and distant metastasis, which is less than 10%[2]. It highlights the significance of effective molecular marker screening in diagnosis and treatment of gastric cancer.

Supervillin (SVIL) is a myosin II- and Actin-binding peripheral membrane protein that is generally expressed in human tissues [3]. Besides regulating cell-substrate adhesion, cell polarization, cell diffusion and metastasis, SVIL also involves in cell division, extracellular matrix degradation, survival signal transduction, Integrin circulation and other functional activities [4]. SVIL has also been reported to participate in liver cancer occurrence and development [5, 6]. However, it is still unclear with respect to the expression of SVIL protein in gastric cancer tissue. In present study, relationships of SVIL gene expression with the prognosis and clinical pathological characteristics of gastric cancer were analyzed by sourcing gastric cancer data from the Gene Expression Omnibus (GEO) database. Meanwhile, via cell experiment with SVIL knockout, this study observed gastric cancer cells' biological activity, providing possible references for further research on SVIL mechanism.

Materials and Methods

Experimental materials

Human gastric cancer SGC-7901 and MGC-801 cells purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). A public gene expression profiling dataset of GSE84437, sourced from the GEO database of National Center for Biotechnology Information (NCBI) in United States (http://www.ncbi.nlm.nih.gov/geo), was collected for data analysis, including the gene expression profiles of 433 gastric cancer samples.

Bioinformatics analysis

GSE84437 was downloaded from the GEO database, with the expression of the control gene through the annotation package of the gene platform. Eligible data were obtained through deletion of missing data, background correction and log2 conversion for further analysis. Dividing as high- and low-levels based on median value of SVIL. Based on statistics of gender, age, T stage and N stage frequency of gastric cancer patients in each group, relationship between SVIL gene level and clinical symptoms calculated through Chi-squared test. Furthermore, by analyzing survival time and survival status of two groups, Kaplan-Meier (KM) survival analysis was used to compare the overall survival differences between groups. Univariate and multivariate Cox regression analyses were further employed to determine the impact of SVIL gene expression on patient survival prognosis. Differentially expressed genes (|Fold change|>1.4-fold, $P<0.05$) were screened in tissues of high- and low-expression groups, with corresponding heat map plotted at the same time. Furthermore, using the clusterProfiler package in Bioconductor, this study plotted the enrichment of differentially expressed genes on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, with bubble diagram generated simultaneously.

Cell culture and grouping

SGC-7901 and MGC-803 cells were cultured in DMEM medium containing 10% Fetal bovine serum in an incubator (37 , 5% CO_2), with the medium changed every 2 days. When cell confluence reached about 80% under the microscope, the cells were digested by trypsin, and then prepared into single-cell suspension

in DMEM medium for passage. The cells progressed four generations in logarithmic growth phase were randomly divided into Normal group, si-NC group, and si-SVIL group. Except for Normal group, si-NC group and si-SVIL group were transfected with si-negative control and si-SVIL, respectively (Keygentec, Nanjing, China). Further experiments were continued after 24 h of cell transfection.

Detection of SVIL gene expression in each group of cells using qRT-PCR

After centrifugation, cells were collected for the extraction of total RNA via the addition of Trizol. With the measurement of RNA concentration and purity, the extracted RNA were reversely transcribed into cDNA by using reverse transcription kit. Using cDNA as a template, PCR reaction was carried out under the following conditions: pre-denaturation at 95 for 5 min, denaturation at 95 for 30 s, annealing at 60 for 30 s, and elongation at 72 for 30 s, with 40 cycles in total. The expression of SVIL was calculated based on the 2 -T method, and the primer sequences are shown in Table 1.

Detection of cell survival rate using MTT assay

The transfected cells were collected and prepared into single-cell suspension at the density of 5 $\times 10^4$ /ml using DMEM medium, after which cells were inoculated on the 96-well plate (200 μl/well). After 24 h of culture in the incubator, 50 μl 5 mg/ml of MTT solution was added into each well for 4 h of further culture. Then, with the supernatant discarded, 150μl DMSO was added into each well and mixed well for 15 min. Finally, the absorbance (A) value was measured using Microplate Reader at a wavelength of 490nm, and the cell survival rate was calculated according to the formula of cell survival rate (%)=(A value of the experimental group/A value of the control group) ×100%.

