

MiR-29c Inhibits The Metastasis of Oral Squamous Cell Carcinoma And Promotes Its Cell Cycle Arrest By Targeting SERPINH1

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Research Article

Keywords: OSCC, SERPINH1, miR-29c, proliferation, metastasis

Posted Date: May 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-521428/v1>

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Abstract

Background: A large number of studies have shown that the imbalance of miRNA and its target genes can promote the development of tumors. The purpose of this study was to investigate the biological role and molecular mechanism of SERPINH1 and its upstream regulator miR-29c in oral squamous cell carcinoma (OSCC).

Material and methods: The expression levels of SERPINH1 and miR-29c were detected by RT-qPCR and Western blotting. The proliferation, apoptosis, metastasis and cell cycle were detected by MTT assay, wound healing assay, transwell assay, flow cytometry and dual luciferase reporter assay.

Results: SERPINH1 is highly expressed in patients with OSCC and can be used as a prognostic biomarker for OSCC. Cell function experiments showed that silencing the expression of SERPINH1 inhibited the proliferation and migration of OSCC cells and caused cell cycle arrest at S phase. Bioinformatics analysis showed that there is a binding site between miR-29c and SERPINH1, indicating that miR-29c may regulate the expression of SERPINH1. In addition, miR-29c overexpression inhibited the proliferation and metastasis of OSCC cells, and subsequent rescue experiment showed that SERPINH1 overexpression can reverse the inhibitory effect of miR-29c in OSCC cell proliferation, migration, apoptosis and cell cycle arrest.

Conclusions: MiR-29c can regulate the proliferation, migration, invasion and cell cycle of OSCC cells by targeting SERPINH1.

Background

In 2018, there are an estimated 800,000 new cases of head and neck squamous cell carcinoma (HNSCC) and more than 450,000 deaths. HNSCC originating from the mucosa of the upper respiratory tract can be classified by its location: laryngeal cancer, hypopharyngeal cancer, tonsil cancer, oropharyngeal cancer and oral cancer according to its location [1]. Among them, oral cancer is a major public health problem in the world, ranking sixth among human malignancies, with a 5-year mortality rate of about 50%. Oral malignancies include cancers that occur in the mouth, lips and pharynx, and oral squamous cell carcinoma (OSCC) accounts for over 90% of oral cancer. In 2018, OSCC accounted for 43.8% of new HNSCC cases and 37.8% of deaths [2]. Oral cancer is characterized by concealed onset, difficult diagnosis, rapid development, and is often accompanied by metastasis and destructive treatment [3]. Therefore, it is necessary to find and develop new accurate diagnosis and effective treatment strategies for OSCC.

Serpin peptidase inhibitor clade H member 1 (SERPINH1), also known as heat shock protein 47 (HSP47), belongs to the Serpin superfamily and is functionally involved in the proper folding and secretion of collagen. The unique characteristics of SERPINH1 in regulating collagen production and its location on the cell membrane of many cancers have led to the utilization of SERPINH1 as a potential biomarker or therapeutic target for many diseases and cancers [4]. The abnormal expression of SERPINH1 is closely

related to a variety of cancers. Increased expression of SERPINH1 can promote cancer progression and is associated with poor survival, such as hepatocellular carcinoma [5], lung cancer[6], renal cell carcinoma[7] and gastric cancer [8]. Comprehensive screening of proteomics and transcriptome showed that SERPINH1 is strongly correlated with the poor prognosis of renal clear cell carcinoma, and it can regulate the expression of epithelial-mesenchymal transition markers [9]. Our current TCGA analysis shows that SERPINH1 is abnormally expressed in OSCC, but its specific regulatory mechanism is still unclear.

In this study, we found that SERPINH1 may play a key role in the occurrence and development of OSCC. Silencing SERPINH1 can inhibit the growth and migration of OSCC cells, and SERPINH1 may be regulated by miR-29c. Therefore, exploring the molecular mechanism of SERPINH1 in OSCC will help us further understand the pathogenesis of OSCC.

Methods

Bioinformatics

The GSE31056 microarray dataset containing gene expression data of 23 OSCC tumors and their normal marginal tissues was downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). The differentially expressed mRNAs between normal group and tumor group in GSE31056 were obtained by R language analysis tool GEO2R. At the same time, public cancer transcriptome database UALCAN (<http://ualcan.path.uab.edu/index.html>) was used to examine the expression and patient survival information of SERPINH1, while TargetScan (http://www.targetscan.org/vert_72/) was used to identify the putative upstream miRNA binding sites. The survival analysis and clinical stage of the target mRNA and corresponding miRNAs were analyzed by LinkedOmic database (<http://www.linkedomics.org/login.php>).

Cell Culture

Human OSCC cell lines (TSCC1, CAL-27, HSC-4, SCC-4, SCC25) and human normal oral mucosal epithelial cells (HL-047) were purchased from Beijing Boehner Culture Center. All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma, USA) containing 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Science, Inc.), 100 µg/mL streptomycin (Gibco, Thermo Fisher Science, Inc.) and 100 µg/mL penicillin (Gibco, Thermo Fisher Science, Inc.) at 37°C in a humidified atmosphere of 5% CO₂.

