

Original Article

Vesicle-mediated transport-related gene SEC23A promotes cell proliferation by regulating cell cycle leading to gastric cancer progression

Kang Wang^{1,2,†}, Shihao Rao^{1,2,†}, Xujin Wei^{2,3}, Wen Xie^{1,2}, Zhijun Hong^{1,2}, Jia Cheng^{1,2}, Xin Chen^{2,3}, Jingjing Hou^{1,2,*}, and Huiqin Zhuo^{1,2,*}

¹Department of Gastrointestinal Surgery, Zhongshan Hospital of Xiamen University, School of Medicine, Xiamen University, Xiamen 361000, China, ²Xiamen Municipal Key Laboratory of Gastrointestinal Oncology, Xiamen 361000, China, and ³The Graduate School of Fujian Medical University, Fuzhou 350000, China

[†]These authors contributed equally to this work.

*Correspondence address: Tel: +86-13656035343; E-mail: jihou@xmu.edu.cn (J.H.) / Tel: +86-13696923094; E-mail: zhuohuiqin@xmu.edu.cn (H.Z.)

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Abstract

Gastric cancer (GC) is a highly prevalent and lethal gastrointestinal cancer. Dysregulation of vesicle-mediated transport-related genes (VMTRGs) is closely associated with tumorigenesis and disease progression. However, the prognostic value of VMTRGs in GC remains unclear. In this study, on the basis of our proteomics data and public databases, we identify differentially expressed VMTRGs in infiltrative-type GC with more metastases and recurrences identified by Ming's classification. Least absolute shrinkage and selection operator (LASSO) regression identifies 3 VMTRGs (*SEC23A*, *RAB31*, and *GABARAPL2*) from 41 infiltrative-associated VMTRGs, based on which a risk model Vesicle-Infiltrative Lasso System (VILS) is constructed, and its effectiveness and potential importance are validated by immune microenvironment analysis and functional enrichment analysis. As an independent prognostic factor for GC, VILS, combined with other clinically independent prognostic factors to form a nomogram, is effective in predicting GC prognosis. The VILS high-risk group has higher M2 macrophage and cancer-associated fibroblast infiltration, and lower infiltration of Th1 cells and natural killer cells. *SEC23A* is highly expressed in GC tissues and cells. The importance of *SEC23A* in GC cells is evaluated by *in vitro* assays including colony formation assay and CCK-8 assay, and by *in vivo* assay using a subcutaneous xenograft mouse model. The results show that *SEC23A* promotes GC cell proliferation and tumor growth through regulation of the cell cycle *in vitro* and *in vivo*. VILS provides excellent prognostic prediction for GC patients and is correlated with antitumor immune cell infiltration. *SEC23A*, the dominant gene of VILS, is highly expressed in GC and promotes GC growth and malignant progression through various molecular mechanisms. Our study reveals the effect of *SEC23A* on the proliferation of gastric cancer cells for the first time. Therefore, *SEC23A* has the potential to be a new therapeutic target for the diagnosis and treatment of GC.

Key words gastric cancer, vesicle-mediated transport-related genes, *SEC23A*, prognostic prediction, immune infiltration

Introduction

Gastric cancer (GC) is a highly prevalent and lethal gastrointestinal cancer worldwide [1]. According to its histological variants and biological behavior, GC can be broadly divided into two categories: expanding and infiltrative types by Ming's classification [2,3]. Studies have shown that infiltrative type GC has a worse prognosis

than expanding type GC [4]. The expanding type eventually forms discrete tumor nodules, whereas the infiltrative type tumor cells lead to diffuse involvement of the stomach [5]. However, the specific mechanism that leads to the difference in biological behavior and prognosis between these two types of GC has not been fully clarified.

Once biosynthesis is complete, each protein must be transported to the appropriate cellular compartment or secreted extracellularly to function. Vesicular transport plays a pivotal role in the transfer of protein molecules and the secretory activities of cells [6]. The set of genes that mediate these functions is known as vesicle-mediated transport-related genes (VMTRGs). The proteins encoded by genes contained within VMTRGs include Ras-related proteins in the brain (Rab) family, the transmembrane emp24 domain (TMED) family, coat protein complex (COP) I, the kinesin superfamily, and COP II proteins [7]. COP I and COP II were first identified as complexes involved in vesicular trafficking between the endoplasmic reticulum (ER) and Golgi complex [8]. The aberrant expressions of VMTRGs have been implicated in human disease and tumors [9–11].

COP II has been reported to mediate the first step of the protein secretion pathway and to play a part in tumors. SEC23A is a core member of COP II [12], and in several tumors, including breast cancer [13], bladder cancer [14], melanoma [15], GC [16], the expression of SEC23A is upregulated. SEC23A was identified as an independent prognostic factor correlated with the MAPK pathway in bladder cancer [14], and it may act as a suppressor of oligometastatic to polymetastatic progression in melanoma [17]. SEC23A is significantly overexpressed in GC. Some studies seem to indicate that SEC23A plays an inhibitory role in malignant development and tumor metastasis [15, 17–19], but one study showed that elevated expression of SEC23A is associated with adverse GC prognosis [16]. Another study showed that SEC23A affects the autophagy of cancer cells [20]. However, the exact role of SEC23A in GC and the exact mechanism are yet unclear.

In this study, we constructed a risk model based on Least Absolute Shrinkage and Selection Operator (LASSO) Cox regression analyses to probe the possible role of aberrant vesicular transport in GC. This risk model, constructed from hub genes *SEC23A*, *RAB31*, and *GABARAPL2*, has good predictive efficacy for patient prognosis. We verified that SEC23A is highly expressed in gastric cancer cells, and promotes the growth and proliferation of GC cells *in vitro* and tumor growth *in vivo*. We also found that it might exert this effect through regulating the cell cycle-related proteins CCND2 and CDK4/6.

Materials and Methods

Data collection and analysis

The stomach adenocarcinoma (STAD) gene expression and clinical features data were obtained from UCSC XENA (<https://xenabrowser.net/datapages/>) and The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>). The validation cohort with expression and prognostic data (GSE62254) was derived from the Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/gds>). The proteomics data of infiltrative type GC and normal stomach tissues were from our previous research [21]. The pathway “Vesicle-mediated transport” includes 752 VMTRGs in Reactome database (<https://reactome.org/>).

VMTRG-based risk model construction and validation

Proteins abnormally expressed in infiltrative GC compared to normal gastric tissues were previously identified [21]. We identified 77 out of 752 VMTRGs that are aberrantly expressed in infiltrative GC by using the intersection. These 77 differential genes were analyzed for enrichment, the top three pathways are Golgi vesicle transport, Endocytosis and coated vesicle, and they contain 41 of

these genes.

Based on the TCGA-STAD data, LASSO regression analysis (“glmnet” package) was used to reduce model overfitting and redundancy [22]. Three genes (*GABARAPL2*, *RAB31*, and *SEC23A*) were eventually selected to construct a risk model for predicting the Overall Survival (OS) of GC patients. The risk score is calculated as follows:

$$\text{Risk score} = \sum_{k=1}^n \text{Coef}_k \times \text{ExpGene}_k$$

We named this prognostic model as Vesicle-Infiltrative Lasso System (VILS). Using the median of risk scores, GC patients were allocated into low- and high-risk groups. Survival differences between two risk groups were measured using the KM method (“survival” package). A time-dependent ROC analysis was carried to evaluate VILS in predicting prognosis (“timeROC” package).

Building and validating of a nomogram

To determine the independence of VILS, univariate and multivariate Cox regression analyses were used (“survival” package) [23]. Ultimately, the indicators VILS, age, TNM stage, and residual tumor were considered independent prognostic factors. We applied the R package “rms” for nomogram construction and used calibration plot to evaluate the difference between nomogram-predicted and actual OS. The feasibility of this nomogram was verified using the dataset GSE62254 from GEO database (<https://www.ncbi.nlm.nih.gov/geo>).