Flow cytometry

Cells in logarithmic growth phase were washed and re-suspend with pre-cooled PBS solution, and then added with 70% ethanol pre-cooled in an ice bath to gently blow evenly, and fix overnight at 4 . The next step was centrifugation at 1,000 r/min for 5 min. With repeated washing with pre-cooled PBS, another centrifugation was performed to precipitate cells. After the removal of the supernatant, 0.5 mL of propidium iodide was added to fully shake and mix, followed by a warm bath at 37 for 30 min, and then placement at 4 . After filtration with 300-mesh nylon mesh, samples were loaded for cell apoptosis detection using flow cytometer.

Wound healing assay

After the digestion of cells in each group with trypsin, cells with adjusted concentration were inoculated into the 6-well plate. The optimal cell concentration should be 80%˜90% of growth on the second day. Sampling of each group was run in triplicate. Cells were cultured with 1640 medium at 37 in an incubator containing 5% CO_2 until a monolayer of cells was formed. Scratches were made using the vertical surface of a 100 μL pipette tip, forming a cell-free growth area. After washing with serum-free medium for three times, the scraped cells were scraped, and fresh serum-free medium were supplemented, with the addition of Mitomycin to inhibit cell division. The relative distance of the scratching area was observed and measured under an inverted microscope.

Transwell assay

The diluted Matrigel was placed into the Transwell insert to form membrane. An amount of 100 μL of diluted cell solution was inoculated into the upper chamber, and 500 μL of culture medium containing 10% fetal bovine serum was added to the lower chamber, both of which were cultured for 36 h in an incubator with 5% CO_2 at 37 . After that, the transwell insert was removed and the cells in the upper layer of the chamber was discarded, followed by cell fixation with 4% paraformaldehyde for 10 min, staining with 0.1% crystal violet, and drying in the air after washing with PBS solution. Under the light microscope, 4 high-power fields were selected randomly for cell counting and averaging.

Western blotting (WB) assay

Cells washed with pre-cooled PBS three times were added with RIPA lysate to lyse cells, and the supernatant

was collected by centrifugation. After protein concentration measurement using the BCA method, cells with adjusted protein concentration were added with loading buffer and boiled for denaturation. Then, 20 μl of protein sample was added to each well of concentrated gel, and electrophoresis at 80V was performed until the protein sample reached the separation gel, followed by electrophoresis at 120V until the protein sample reached the bottom of the separation gel. After that, wet transfer was carried out at a constant current of 0.3A for 2 h, and the PVDF membrane was taken out for sealing at room temperature for 1 h after washing three times with TBST. The primary antibodies of anti-SVIL (1:1,000) and anti-GAPDH (1:1,000) were added for incubation overnight at 4 , and the secondary antibody (1:5,000) was supplemented at room temperature for 1 h of incubation after another washing with TBST (×3). Following visualization using ECL, Image J was used to analyze the grayscale values of the bands. The relative expression level of the target protein was generated via dividing the grayscale value of the target protein bands by that of GAPDH bands.

Statistical analysis

SPSS 22.0 statistical software was used for data analysis of this study. The measurement data were all expressed in Mean±SD, and the comparison of multivariate measurements was conducted using one-way analysis of variance. Further pairwise comparison employed LSD-t-test, and the comparison of two samples applied paired t-test. $P<0.05$ meant that difference was statistically significant.

Results

The relationship between SVIL gene expression and clinical pathological parameters in gastric cancer patients

For analyzing SVIL gene expression of 433 gastric cancer patients' tissues, all samples were classified into high-expression group (n=216) and low-expression group (n=217) based on the median value of SVIL expression. Statistical analysis showed that the expression level of SVIL was associated with age (χ^2=5.580, P=0.018) and T staging (χ^2=19.800, P<0.001), but had none obvious relationship with gender (χ^2=0.805, P=0.370) and N staging (χ^2=6.500, P=0.090). There would be a higher expression level of SVIL gene in patients with increased T staging, as shown in Table 2.

The relationship between SVIL gene and survival prognosis of gastric cancer patients

Survival analysis using KM revealed a negative correlation between SVIL gene expression and patient survival in the dataset. In other words, the survival time of patients with high expression was significantly lower than that of patients with low expression ($P < 0.05$; Figure 1).