Cellular lentivirus infection

The lentivirus infection of Mimic NC, miR-29c mimic, sh-NC and sh-SERPINH1, ov-NC and ov-SERPINH1 were obtained from Shanghai Genechem Company (Shanghai, China) and infected SCC25 cell lines according to the manufacturer's instructions.

RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Total RNAs were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) and RT-qPCR were performed using the PrimeScript RT reagent kit (Takara, Dalian, China) and SYBR Prime Script RT PCR kit (Takara, Dalian, China), respectively. RNU6-1 (U6) and GAPDH were used as internal control for miR-29c and SERPINH1, respectively. The results were calculated using the $2^{-\Delta\Delta C_t}$ method. The primer sequences used for RT-qPCR were listed in Table 1.

Table 1, Primer Sequences

Target Gene	Primer (5'-3')
miR-29c	F: 5'-GCAGTAGCACCATTTGAAATC-3'
	R: 5'-GGTCCAGTTTTTTTTTTTTTTTAACC-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3'
	R: 5'-AACGCTTCACGAATTTGCGT-3'
SERPINH1	F: 5'-CAGAAGTTTCTCGGGACGGG-3'
	R: 5'-GCCTGCCTTTTTCATTCTGGG-3'
GAPDH	F: 5'-GGAGCGAGATCCCTCCAAAAT-3'
	R: 5'-GGCTGTTGTCATACTTCTCATGG-3'

Western blotting analysis

Proteins for immunoblotting were resolved by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% skimmed milk and then incubated with SERPINH1 rabbit polyclonal antibody (ab109117, Abcam, Cambridge, UK; 1:2000 dilution) and GAPDH rabbit polyclonal antibody (#2118, Cell Signaling Technology, Boston, USA; 1: 5000) at room temperature. The membrane was incubated with horseradish peroxidase-conjugated sheep anti-rabbit IgG secondary antibody (ab6721, Abcam, Cambridge, UK; 1:2000 dilution) at room temperature for 2 hours, and then washed 3 times with PBST buffer (PBS contains 0.1% Tween 20) for 20 minutes. Chemiluminescent signal was visualized using a Clarity™ Western ECL Substrate (#170-5061, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and detected by Tanon 5200 full automatic chemiluminescence image analysis system (Tanon Science and Technology Co., Ltd., Shanghai, China).

Cell viability and proliferation assay

Cell viability was measured by the MTT assay. Forty-eight hours after transfection, cells were trypsinized and seeded onto the 96-well plate (5×10^3 cells per well). After 72 hours of incubation, 10 μ L of tetrazolium blue reagent (5 mg/mL) was added to each well and incubated for 4 hours at 37°C. After the supernatant was removed, 200 μ L of dimethyl sulfoxide was added, and the resulting colored solution was quantified by measuring the absorbance at the 490nm using a mini tablet reader (Bio-Rad Laboratories).

Cell migration assay

Cell migration ability was determined by wound healing assay and transwell assay. For wound healing assay, cells were seeded in six-well plates and transfected with Mimic NC, miR-29c mimic, sh-NC and sh-SERPINH1, ov-NC, or ov-SERPINH1. A linear scratch wound was created on cell monolayers by using a sterile 200 μ L pipette tip, and the scratched area was photographed at $\times 100$ magnification using a Leica DMI3000B computer-assisted microscope (Leica, Buffalo Grove, United States). After scratching, images were captured at 0, 24, 48, and 72 h and, the gap area was analyzed using Image-Pro Plus v6.0 image analysis software (Media Cybernetics, Rockville, MD, United States). Transwell assay was performed using Transwell chambers (Corning Inc., Corning, NY, USA) with a polycarbonate membrane. Cells (1×10^5 cells) were seeded onto upper transwell insert in serum-free DMEM, and the lower chamber contained 10% FBS. After incubating for about 10 h, the cells in the upper chambers were wiped off, and the cells in the lower chamber were further stained with crystal violet for 1 min at 25°C. The stained cells were observed under a light microscope (Nikon, 100 \times) and counted in 5 randomly selected fields.

Cell cycle analysis

Briefly, the analyzed cells were collected, trypsinized, washed with PBS and fixed with cold ethanol. The cells were subsequently stained with propidium iodide (PI, Sigma, StLouis, MO, USA) for 15 mins, and the percentage and proportion of cells in G0/G1, S and G2M phase were analyzed by flow cytometry (BeckmanCoulter, Brea, CA).