Functional enrichment and immune infiltration analysis

Xiantao (<https://www.xiantaozi.com/>) is a comprehensive bioinformatics tool platform based on R language that has realized online analysis and visualization through front-end technology to conduct analysis of public database data [16], we applied this tool for subsequent analyses. The Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses and Gene Set Enrichment Analysis (GSEA) were performed using the “clusteRfilter” package in R, and the “ggplot2” package was applied to visualize enrichment results. For the assessment of the abundance of immune cell infiltration in the GC cohort, we used several methods from the IOBR package, including CIBERSORT, IPS, MCPCounter, QUANTISEQ, EPIC, TIMER, and xCell.

Cell culture

The human GC cell lines MKN45, HGC-27, MGC803, SGC-7901, BGC823 and the gastric epithelial cell line GES-1 were obtained from the Cellbank of the Chinese Academy of Sciences (Shanghai, China). XGC-2 (cell line of expanding GC) and XGC-1 (infiltrative GC cell line, China patent number: CN103396994A) were previously constructed in our lab [24,25]. The culture conditions were consistent with a previous study [21]. DMEM medium was used to culture the human embryonic kidney 293T cells (Cellbank of the Chinese Academy of Sciences), other culture conditions were identical.

Overexpression or knockdown of SEC23A

The entire sequence of human *SEC23A* gene (NM_006364) was cloned into the PLVX-Puro vectors (HanYibio, Guangzhou, China). A lentivirus was produced in collaboration with pMD2.G and psPAX2 (Addgene, Watertown, USA) to allow stable expression of SEC23A in GC cell lines. Lentivirus suspension (1 mL) and culture

medium (1 mL) were mixed with 2 µL of polybrene (Beyotime, Shanghai, China) to infect the gastric cancer cells. The pLKO.1-Puro vectors (HanYibio) were used to introduce short hairpin RNA (shRNA) targeting the *SEC23A* gene (shSEC23A). The target sequences are as follows: shRNA negative control (shNC): GTCTCCGAACGTGTCACGT; SEC23A-sh#1: GCTACAACTGGC CATGTTATT; SEC23A-sh#2: GGAGGCAGCTGCCATTCTTAT. Specific PCR primer sequences with are shown in Table 1. Finally, transfection efficiency was verified using quantitative Real-Time reverse transcription PCR (RT-qPCR) or/and western blot analysis.

RNA-seq and data analysis

GC cells were lysed with TRIzol (Thermo Fisher Scientific, Waltham, USA) and submitted to Bioprofile (Shanghai, China) for whole transcriptome sequencing. As a result, a differentially expressed gene set between the *SEC23A*-knockdown cell line and the normal cell line were screened. The functions and pathways of *SEC23A* in GC cells were explored using the functional enrichment analyses as mentioned above.

RT-qPCR analysis

TRIzol was applied to extract the total RNA from cells. Using HiFi-MMLV cDNA Kit (CWbio, Taizhou, China), total RNA (1 µg) was then reverse transcribed to cDNA. The CFX96 Real-Time System (Bio-Rad, Shanghai, China) was used to run the RT-qPCR using UltraSYBR Mixture (CWbio). In the reverse transcription stage, the RNA template was reverse transcribed to cDNA by primers under the action of reverse transcriptase at 65°C. Subsequently, in the PCR amplification stage, the cDNA was used as the template, and amplified by DNA polymerase under the cyclic conditions of denaturation at 95°C, annealing at 65°C, and extension at 72°C, and the target DNA product was obtained after 30 cycles. The sequences of primer are listed in Table 1. The housekeeping gene *GAPDH* was used as the internal reference.

Western blot analysis

Collected cells were lysed utilizing RIPA buffer (Solarbio, Beijing, China) supplemented with a protease inhibitor cocktail (1:100 dilution; Lablead, Beijing, China). Protein bands were transferred to PVDF membranes (Bio-Rad) after been separated by 8%-10% SDS-PAGE. After 1-h blocking with 5% skimmed milk, the PVDF membranes were sequentially incubated with the primary antibody overnight at 4°C, followed by incubation with the appropriate secondary antibody at room temperature for 1 h. Then the membranes were visualized using an enhanced chemiluminescence (ECL) kit (Bio-Rad). The antibodies used in the study are detailed in Table 2.

Colony formation and cell counting kit-8 (CCK-8) assays

Cell suspensions were plated into 6-well plates with a suitable density (1000 cells/well) and cultured for 7 to 10 days to form colonies, with medium change every 3 days. After 15 min of fixation with 4% paraformaldehyde, staining was performed with crystal violet solution at room temperature for 15 min. Cells in the 6-well plates were photographed with a camera (R100; Canon, Tokyo, Japan) and the number of colonies was counted.

Cells were plated into 96-well plates with a suitable density (1000 cells/well). CCK-8 solution (10 µL; Lablead) was added to each well after cell attachment. After 1 h of incubation, the absorbance at

450 nm was recorded by using the microplate reader (Thermo Fisher Scientific).

EdU cell proliferation assay

An EdU-488 cell proliferation kit (Beyotime) was used for this assay. Cells were plated into 6-well plates and cultured for 2–4 h after addition of 1× EdU solution at approximately 80% density. EdU staining and nuclear staining were performed sequentially according to the reagent instructions. Cells were cultured and labelled with Edu reagent (usually at 10 µM). After labelling, the cells were fixed with 4% paraformaldehyde and permeabilised with 0.5% Triton X-100. A Click reaction was then performed using a reaction buffer containing a fluorescent dye to observe the incorporation of Edu. The nuclei were then stained with Hoechst 33342 for localization. The cells were then photographed using a DM3000 fluorescence microscope (Leica, Wetzlar, Germany) and the percentage of proliferating cells was calculated.

Immunohistochemistry (IHC) analysis

IHC assay was performed as described preciously [21]. The antibodies used are shown in Supplementary Figure S1. A tissue microarray (HStmA180Su19) produced by Shanghai Outdo Biotech Company (Shanghai, China) was used. First, tissue samples were fixed in 4% paraformaldehyde or 10% neutral buffered formalin, dehydrated through an ethanol gradient (100%, 95%, 80%, and 70%), cleared with xylene, and embedded in paraffin wax. Sections are cut at 4–6 µm thickness and mounted on glass slides. The slides are then deparaffinized in xylene and rehydrated through a descending ethanol series. Antigen retrieval is performed using citrate buffer (pH 6.0) or EDTA buffer (pH 9.0) with heat-induced methods (microwave or pressure cooker) or enzymatic methods (proteinase K or trypsin). Endogenous peroxidase activity was

Table 1. Sequences of primers used in the study

Accession	Primer	Sequences (5'→3')	Exon
NM_002046	GAPDH-F	ACAACCTTTGGTATCGTGGAAGG	7
	GAPDH-R	GCCATCACGCCACAGTTTC	8
NM_006364	SEC23A-F	CTTGCCGTGCAGTTTGAATC	2
	SEC23A-R	AGGACCACGCAGAACTACATATT	4, 5
NM_001759	CCND2-F	TCCTGGCCTCCAAACTCAAA	2
	CCND2-R	AAGTCATGAGGAGTGACAGC	3

Table 2. Antibodies used in the study

Target	Cat. No.	Company
PCNA	ab92552	Abcam
SEC23A	A8613	ABclonal
GAPDHAb	AC002	ABclonal
β-Actin	AC026	ABclonal
DDDDK-Tag	AE005	ABclonal
Cyclin D2	10934-1-AP	Proteintech
CDK4	11026-1-AP	Proteintech
CDK6	14052-1-AP	Proteintech
SEC23A	MA5-34630	Thermo Fisher Scientific
Rabbit IgG(H+L)	31460	Thermo Fisher Scientific
Mouse IgG(H+L)	31430	Thermo Fisher Scientific

blocked with 3% hydrogen peroxide. Nonspecific binding sites were blocked with 5% bovine serum albumin (BSA) or normal goat serum. Primary antibodies, diluted in antibody diluent, were applied and incubated overnight at 4°C or for 1–2 h at room temperature. The corresponding secondary antibodies (HRP-conjugated anti-mouse or anti-rabbit IgG) were then applied. Chromogenic detection was performed using DAB (3,3'-diaminobenzidine) for brown staining or AEC (3-amino-9-ethylcarbazole) for red staining. The nuclei were counterstained with hematoxylin, differentiated in 1% acid alcohol, and blued in ammonia water. Finally, the sections were dehydrated through an ethanol series, cleared in xylene, mounted with neutral resin, and examined under the DM3000 fluorescence microscope.