According to Cox regression analysis (Table 3), univariate analysis indicated that age, T stage, N stage, and SVIL gene expression levels were closely related to the overall survival of patients. While the results of multivariate analysis suggested that SVIL gene expression was an independent risk factor for prognosis in gastric cancer patients [hazard ratio (HR)=1.30, 95% confidence interval (CI)=1.11 ˜ 1.52, $P < 0.001$]. SVIL can be considered as a biomarker for predicting the overall survival of gastric cancer patients.

Screening of SVIL-associated differentially expressed genes

Statistical analysis was conducted on microarray data of 433 samples, with the screening of 823 differentially expressed genes, including 697 genes with high expression and 126 genes with low expression. The heat map of the top-20 differentially expressed genes in SVIL high-expression and low-expression groups was plotted in Figure 2. The expression of these genes may be related to the role of SVIL.

GO and KEGG pathway enrichment analyses

GO and KEGG pathway enrichment analyses were performed to further explore the potential mechanism of SVIL-associated genes regulating the occurrence and development of gastric cancer. Through GO analysis, SVIL and its associated genes showed an intimate association with extracellular structures and tissues, extracellular matrix (ECM), muscular system processes, cell-substrate adhesion, muscle contraction and other molecular mechanisms (Figure 3). Meanwhile, KEGG analysis (Figure 3) found that among 823 genes

related to SVIL, 17 signal pathways were mainly involved, including local adhesion, phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (Akt) signaling pathway, ECM receptor interaction, vascular smooth muscle contraction, protein digestion and absorption, etc.

The effect of SVIL knockout on cell proliferation and apoptosis

The results of MTT assay showed that compared with Normal group, si-SVIL group had significantly reduced cell proliferation rate of SGC-7901 and MGC-803 cells (P<0.001, Figure 4A&4B). Meanwhile, as indicated by the results of flow cytometry, the apoptosis rates of SGC-7901 and MGC-803 cells in si-SVIL group was significantly increased than those in Normal group (P<0.001, Figure 4C&4D).

The effect of SVIL knockout on cell invasion and migration

According to the results of Transwell assay, compared with Normal group, si-SVIL group had significantly reduced count of invasive cells of SGC-7901 and MGC-803 (P<0.001, Figure 5A&5B). Wound healing assay revealed that compared with Normal group, SGC-7901 and MGC-803 cells in si-SVIL group had significant decrease in scratch healing rates at 24 and 48 h, respectively (P<0.001 and P<0.001, Figure 6A&5B).

SVIL gene and protein expressions

The results of qRT PCR and western blot revealed that the gene and protein expressions of SVIL in SGC-7901 and MGC-803 cells of si-SVIL group were significantly reduced than those in Normal group (P<0.001, Figure 7A-6D).

Discussion

In occurrence, gastric cancer development, and prognosis, there is an involvement of the activation of numerous molecular signaling pathways, among which expressions of key molecules (HER-2, PDL1, etc.) can serve as markers for gastric cancer and molecular targets for targeted therapy of this cancer. It suggests the importance of searching for novel and more effective molecular markers. In our research, SVIL negatively correlated with prognosis of gastric cancer and was an independent prognostic indicator for this cancer. Further screening discovered 823 differentially expressed genes associated with SVIL. Through KEGG and GO analysis, these associated genes were involved in the tumorigenesis and development processes, such as extracellular structures and tissues, ECM, muscle contraction process, cell-substrate adhesion, etc. Furthermore, 6 core genes were screened through relevant software package, and validation in database revealed that ITGAV and LAMC1 were highly expressed in gastric cancer tissue, with statistically significant differences. Meanwhile, the expression levels of ITGA1, ITGAV, and LAMB1 were negatively correlated with the prognosis of gastric cancer. Collectively, findings in this study may provide reference for further research on the mechanism of SVIL.