Dual-luciferase reporter assay

For dual-luciferase reporter assay, the 3-UTR of SERPINH1 containing wild-type or mutated putative miR-29c binding site was constructed. SCC25 cells were cultured in 24-well plates and then co-transfected with above luciferase reporter plasmids and miR-29c mimic (or mimic NC for control group) using Lipofetamine 2000 reagent (Invitrogen). After transfection for 48 h, the cell lysates were collected and the luciferase activity was detected by dual luciferase reporter gene detection system (Promega).

Statistical Analysis

All statistical Data were analyzed using SPSS 20.0 software. All data were presented as mean \pm standard deviation (SD). The difference between groups was analyzed by t-test. Pearson χ^2 test was used to analyze the relationship between expression of miR-29c and SERPINH1. All experiments in the study were independently repeated three times. A $P < 0.05$ or $P < 0.01$ was considered statistically significant.

Results

The high expression of SERPINH1 in OSCC is related to poor prognosis

As shown in Fig. 1A-B, the gene expression in each sample was symmetrically distributed with other samples. The median-centered values in all samples are indicative, thus the samples are normalized and cross-comparable. In other words, subsequent bioinformatics analysis can be continued, and the data is

valuable for further screening. Through the analysis of GEO2R in the GSE31056 dataset, we found that SERPINH1 was highly expressed in tumor tissues (Fig. 1C). In addition, the analysis of the UALCAN database further showed that SERPINH1 was highly expressed in OSCC patients (Fig. 1D), and patients with high SERPINH1 expression were significantly associated with poor survival (Fig. 1E). Combined with the clinical data of the patients in UALCAN database, there were significant differences between individual cancer (Fig. 1F), tumor grade (Fig. 1G) and nodal metastasis status (Fig. 1H) of OSCC, and the expression level of SERPINH1 increased with the increase of tumor stages. Furthermore, the expression of SERPINH1 in the OSCC cell line was significantly higher than that in the human normal oral mucosal epithelial cell line HL-047 (Fig. 1I and 1J). Taken together, SERPINH1 may play an oncogene role in OSCC, and its high expression is associated with poor prognosis.

Down-regulation of SERPINH1 inhibited the proliferation and migration and promoted cell cycle arrest and apoptosis in OSCC cell line

The mRNA and protein levels of SERPINH1 in SCC25 cells transfected with sh-SERPINH1 were significantly decreased (Fig. 2A and B). The results of MTT assay showed that after inhibiting the expression of SERPINH1, the cell viability of SCC25 cells decreased significantly (Fig. 2C). The results of wound-healing assay and Transwell assay showed that the decreased expression of SERPINH1 reduced the migration ability of SCC25 cells (Fig. 2D and E). The results of DAPI staining further showed that down-regulation of SERPINH1 expression reduced cell proliferation and promote apoptosis (Fig. 2F). The results of cell cycle analysis showed that the proportion of G0/G1 phase cells in sh-SERPINH1 group was significantly lower than that in control group, while the proportion of S phase cells was significantly higher than that in the sh-SERPINH1 group (Fig. 2G). These results indicate that the reduced SERPINH1 expression inhibited the proliferation of OSCC tumor cells.

SERPINH1 as the downstream target gene of miR-29c

In order to understand the underlying regulatory mechanism of SERPINH1 in OSCC, we excavated and analyzed its upstream regulatory factors. TargetScan database was used to analyze the putative binding microRNAs on SERPINH1 and identified that miR-29c may act as the potential regulator of SERPINH1 (Fig. 3A). LinkedOmic database analysis of miR-29c showed that there were differences among different clinicopathological grades (Fig. 3B), different individual cancer stages (Fig. 3C) and lymph node metastatic status stages (Fig. 3D). In addition, LinkedOmic database survival analysis showed that patients with high miR-29c expression were associated with better survival outcome than patients with low miR-29c expression (Fig. 3E). Pearson correlation analysis further showed that there was a negative correlation between miR-29c and SERPINH1 (Fig. 3F). In addition, the expression of miR-29c in OSCC cell line was significantly lower than that in HL-047 cell line (Fig. 3G), while the expression of SERPINH1 in these two cell lines was opposite. In order to validate the targeted regulation relationship between miR-29c and SERPINH1, we first examined the mRNA and protein expression of SERPINH1 in miR-29c mimic-transfected OSCC cells. The results showed that when, the expression of SERPINH1 significantly down-regulated in miR-29c overexpressed cells (Fig. 3H and I). The results of dual luciferase reporter assay

showed that miR-29c exerted a significant inhibitory effect on luciferase activity of the SERPINH1-WT group (Fig. 3J). Taken together, these results indicate that SERPINH1 is the downstream target of miR-29c and is negatively regulated by miR-29c in OSCC.