Subcutaneous xenograft mouse model

SGC-7901 cells with stably knockdown of *SEC23A* (a dose of 100 μ L, 10^6 cells/100 μ L) were injected subcutaneously into male BALB/c-nu mice (4 to 5 weeks old and weighing 15–20 g) (5 per group). The mice were obtained from Experimental Animal Center of Xiamen University (Xiamen, China). All the animals were housed in an environment with a temperature of $22 \pm 1^\circ\text{C}$, a relative humidity of $50\% \pm 1\%$. All experimental procedures involving animals were performed using protocols approved by the Institutional Animal Use and Care Committee of Xiamen University (protocol code: XMULAC20190624 and date of approval: June 23, 2019). Tumor size was measured along both vertical axes with a vernier caliper every 4 days starting on day 10 after injection. The volume of each tumor was calculated by the following formula: volume = length $\times$ width² $\times$ 0.52. The tumor volumes and weights were measured after 4 weeks. This study was approved by the Research Ethics Committee of Xiamen University (Approval No. XMULAC20220268).

Statistical analysis

All data analyses were carried out using the R software (version 4.2.2). The heatmap and volcano plot were plotted with “ggplot2” package in R. Differences in KM survival curves were calibrated using log-rank test. $P < 0.05$ indicated significant difference.

Results

Construction of VMTRG-based prognostic model for GC

In order to screen key genes associated with prognosis and construct a prognostic model to predict the survival of gastric cancer patients, we performed data screening and analysis of proteins abnormally expressed in invasive GC, gene expression analysis, and model construction and validation. According to a previous study by our team [21], there are 1735 aberrantly expressed proteins in infiltrative GC, and VMTRGs contains a total of 752 components. Taking the intersection of abnormal proteins in infiltrative GC and VMTRG by means of Venn diagrams, the results showed that the intersection of abnormal proteins in infiltrative GC with VMTRG contains 77 genes (Figure 1A). GO/KEGG analysis [26] indicated that the 77 genes are mainly enriched in three pathways: Golgi vesicle transport, endocytosis, and coated vesicle (Figure 1B). Heatmap showed that 19 of the 41 genes included in these three pathways are highly expressed in infiltrative GC, and 22 are poorly expressed (Figure 1C).

We developed the VILS prognostic model using LASSO analysis to screen for the optimal candidate genes. This model consists of three genes with non-zero coefficients, all of which act as hazard factors:

GABARAPL2, RAB31, and SEC23A (Supplementary Figure S1A,B). Elevated levels of these factors are associated with poor survival ($P < 0.001$; Supplementary Figure S1C–E). The risk score was calculated as follows: Risk score = $0.20859 \times \text{GABARAPL2} + 0.01991 \times \text{RAB31} + 0.01463 \times \text{SEC23A}$ (Figure 1D). The median of risk scores were used to divide the GC patients into two groups, a low-risk group and a high-risk group. Kaplan-Meier (KM) survival analysis [27] showed that members in high-risk group had worse prognostic outcomes than those in low-risk group ($P = 0.009$; Figure 1E). To evaluate the prediction accuracy of VILS, the receiver operating characteristic (ROC) curves [27] were employed. The area under the ROC curve (AUC) for the GC cohort at 3-, 5-year were 0.651, and 0.684, respectively (Figure 1F). The distribution of gene expression patterns, survival time and status, and risk scores were determined (Figure 1G). The same conclusion was validated for this VILS by using the external dataset GSE62254 (Supplementary Figure S1F–K). These results demonstrated the prognostic efficacy of VILS model in GC.

Construction and evaluation of a nomogram

In order to establish VILS as an independent prognostic factor for gastric cancer patients and to construct a valid prediction model, we performed univariate and multivariate Cox regression analyses, nomogram construction, calibration plot and AUC analysis, and decision curve analysis. Univariate and multivariate Cox regression analyses were used to determine whether the VILS is an independent prognostic factor for OS of GC patients. Univariate analyses showed that age ($HR = 1.55$, $P = 0.012$), TNM stage (stage III: $HR = 1.75$, $P < 0.001$; stage IV: $HR = 2.81$, $P < 0.001$), residual tumor ($HR = 3.79$, $P < 0.001$), and VILS ($HR = 20.84$, $P < 0.001$) are the prognostic factors for GC patients (Supplementary Figure S2A). Multivariate analyses also revealed that age ($HR = 1.51$, $P = 0.034$), TNM stage (stage III: $HR = 1.53$, $P = 0.044$; stage IV: $HR = 2.36$, $P = 0.006$), residual tumor ($HR = 2.59$, $P < 0.001$), and VILS ($HR = 8.91$, $P = 0.033$) are the independent prognostic factors for GC patients (Supplementary Figure S2B).

The nomogram constructed using VILS and clinical indicators including TNM stage, age, and residual tumor. The nomogram's effectiveness in predicting 1-, 3-, and 5-year OS was evaluated individually (Figure 1H). The coefficients for each factor in calculating the nomogram-based risk score are shown in Supplementary Figure S2C. The calibration chart showed that the prediction effectiveness of this nomogram was exceedingly accurate (Figure 1I). The AUC for the GC cohort at 1-, 3-, 5-year were 0.716, 0.685, and 0.659, respectively (Figure 1J). The decision curve analysis (DCA) [27] results revealed that this nomogram outperformed TNM staging system when predicting 1-, 3-, and 5-year OS, respectively (Supplementary Figure S2D–F). The same conclusion was validated by using an external dataset (GSE62254) to validate this nomogram (Figure 1K and Supplementary Figure S2G–H). These data indicated that VILS has potential clinical applications for guiding the individualized treatment and prognostic assessment of gastric cancer patients.

The VILS high-risk group has a higher abundance of immune cell infiltration

Different types of immune cells play specific roles in the processes of antitumor and immune escape in the tumor immune micro-environment (TIME). Tumor growth, cell invasion and metastasis