SVIL has been confirmed to be involved in multiple physiological processes of tumors. As a peripheral membrane protein that regulates cell movement, SVIL can interact with myosin II, F-Actin and other proteins to transmit extracellular signals intracellularly, thereby promoting cell contraction and movement. For instance, in animal and cell experiments, Chen X et al.[7] discovered that a novel splicing variant of SVIL, Superllin (SV5), could promote the proliferation and migration of tumor cells. Other researchers also detected high expression of SVIL in liver cancer tissue. Under hypoxia, SVIL would be up-regulated, which would eventually promote the occurrence of liver cancer cells as well as cell migration and invasion owing to epithelial-mesenchymal transition (EMT) induced by activating extracellular signal regulated kinase (ERK)/p38 pathway in the downstream of ras homologous family member A (RhoA)/Rho-associated coiled-coil-forming protein kinase (ROCK) signaling pathway [6].

In this study, there was an intimate correlation between high expression of SVIL and poor prognosis in gastric cancer patients. The experiment of SVIL knockout in gastric cancer cells was performed for further verification. Consequently, the proliferation, invasion, and migration abilities of gastric cancer cells were significantly inhibited after knocking out SVIL. GO and KEGG enrichment analyses of SVIL-associated genes indicated that these associated genes were significantly enriched in extracellular structures and tissues,

ECM, muscle contraction process, cell-substrate adhesion and other pathways. It also offers a new insight into the study of SVIL in gastric cancer.

Additionally, the hub genes screened in this study might explain the role of SVIL to some extent. It has been reported that integrin was involved in the occurrence, metastasis, proliferation and angiogenesis of multiple tumors. Specifically, ITGAV was highly expressed in breast cancer and can be considered as a poor prognostic marker[8]. ITGA1 might be associated with the occurrence and heterogeneity of colorectal cancer[9]. Laminin is an important component of extracellular matrix, with different tissue distributions and functions concerning different tissue subtypes. Amogn them, LAMB1 and LAMC1 were respectively confirmed to be highly expressed in colorectal cancer and endometrial cancer, both of which were recognized to be prognostic factors and potential therapeutic targets[10,11]. Growth arrest specific gene protein 6 (GAX6) is a ligand of Tyro3/Axl/Mer receptor tyrosine kinase in the receptor tyrosine kinase subfamily. Relevant research supported that Gas6/Axl (Anexelekto)/zinc finger E-box binding homeobox 1 (ZEB1) signaling pathway might be involved in regulating EMT, invasion, and proliferation in gastric cancer cells, which may be a potential therapeutic target for gastric cancer treatment[12]. Besides, PENK had a low expression in bone tumor, and it could inhibit the metastasis of osteosarcoma cells through PI3K/Akt signaling pathway[13]. With respect to the above, these core genes may be critical in the functioning of SVIL.

In conclusion, this study was conducted by analyzing microarray data sourced from public platform, screening genes with statistical significance, and verifying through cell experiments. Findings in this study may contribute to the preliminary exploration of the feasibility of subsequent projects, predicting results, and providing corresponding theoretical evidence. This study suggests that early identification of patients with high SVIL expression may improve the survival of gastric cancer patients through early intervention or combined therapy. To sum up, SVIL and its associated proteins can serve as important targets for researching prognostic molecular markers in gastric cancer patients, providing new directions for future research.

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Figure legends

Figure 1. Curve of the relationship between SVIL gene expression level and survival time

Figure 2. Heat map of differentially expressed genes

Figure 3. Results of GO (A) and KEGG enrichment analysis of related genes (B)

Figure 4. The effect of SVIL knockout on cell proliferation and apoptosis

Normal: The cells were treated with normal; si-NC: The cells were transfected with si-negative control (NC); si-SVIL: The cells were transfected with si-SVIL

1. Cell proliferation rate in different MGC803 cell groups
2. Cell proliferation rate in different SGC-7901 cell groups
3. Apoptosis rate in different MGC-803 cell groups
4. Apoptosis rate in different SGC-7901 cell groups

***: P¡0.001, compared with Normal group

Figure 5. The effect of SVIL knockout on cell invasion and migration

Normal: The cells were treated with normal; si-NC: The cells were transfected with si-negative control (NC); si-SVIL: The cells were transfected with si-SVIL

Invasion MGC-803 cell number

Invasion SGC-7901 cell number

***: P¡0.001 compared with Normal

Figure 6. The effect of SVIL knockout on cell migration

Normal: The cells were treated with normal; si-NC: The cells were transfected with si-negative control (NC); si-SVIL: The cells were transfected with si-SVIL