Overexpression of miR-29c inhibits the proliferation and migration and promote cell cycle arrest and apoptosis in OSCC cells, and promotes apoptosis

Since miR-29c can regulate the expression of SERPINH1, we further studied the effect of miR-29c overexpression on cell function. The transfection efficiency of miR-29c was shown in Fig. 4A-C. The results of MTT assay (Fig. 4D), wound healing assay (Fig. 4E), transwell assay (Fig. 4F) and DAPI staining (Fig. 4G) showed that miR-29c overexpression significantly inhibited the proliferation and migration of OSCC cells and promote apoptosis. Moreover, the overexpression of SERPINH1 significantly reduced the inhibitory effects of miR-29c on cell migration, invasion and apoptosis. In addition, overexpression of miR-29c caused cell cycle arrest at S phase, which may be the cause of the inhibition of OSCC cell proliferation (Fig. 4H). In summary, these results indicate that miR-29c overexpression can inhibit the cellular function of OSCC, and SERPINH1 overexpression can reverse the inhibitory effect of miR-29c.

Discussion

In the present study, we found that the expression of SERPINH1 was significantly up-regulated in OSCC. Cell biology experiments showed that silencing SERPINH1 expression inhibited the proliferation and metastasis of OSCC cells. Mechanism studies further showed that miR-29c can regulate the expression of SERPINH1. In addition, bioinformatics analysis of GSE31056 dataset revealed that the expression of SERPINH1 expression were significantly upregulated in OSCC tumor tissues. At the same time, this study also confirmed the increased mRNA and protein expression of SERPINH1 in OSCC cell lines.

Another bioinformatics analysis supported our finding that the expression of SERPINH1 significantly increased in head and neck squamous cell carcinoma (HNSC) and associated with poor overall survival rate (OS), indicating that SERPINH1 is a potential clinical target and prognostic marker of HNSC [10]. Genome-wide analysis of alternative splicing revealed the specific expression of SERPINH1 in human hepatocellular carcinoma [11], and another study also showed that the expression of SERPINH1 is related to the development and prognosis of in pancreatic ductal adenocarcinoma [12]. Therefore, SERPINH1 may play an important role in the OSCC tumorigenesis and development. In this study, UALCAN database was used to examine the association between the expression of SERPINH1 and the survival and clinical staging of OSCC patients. We found that high SERPINH1 expression is significantly associated with poor survival. In addition, the expression of SERPINH1 differs significantly in different individual cancer stages, different clinicopathological grades, and different lymph node metastasis stages [13, 14]. Thus, we recommend using SERPINH1 as a prognostic marker for survival and early diagnosis of OSCC. In addition, we also verified the effect of SERPINH1 on cell function by silencing the expression of SERPINH1 in SCC25 cells, and found that silencing SERPINH1 not only significantly inhibited cell

proliferation and migration, but also significantly increased the proportion of cells in S phase. Thus, our study further clarified the crucial function of SERPINH1 in OSCC.

It is known that miRNAs plays an important role in biological processes, such as cell migration, differentiation, and apoptosis as well as chemoresistance of tumor cells [15]. It can directly bind to the target gene mRNA to cause translational inhibition or post-transcriptional degradation of targeted mRNA [16]. In order to further explore the upstream regulation mechanism of SERPINH1, we analyzed the TargetScan and LinkedOmic databases through bioinformatics, and found that miR-29c may be putative binding site located on the 3'-UTR of SERPINH1 mRNA. A series of experimental analysis showed that miR-29c can specifically down-regulate the expression of SERPINH1 in OSCC cells, suggesting that miR-29c may act as a tumor suppressor to regulate the tumorigenicity of OSCC [17]. A study by Lopes et al. showed that compared to non-cancerous tissues of healthy volunteers, miR-29c is significantly expressed in oral cancer tissues and adjacent normal tissues [18]. The miR-29c-KIAA1199 axis regulates gastric cancer migration by binding with WBP11 and PTP4A3 [19]. In addition, miR-29c can inhibit the expression of FBXO31 by directly binding to the 3'UTR of FBXO31, resulting in downstream activation of p38 MAPK and subsequent 5-FU chemoresistance of esophageal squamous cell carcinoma (ESCC) [20]. Up-regulation of long non-coding RNA TUG1 can inhibit miR-29c and subsequently promotes the proliferation, migration and invasion of bladder cancer cells [21]. In breast cancer cells, miR-29c acts as a tumor suppressor miRNAs by targeting TIMP3/STAT1/FoxO1 signaling pathway [22]. In order to further verify whether miR-29c also plays a tumor suppressor in the tumorigenesis of OSCC, we overexpressed miR-29c in OSCC cell lines, and found that miR-29c overexpression significantly inhibited the proliferation and metastasis of OSCC cells. Moreover, when SERPINH1 was further overexpressed, the tumor suppressor effect of miR-29c was reversed, which is consistent with the role of miR-29c in other tumors. It is suggested that miR-29c may play an important role in tumor treatment. In the future, miR-29c/SERPINH1 axis may be used as the therapeutic target for OSCC to improve the therapeutic effect and prognosis of OSCC.