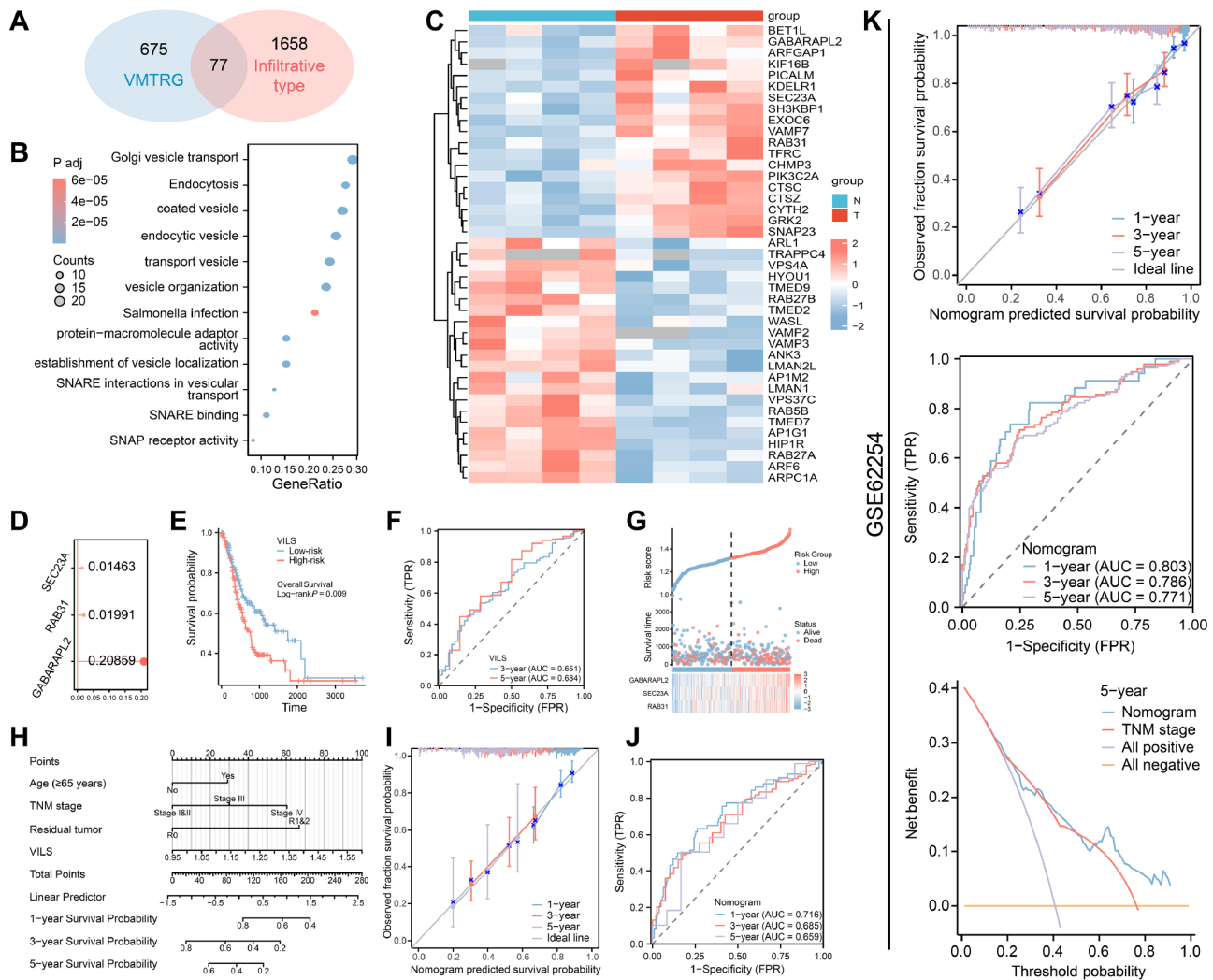


Figure 1. Construction of vesicle-infiltrative Lasso system (VILS) prognostic model (A) Intersection of differentially expressed genes in infiltrative gastric cancer with vesicle-mediated transport-related genes. (B) Enrichment analyses of intersection genes. (C) Visual representation of intersection genes in a heat map. (D) Three hub gene coefficients. (E) TCGA-STAD set-based prognostic analysis of low- and high-risk VILS groups. (F) Time-dependent ROC curve analysis of the VILS using the TCGA-STAD set. (G) Distribution of survival status, risk scores, and expression profiles of three hub genes in TCGA-STAD set. (H) Construction of nomogram for predicting the OS of GC patients at several time points. (I) The calibration chart of the nomogram at several time points. (J) The nomogram is evaluated by time-dependent ROC curve analysis. (K) The calibration chart, time-dependent ROC curve and decision curve analysis of nomogram in GSE62254 dataset.

are strongly associated with infiltration of immune cells. Therefore, we explored the association between VILS risk scores and TIME. The results showed that low infiltration of B cells, Th1 cells and natural killer (NK) cells, but a high infiltration of M2 macrophages, cancer-associated fibroblasts (CAFs) in VILS high-risk group (Figure 2A). These results revealed that B cells, cytotoxic cells, NK CD56dim cells, and T cells are more enriched in the VILS low-risk group by ssGSEA analysis (Figure 2B). These data indicated that immune cell infiltration in the tumor immune microenvironment has an important impact on tumor development and prognosis.

These function and signaling are significantly different between different risk groups

To reveal the molecular differences between low and high risk groups and identify key biological pathways and functions

through gene expression analysis, we screened differentially expressed genes, and performed gene set enrichment analysis, functional analysis and pathway analysis. Differentially expressed genes (DEGs) in the low- and high-risk groups were screened for map potential molecular signatures of these two groups. The results showed 484 upregulated and 604 downregulated in all DEGs (Figure 3A). GSEA analyses of the upregulated group revealed that the DEGs are mainly enriched in the ECM-receptor interaction, pathways in cancer, focal adhesion, MAPK signaling, and Wnt signaling pathway (Figure 3B), whereas DEGs in the downregulated group are primarily concentrated in keratinization, CD22-mediated BCR regulation, fatty acids and nitrogen metabolism, while SIRT1 negatively regulates rRNA expression (Figure 3C).

GO functional analyses of the upregulated group showed that DEGs are enriched in cell growth, focal adhesion, cellular