Wound healing rate in different MGC-803 cell groups

Wound healing rate in different SGC-7901 cell groups

***: P¡0.001 compared with Normal

Figure 7. SVIL gene and protein expressions

Normal: The cells were treated with normal; si-NC: The cells were transfected with si-negative control (NC); si-SVIL: The cells were transfected with si-SVIL

1. SVIL gene expression in different MGC-803 cell groups
2. SVIL gene expression in different SGC-7901 cell groups
3. SVIL protein expression in different MGC-803 cell groups
4. SVIL protein expression in different SGC-7901 cell groups

***: P¡0.001 compared with Normal

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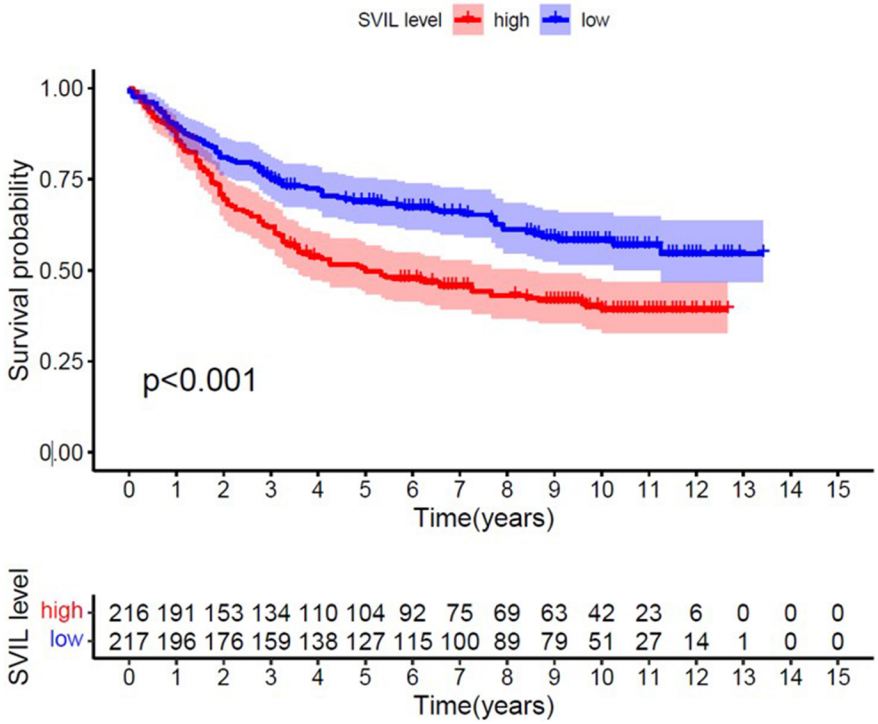