Conclusion

SERPINH1 plays an important role in the proliferation and migration of cancer cells. This study explored the mechanism by which miR-29c regulates the expression of SERPINH1 in the malignant process of OSCC. SERPINH1 can not only be used as a marker for OSCC diagnosis, but also can be used as therapeutic target to improve survival of patients with OSCC. This may be a new direction for our future research. In conclusion, this study provides a new theoretical basis for molecular targeted therapy of OSCC in the future.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets analysed in the study GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), public cancer transcriptome database UALCAN(<http://ualcan.path.uab.edu/index.html>), TargetScan (http://www.targetscan.org/vert_72/), LinkedOmic database (<http://www.linkedomics.org/login.php>) .

Competing interests

The authors declare that there are no conflicts of interest.

Funding

This study was supported by the grants of Liaoning Province Key Scientific and Technological Project (No. 20180550035).

Authors' Contributions

Chuanning Wang, Xiaoping Lin: data curation; formal analysis; Writing-original draft. Zhiming Wang, Liping Zhang: methodology; project administration; resources; writing-review and editing.

Acknowledgments

Not applicable

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Figures

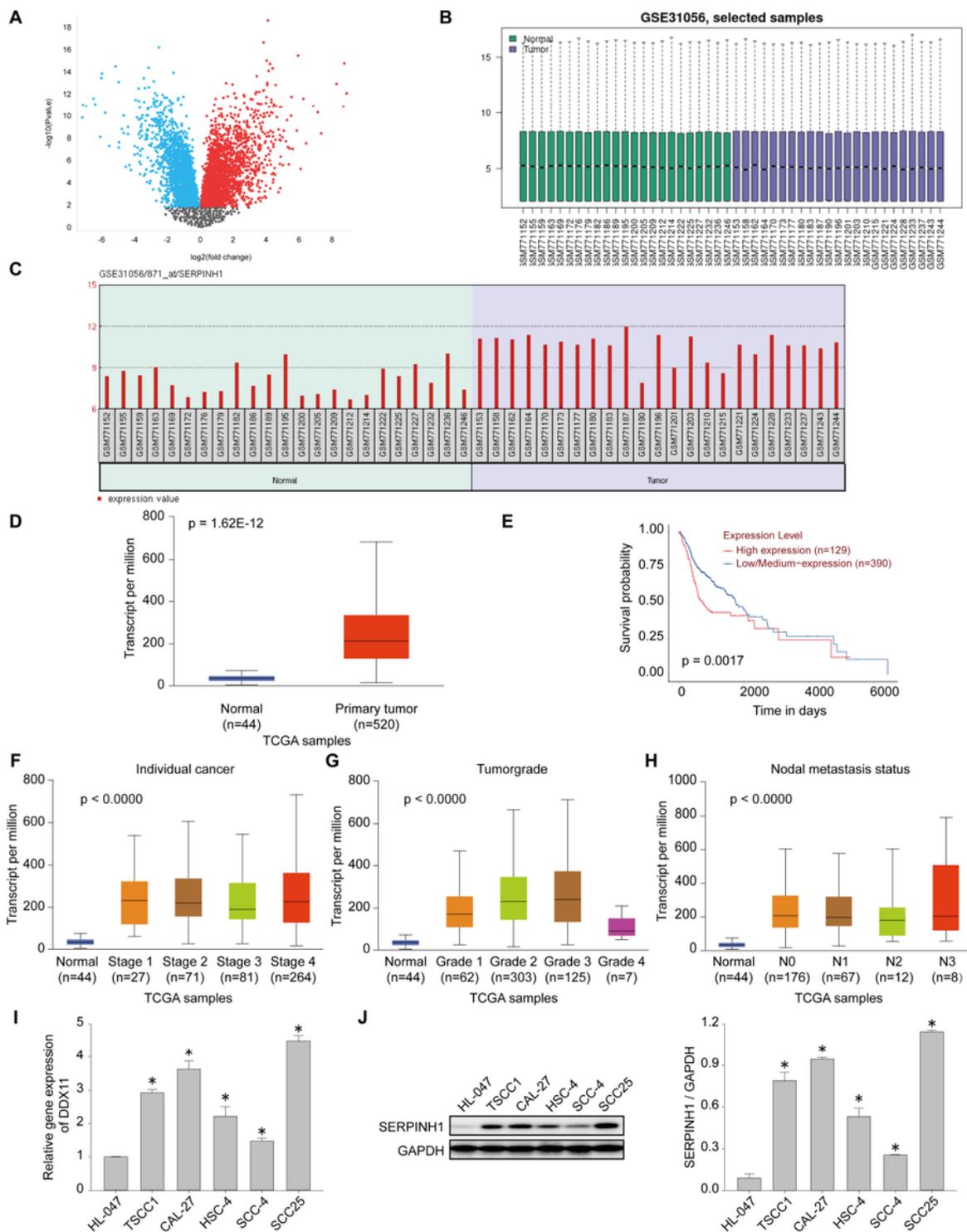


Figure 1

Expression profile and prognosis of SERPINH1 in OSCC. A The volcano map of GSE31056 dataset of differentially expressed genes between normal and tumor tissues. B The box diagram of the distribution of the values of the 23 para-cancerous tissues and 23 OSCC tumor tissues. C-D GEO database. The low expression of SERPINH1 in normal tissues (blue) and the high expression of SERPINH1 in tumor tissues (red). E Survival probability of SERPINH1 expression based on UALCAN database. Red line indicates