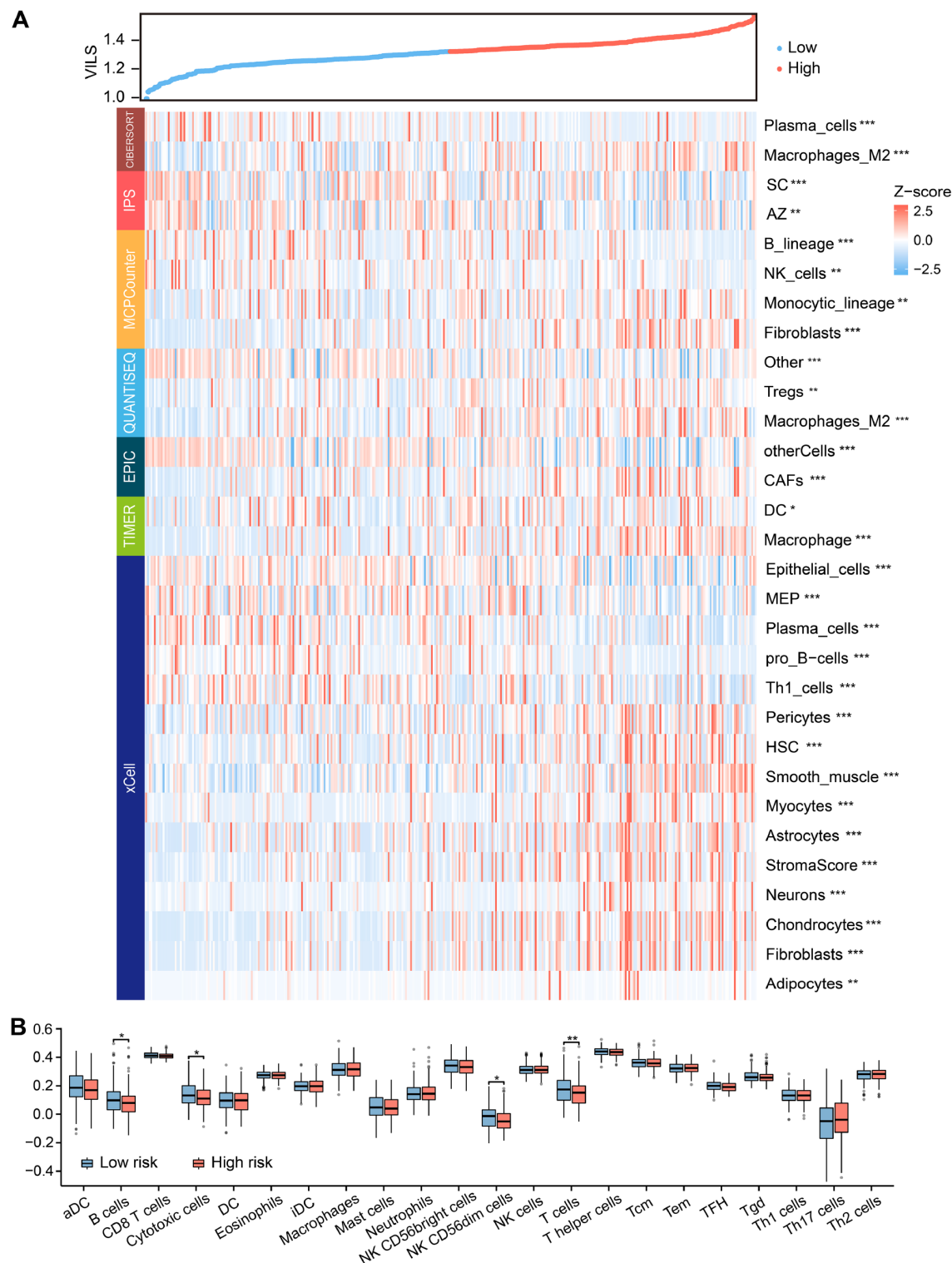


Figure 2. Correlation of VILS with infiltration of immune cells in TCGA-STAD cohort (A) Seven algorithms were run to determine the correlation between the tumor immune microenvironment and VILS risk score. (B) ssGSEA analysis of the two groups in VILS. * $P < 0.05$, ** $P < 0.01$.

component assembly involved in morphogenesis, cell-cell adhesion, and actin binding. The results of KEGG analyses showed that the main pathways are cGMP-PKG signaling, calcium signaling, cell adhesion molecules, neuroactive ligand-receptor interaction, and cAMP signaling pathway (Figure 3D). GO functional analyses revealed that DEGs in the downregulated group are enriched in keratinization, cornified envelope, and structural constituent of skin epidermis. The results of KEGG analyses showed that the main

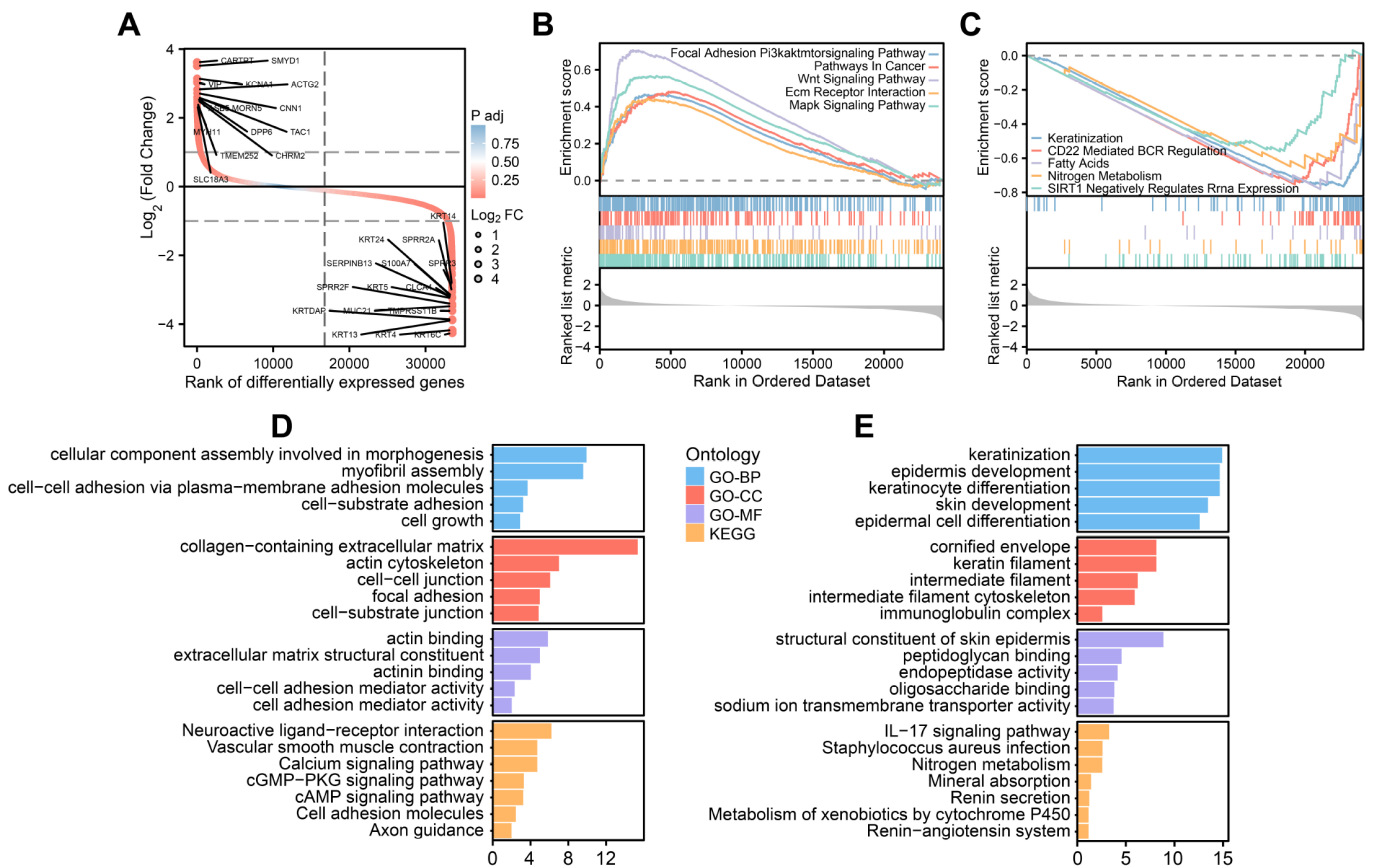


Figure 3. Functional enrichment analysis between VILS low- and high-risk groups (A) DEGs between the VILS low- and high-risk groups. (B) GSEA analyses of the upregulated group in DEGs. (C) GSEA analyses of the downregulated group in DEGs. (D) GO and KEGG analyses of the upregulated group in DEGs. (E) GO and KEGG analyses of the downregulated group in DEGs.

pathways are staphylococcus aureus infection, IL-17 signaling pathway, nitrogen metabolism, mineral absorption, and renin secretion (Figure 3E).

Knockdown of SEC23A inhibits the proliferation of gastric cancer cells

SEC23A regulates the first step of the protein secretion pathway as a core component of COP II and is also a key gene in the VILS. Therefore, exploring the expression pattern and function of SEC23A in GC may help to understand the above analysis results of VILS. Here, we compared differences in SEC23A protein expression between different GC cell lines and the human gastric epithelial cell line GES-1. The western blot analysis results showed that most of GC cell lines expressed higher level of SEC23A (Figure 4A). To investigate the role of SEC23A in human gastric cancer, we used lentiviruses with shSEC23A or shNC for transfection of human gastric cancer cell lines HGC-27 and SGC-7901. Knockdown of SEC23A was performed in two types of human gastric cancer cells. To validate the knockdown efficiency, we analyzed SEC23A mRNA level and protein expression by RT-qPCR and western blot analysis respectively. The results showed that lentiviral transfection significantly suppressed SEC23A mRNA and protein expression levels in gastric cancer cells (Figure 4B and Supplementary Figure S3A,B). To reveal whether SEC23A plays a role in the development of gastric cancer, we did transcriptome sequencing. The results showed the 67 DEGs in SEC23A-knockdown GC line (HGC-27)

screened by RNA-seq, including 16 downregulated genes and 52 upregulated genes (Figure 4C). Enrichment analysis of the DEGs indicated they are mainly enriched in cell growth, focal adhesion, ECM-receptor interaction, PI3K-AKT signaling, and negative regulation of canonical WNT signaling pathway, which is in agreement with the results of the analyses and experiments referred to above (Figure 4D). These results suggested that SEC23A may be the dominant gene in VILS and may act through the above-mentioned pathways in GC.