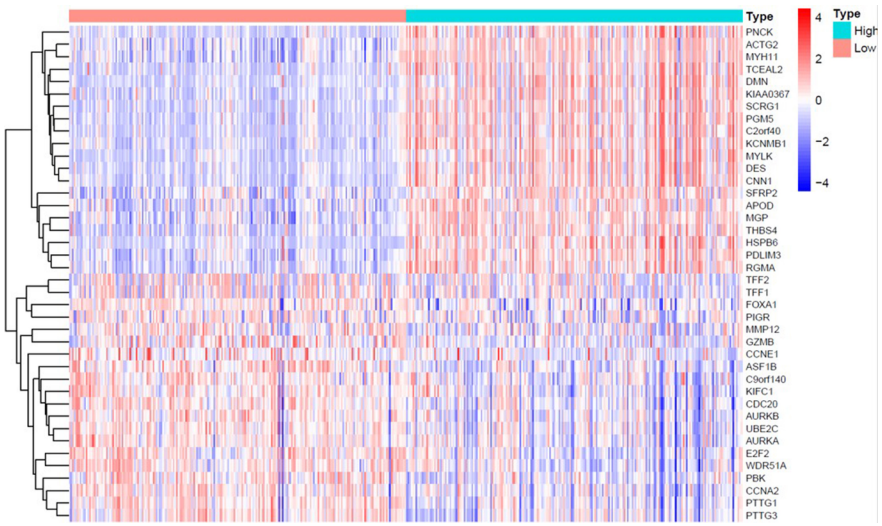

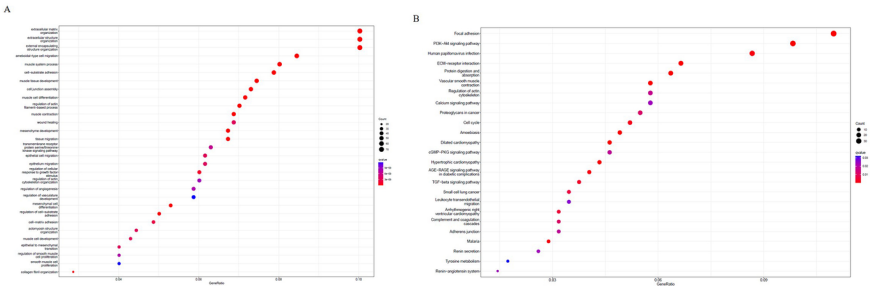

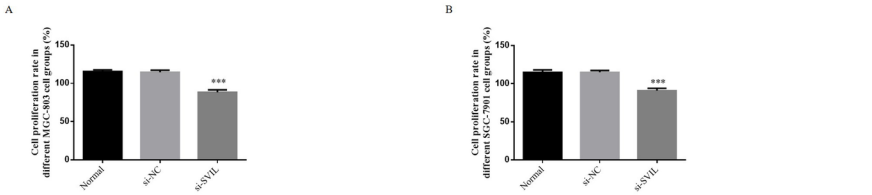

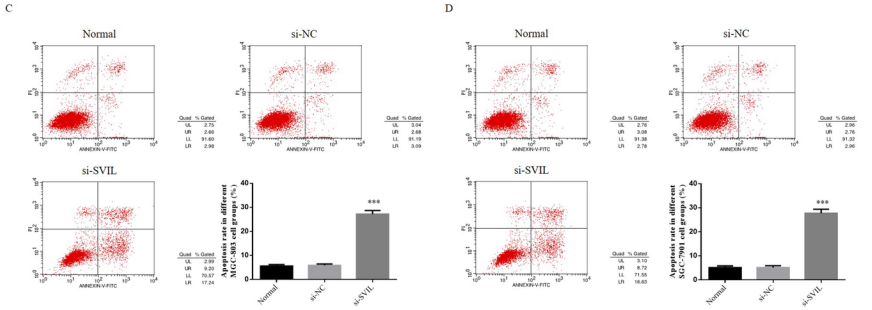