patients with high expression of SERPINH1, and blue line indicates patients with low expression of SERPINH1. F-H Box map shows the expression of SERPINH1 in different individual cancer stages, clinicopathological grades and lymph node metastatic status stages of OSCC. I-J SERPINH1 mRNA and protein levels in HL-047 and OSCC cell lines (TSCC1, CAL-27, HSC-4, SCC-4, SCC25). *P < 0.05 vs. HL-047.

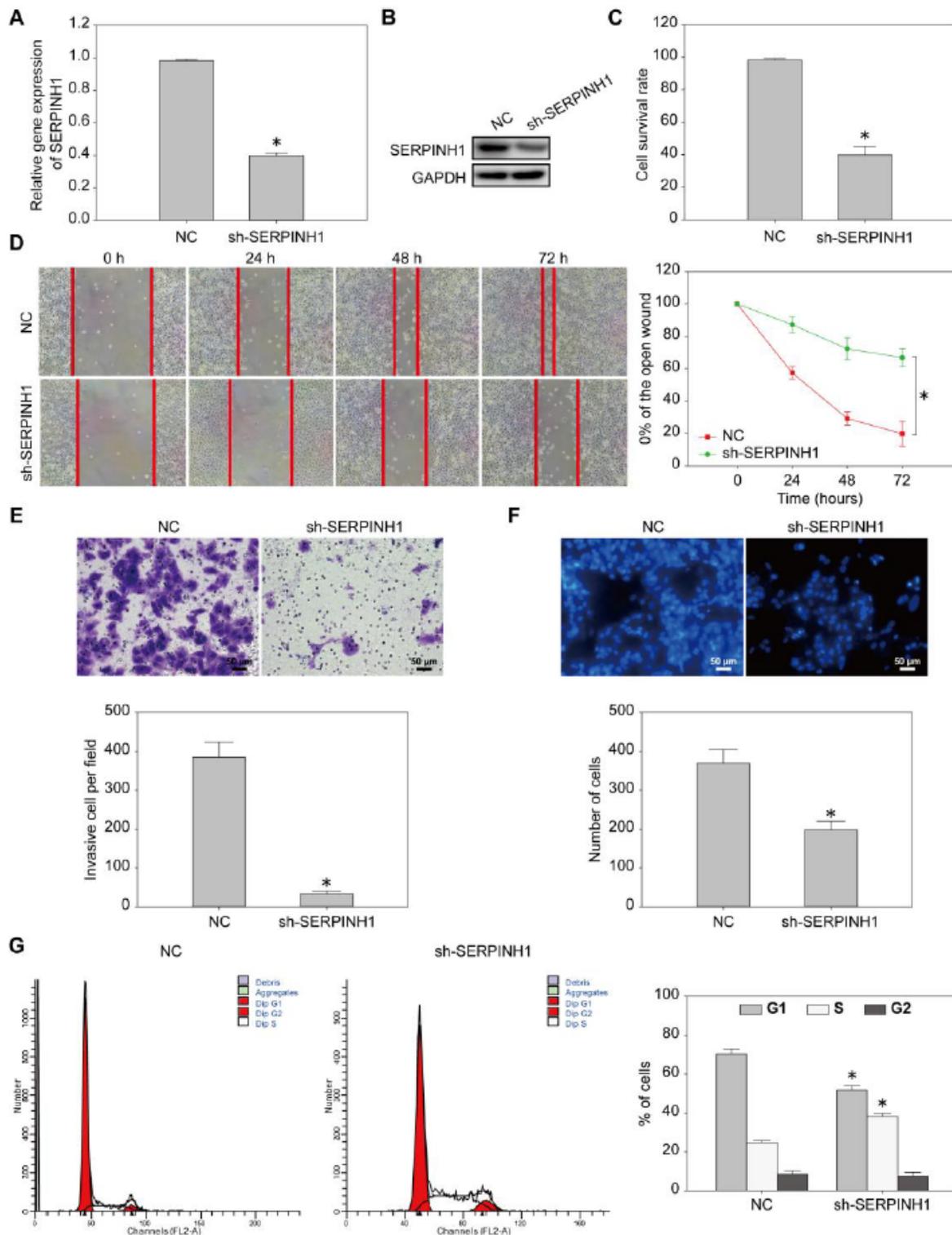


Figure 2

The low expression of SERPINH1 inhibits the proliferation and migration of OSCC cells and promotes cell cycle arrest. A-B SERPINH1 mRNA and protein levels in OSCC cells before and after silencing the expression of SERPINH1. C MTT colorimetric assay of the cell viability of OSCC cells after silencing the expression of SERPINH1 for 72 h. D Wound-healing assay and E Transwell assay were used to measure the migration ability of OSCC cells. F DAPI staining was used to examine the apoptosis of OSCC cells. G Flow cytometry was used to analyze the cell cycle state of OSCC cells. *P <0.05 vs. NC.