Next, the plate clone formation assay was performed to detect the colony-forming ability of gastric cancer cells. Compared with the control group, the number and size of colonies were significantly reduced after knockdown of SEC23A (Figure 4E). In addition, the effects of SEC23A knockdown on the proliferative capacity of gastric cancer were detected using the CCK-8 assay and EdU assay. CCK-8 assay showed that cell proliferation ability was significantly decreased in HGC-27 and SGC-7901 cells in the shSEC23A group (Figure 4F). EdU assay showed that the DNA synthesis ability was significantly slowed down in gastric cancer cells with SEC23A knockdown (Figure 4G). In summary, knockdown of SEC23A significantly inhibited the cell proliferation of gastric cancer cells.

SEC23A promotes cell proliferation by regulating cycle-related proteins

To further validate the role of SEC23A in GC cells, we established

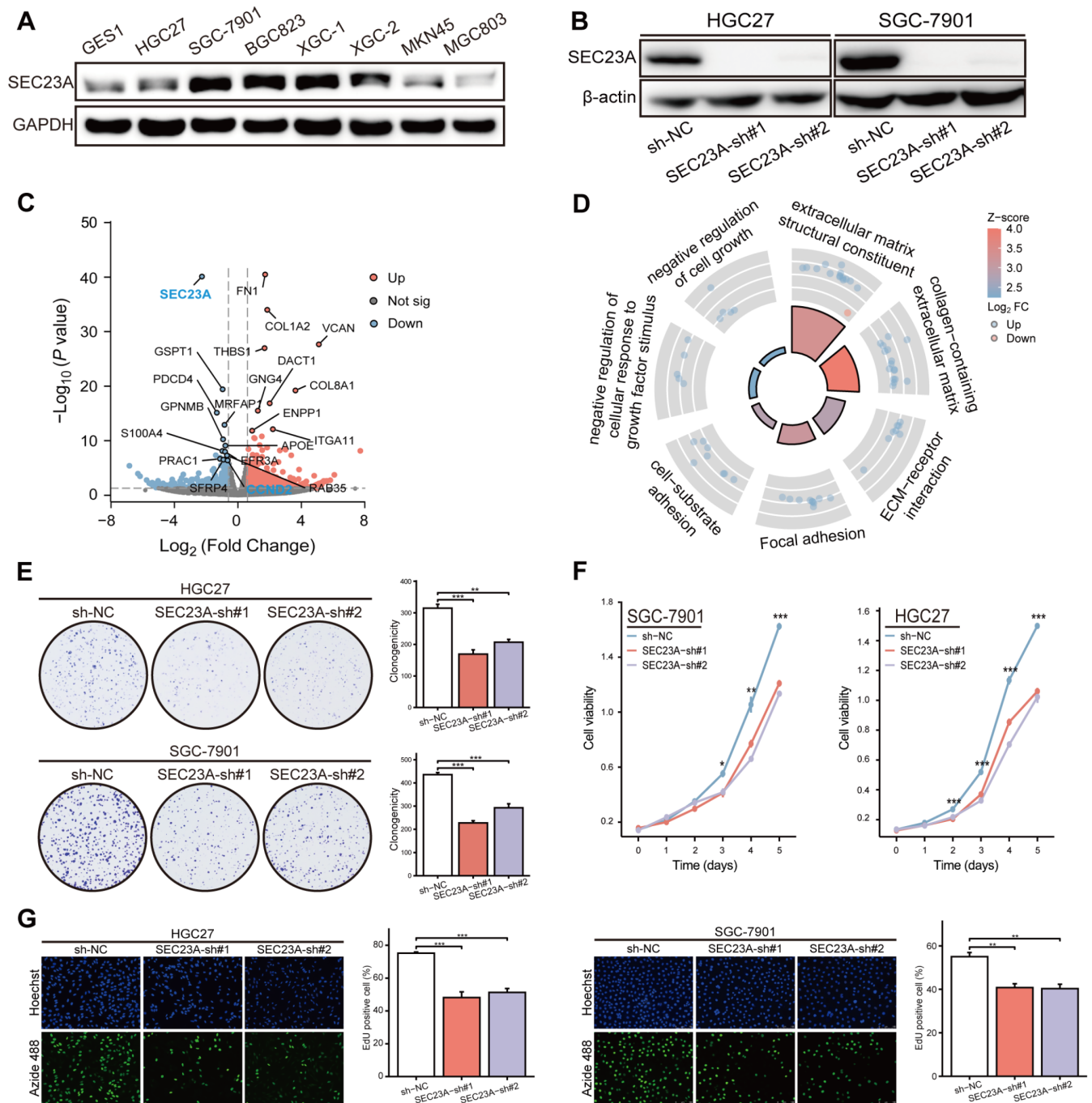


Figure 4. SEC23A knockdown inhibits the proliferation of human gastric cancer cells (A) SEC23A protein expression was upregulated in several GC cell lines. (B) SEC23A protein levels in HGC-27 and SGC-7901 cells after SEC23A knockdown. (C) Volcano map of DEGs in SEC23A-knockdown cells. (D) Enrichment analysis of DEGs in SEC23A-knockdown cells. (E) SEC23A knockdown inhibited the colony formation of GC cells. (F) SEC23A knockdown inhibited GC cell viability as detected by CCK-8 assay. (G) SEC23A knockdown inhibited GC cell proliferation as detected by EdU assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

SEC23A-overexpressing MKN45 and MGC803 cell lines by lentiviral transfection. We verified the overexpression efficiency of SEC23A in both cell lines by RT-qPCR and western blot analysis. The results showed that lentiviral transfection significantly increased the mRNA and protein expressions of SEC23A in gastric cancer cells (Figure 5A and Supplementary Figure S3C,D). Similarly, plate clone formation assays were performed using these two cell lines with

SEC23A overexpression, which showed a significant increase in the number and size of colonies in the SEC23A overexpression group as compared to the control group (Figure 5B,C). In addition, the effects of SEC23A overexpression on the proliferative capacity of gastric cancer were detected by CCK-8 assay and EdU assay. The results showed that the cell proliferation ability was significantly increased in MKN45 and MGC803 cells which SEC23A overexpression