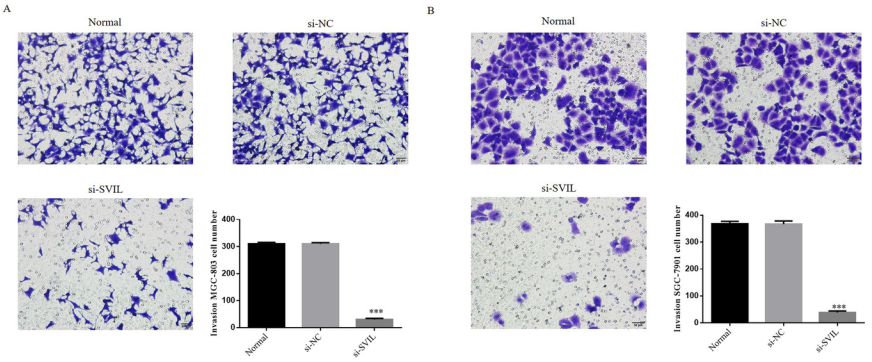

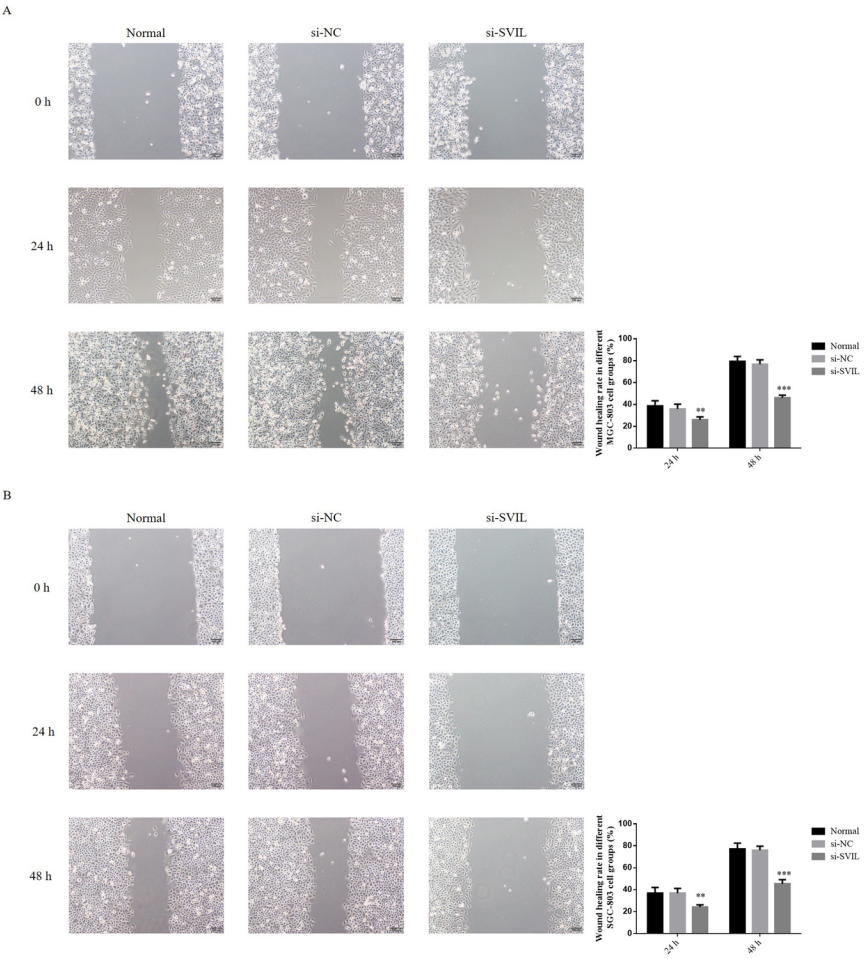

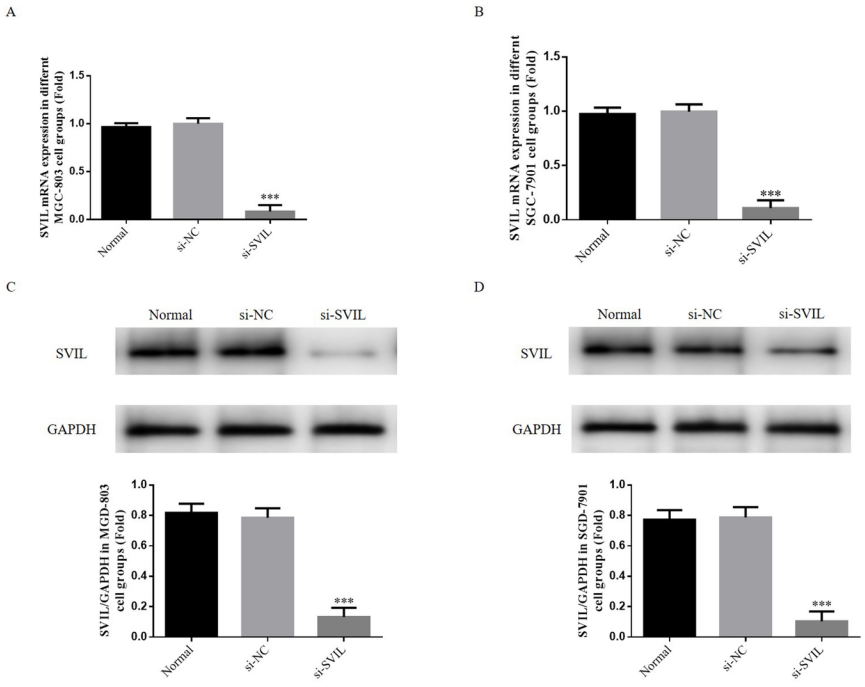

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