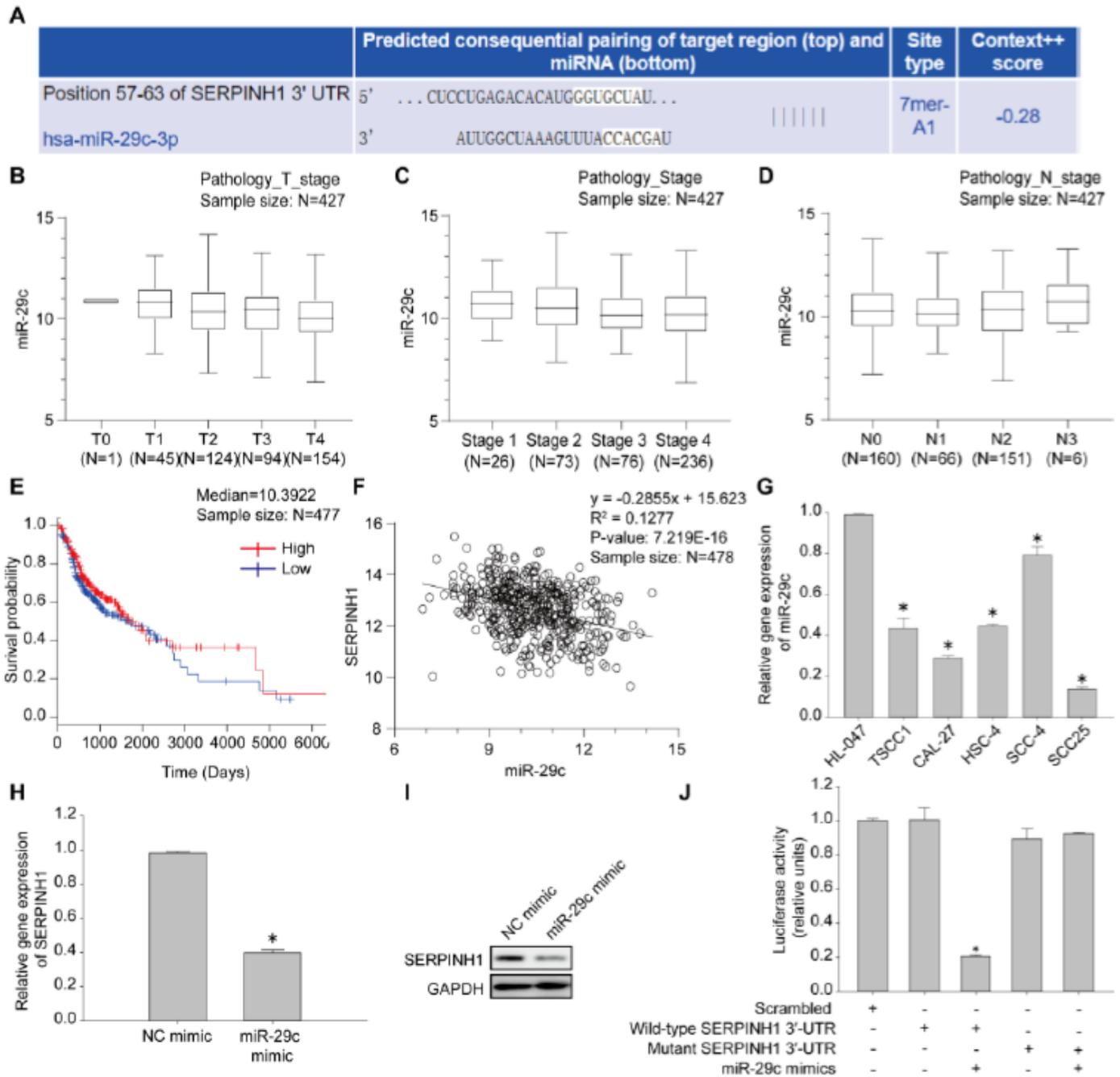


Figure 3

Overexpression of miR-29c down-regulates the expression of SERPINH1. A TargetScan analysis predicted the putative miR-29c binding sites on the 3'-UTR of SERPINH1. Results of LinkedOmic database analysis

of the expression profile of miR-29c in different clinicopathological grades B, individual cancer stages C, and lymph node metastasis stages D. E Survival probability of miR-29c expression in 477 patients with SCC. Red line denotes patients the high miR-29c expression, while blue line represents patients with low miR-29c expression. F Pearson correlation analysis of the relationship between SERPINH1 expression and miR-29c expression. G Relative miR-29c expression in HL-047, TSCC1, CAL-27, HSC-4, SCC-4, and SCC25 cells. H Overexpression of miR-29c suppressed the mRNA level of SERPINH1. I Overexpression of miR-29c suppressed the protein level of SERPINH1. J Dual luciferase reporter assay was used to determine the targeted binding of miR-29c and SERPINH1. Differences were found to be statistically significant at *P <0.05.