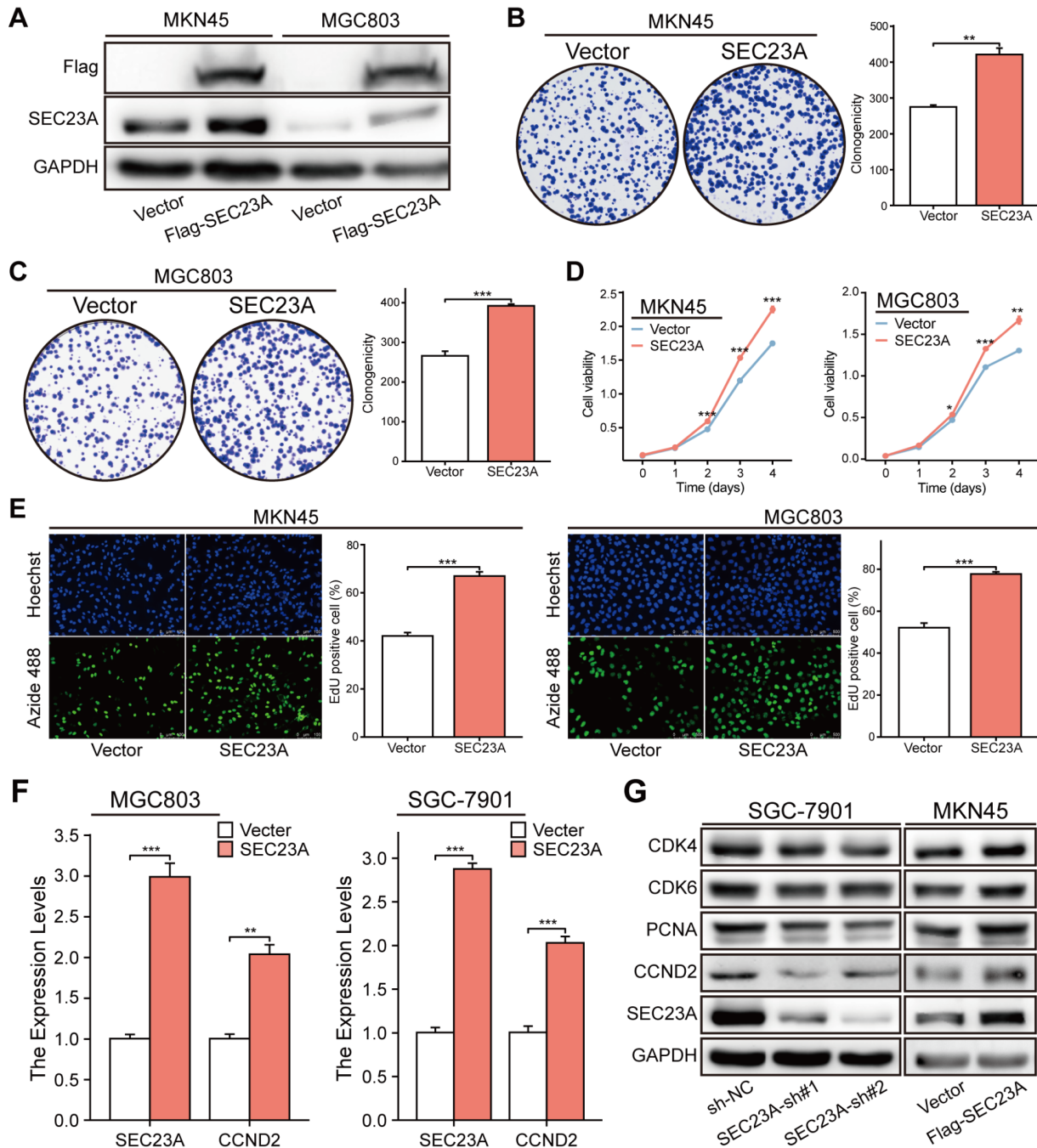


Figure 5. SEC23A promotes cell proliferation by regulating the cycle-related protein (A) The protein levels of SEC23A in MKN45 and MGC803 cells after its overexpression. (B,C) SEC23A overexpression significantly increased GC cell clonogenicity. (D) SEC23A overexpression significantly increased GC cell viability as detected by CCK-8 assay. (E) SEC23A overexpression significantly increased GC cell proliferation as detected by EdU assay. (F) SEC23A upregulated the expression of CCND2 as detected by qPCR. (G) The expression levels of cell cycle-related proteins were detected by western blot analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

compared with that of the control group (Figure 5D). The EdU assay showed that the DNA synthesis ability of gastric cancer cells overexpressing SEC23A was significantly increased (Figure 5E). The above results indicated that overexpression of SEC23A significantly increased the cell proliferation of gastric cancer cells.

The results of RT-qPCR and western blot analysis revealed that CCND2 was significantly elevated at both transcriptional and translational levels in SEC23A-overexpressing cells (Figure 5F,G).

Cyclin dependent kinase (CDK) 4/6, the downstream targets that function as CCND2, were also altered accordingly; meanwhile, the expression level of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, also changed in the same direction as the expression of SEC23A altered (Figure 5G). These results suggest that SEC23A is involved in cell cycle regulation by specifically regulating the expressions of CCND2 and CDK4/6 proteins.

Knockdown of *SEC23A* inhibits gastric cancer cell growth *in vivo*

To explore the effect of *SEC23A* on gastric cancer tumorigenesis *in vivo*, we established a subcutaneous xenograft mouse model. SGC-7901 cells with stable knockdown of *SEC23A* or negative control cells were subcutaneously inoculated into nude mice, then tumor volume and weight were measured after 4 weeks. A significant reduction in mean tumor volume and growth rate could be observed

in the sh*SEC23A* group compared to the control group (Figure 6A–C). Similarly, the tumor weight in the sh*SEC23A* group was significantly lower than that in the control group (Figure 6D). Furthermore, we compared the levels of *SEC23A* expressed in GC tissues with those in different growth patterns, and the results suggested that *SEC23A* had higher level in infiltrative GC tissues (Figure 6E). Thus, these results demonstrated that *SEC23A* knockdown could effectively inhibit the growth of tumor *in vivo*.