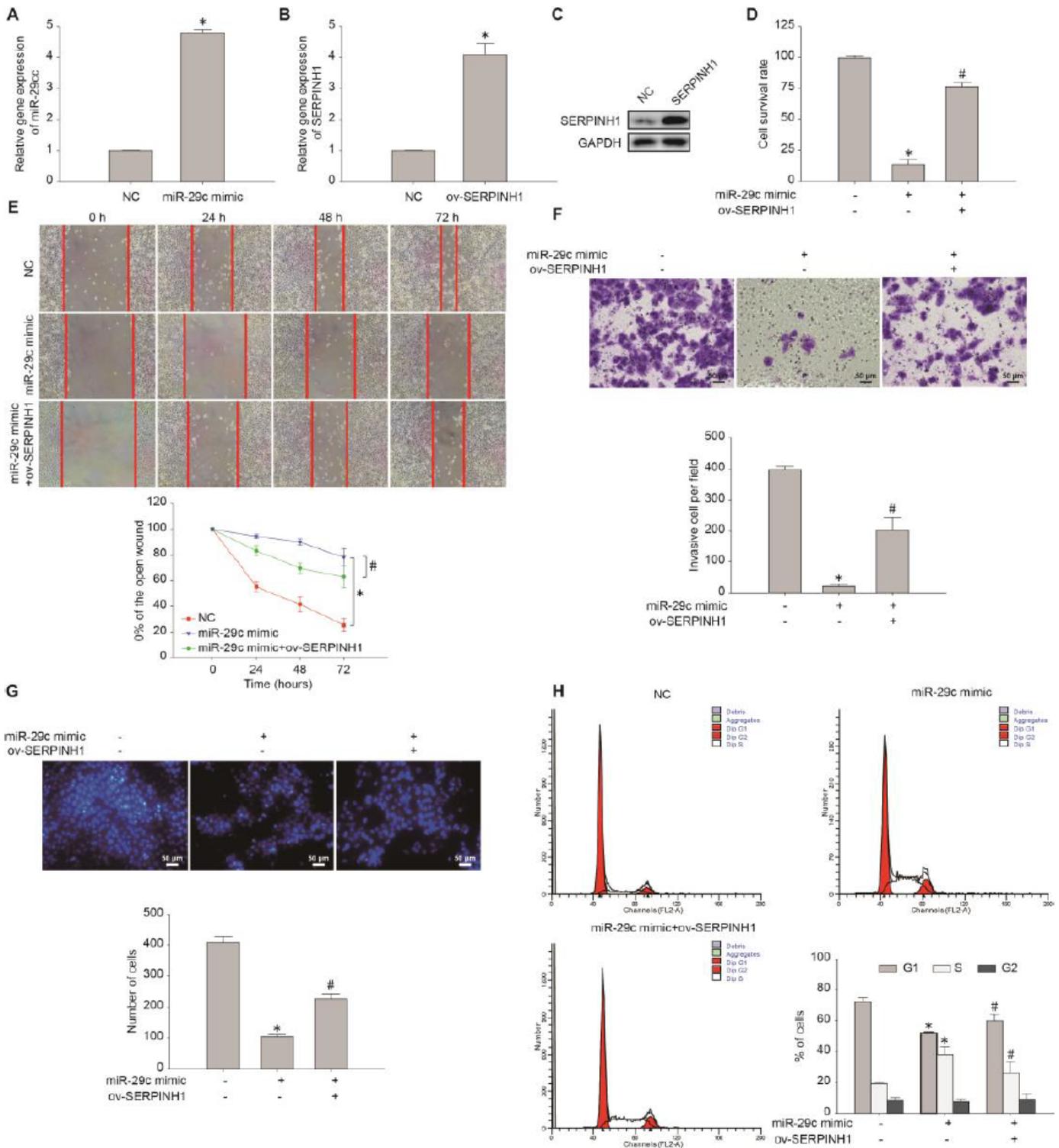


Figure 4

SERPINH1 overexpression reversed the inhibitory effects of miR-29c on OSCC proliferation, migration, apoptosis and cell cycle arrest. A Relative expression level of miR-29c after transfection of miR-29c mimic. B-C Relative mRNA and protein levels of SERPINH1 in OSCC cells after SERPINH1 overexpression. D The viability of OSCC cells at 72 h was detected by MTT colorimetric assay. E-F The migration and invasion ability of OSCC cells was measured by wound healing assay and transwell assay, respectively. G

The apoptosis of OSCC cells was detected by DAPI staining. H The proportion of cells in G1, S and G2 phases was analyzed by flow cytometry. *P <0.05,vs. NC; #P <0.05, vs. miR-29c mimic.