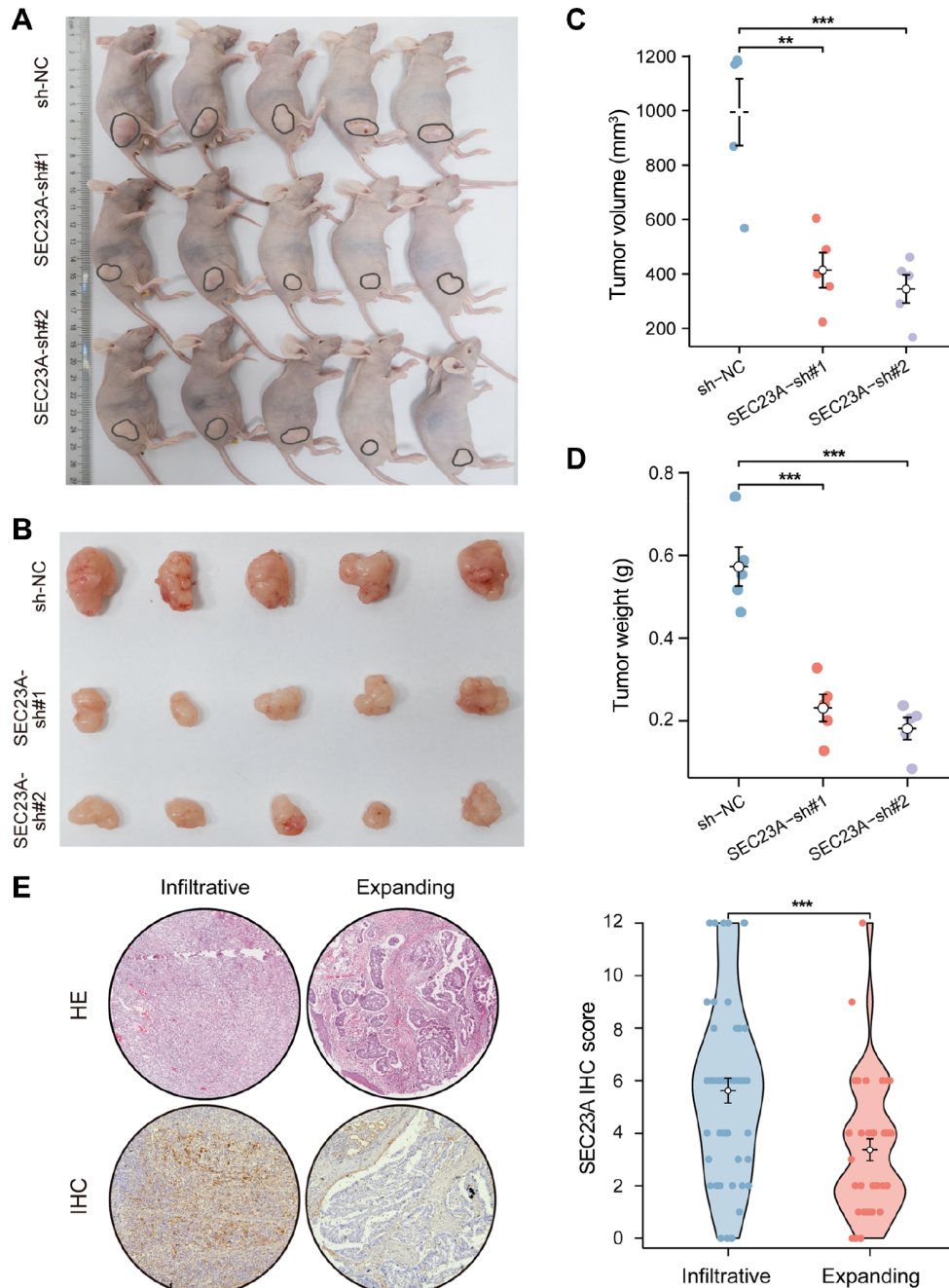


Figure 6. *SEC23A* knockdown inhibited tumor growth *in vivo* (A) The images showing the tumors in the shNC group and the sh*SEC23A* group of mice *in vivo*. (B) Images of tumors removed from mice. The tumor volumes in the sh*SEC23A* group were significantly smaller than those in the control group. (C) The tumor volumes of the sh*SEC23A* group were significantly smaller than those in the shNC group. (D) The tumor weights of the sh*SEC23A* group were significantly lower than those in the shNC group. (E) The protein expression of *SEC23A* was significantly upregulated in infiltrative type GC. ** $P < 0.01$, *** $P < 0.001$.

Discussion

Vesicles are functionally important structural components that are integral to the intracellular membrane system. The appearance of vesicles is accompanied by a process of intracellular transport of substances, also known as vesicular transport, which is important in the development of cancer [28,29]. Some VMTRGs may promote the development of several cancers, as several studies have shown. For example, RAB31 promotes GC metastasis and cisplatin resistance through Twist1-mediated EMT [30], TMED 3 targets CDCA8 and regulates the PI3K/Akt pathway to promote malignant melanoma [31]. However, whether VMTRG can be used as a prognostic predictor for GC and its predictive efficacy have not been investigated. Therefore, we aim to identify key genes in VMTRGs that are associated with GC and discover VMTRG-related targets for treatment and clinical prediction.

Lasso regression can screen out features with greater predictive power for the target variables while ensuring the best fitting error, selecting some proteins that are most important for the diagnosis or prediction of the disease, as well as improving the ability of model generalization. In this study, we established a VMTRG-based prognostic model (VILS) using LASSO regression analysis and investigated the function characteristic and molecular mechanism of the core gene of VILS in GC. The VILS contains 3 VMTRGs, *SEC23A*, *RAB31* and *GABARAPL2*. Cox regression analyses showed that TNM stage, age, residual tumor, and VILS are the independent prognostic factors for GC. The resulting constructed nomogram demonstrated excellent differentiation of patient prognosis, the results of calibration chart, DCA and ROC analysis also verified this. The validation cohort GSE62254 reached the same conclusion.

Several studies have reported that TIME, composed of immune and inflammatory cells, is associated with tumor cell invasion and metastasis and is strongly correlated with tumor prognosis. CAFs secrete a variety of secretory factors that have been reported to promote tumor metastasis and are a critical component of the stroma [21,32]. A variety of immune cells that infiltrate the tumor, such as granulocytes, macrophages, and lymphocytes, have also been reported to promote tumor metastasis [33]. As the key innate immune cells in TIME, the phenotype M2 of tumor-associated macrophages (TAMs) acts as a tumor promoter [34]. NK cells act as cytotoxic lymphocytes of the innate immune system that kill cancerous cells [35]. A previous study showed that high infiltration of plasma cells and Th1 cells are associated with favorable prognosis in cancer patients [36]. The results of multiple immune infiltration algorithms used in the study demonstrated that high-risk group is positively correlated with CAFs and macrophages (M2), but negatively correlated with plasma cells, NK cells, and Th1 cells. Thus, the infiltration characteristic of immune cell of the high-risk group may facilitate GC progression.

By studying the individual components of VILS, we evaluated the possible role of VILS in the development of GC. RAB31 belongs to the Rab family and has been shown to be aberrantly expressed in human cancers in several expression profiling analyses [37]. Increased RAB31 promotes the progression of several cancers, including colon cancer [38], cervical cancer [39], gastric cancer [30], and pancreatic cancer [40]. GABARAPL2 is a member of GABARAP family [41], which is the major player of the autophagy pathway [42]. It has been shown that *GABARAPL2*, as a hazardous gene, is a predictor of clinical prognosis in GC patients [43], and may also be a potential therapeutic target [44]. *SEC23A* is the core

component of COP II, and plays a role in inhibiting cancer progression in several cancers [14,15,17,18]. A recent study revealed *SEC23A* as an independent prognostic factor associated with disease progression, patient prognosis, and immune infiltration in GC by bioinformatics analysis [16]. However, the exact function and mechanism of this is not yet known.

A previous study showed that the expression of *SEC23A* in STAD is significantly higher than that in tumor-adjacent normal tissue [16]. This conclusion is further supported by our finding that *SEC23A* expression is high in GC cell lines. Knockdown of *SEC23A* reduced cell proliferation, possibly by inhibiting the cell cycle, according to *in vitro* functional assays. *In vivo* assays demonstrated that high expression of *SEC23A* promoted subcutaneous tumor growth. In comparison, *SEC23A* overexpression promoted GC cell proliferation.

The western blot analysis experiments showed that the expression levels of CDK4/6, CCND2 and *SEC23A* varied in the same direction, confirming the experimental data that high expression of *SEC23A* promotes cell cycle and proliferation. The western blot analysis results confirmed that the PCNA expression level decreased in *SEC23A*-knockdown cells and increased in *SEC23A*-overexpressing cells, further indicating that high expression of *SEC23A* promotes cell proliferation. On the basis of the results of existing studies and enrichment analyses, we speculate that *SEC23A* may affect cell cycle regulation and GC cell proliferation, which drives the malignant progression of GC.

Nevertheless, a limitation of this study is that we did not verify whether *SEC23A* affects the cell cycle by regulating cycle-associated proteins in our experiments, and we will continue to carry out this part of related research in the future. In addition, we need to further explore and verify whether *SEC23A* regulates the expression of cycle-related proteins through other molecular mechanisms, and whether it specifically activates a certain signaling pathway to promote the growth and proliferation of gastric cancer cells. *SEC23A* is an oncogene in GC but a tumor suppressor gene in other cancers, and we did not elucidate the mechanism responsible for this difference. Although *in vitro* and *in vivo* experiments have demonstrated that *SEC23A* promotes GC development by promoting cell proliferation, the molecular mechanisms need to be further investigated. Moreover, we would like to search for drugs that may bind to *SEC23A* for further *in vivo* experiments in order to directly target it for the treatment of gastric cancer. We will also do further experiments in other tumors in our future work.

In conclusion, the VMTRG-based risk model (VILS), including three genes *SEC23A*, *RAB31* and *GABARAPL2*, provides accurate prognostic prediction for GC patients, and the risk score of VILS correlates with immune cell infiltration. These three hub genes may help develop individualized therapeutic strategies for GC patients by targeting GC progression. Our study experimentally demonstrated that *SEC23A* is highly expressed in gastric cancer and promotes the growth and proliferation of gastric cancer cells. In addition, we also demonstrated that *SEC23A* could regulate cycle-associated proteins and influence the occurrence and development of gastric cancer. On this basis, we believe that *SEC23A* has great potential as a new GC diagnostic biomarker and therapeutic target in the future, and its molecular mechanism deserves further investigation.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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