

Research article

SLC8A1, a novel prognostic biomarker and immunotherapy target in RSA and UCEC based on scRNA-seq and pan-cancer analysis

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ABSTRACT

Background: The field of gynaecological immunology has increasingly focused on recurrent spontaneous abortion (RSA). The complex mechanisms underlying the interaction between RSA and cancer are not well understood.

Methods: Weighted gene coexpression network analysis (WGCNA), single-cell RNA sequencing (scRNA-seq), and machine learning algorithms were used for the analysis of RSA decidua samples to identify the hub genes. The expression and distribution of the hub genes were subsequently investigated via the pancancer database TCGA. A prognostic prediction was made to assess the impact of the hub genes on the cancer response, mutation burden, immune microenvironment, immune checkpoint, and chemotherapy. *In vitro* assays were performed to determine whether SLC8A1 influences HTR-8/SVneo cell proliferation, apoptosis and the concentration of calcium ions.

Results: SLC8A1 was identified as a hub gene within RSA and was highly expressed in uterine corpus endometrial carcinoma (UCEC). The efficacy of SLC8A1 as a predictive marker was substantiated by calibration curves and the concordance index. The mutation rate of SLC8A1 was found to be 6 % on the basis of the waterfall plot. Immune analysis revealed notable differences in the fractions of T cells and macrophages between the high- and low-expression groups. Patients classified in the low-risk group exhibited enhanced responsiveness to osimertinib, dasatinib, and ibuprofen. The results of *in vitro* experiments revealed that SLC8A1 promotes proliferation and inhibits the apoptosis and concentration of calcium ions in HTR-8/SVneo cells.

Conclusion: These findings suggest that SLC8A1 may serve as a promising prognostic biomarker and potential target for immunotherapy in the context of RSA and UCEC.

1. Introduction

Recurrent spontaneous abortion (RSA) refers to the unintentional termination of two or more consecutive pregnancies prior to the

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20th week of gestation, thereby posing a significant threat to overall reproductive health [1,2]. The intricate pathogenesis of RSA is influenced primarily by genetic factors, the immune system, the endocrine system, and the presence of a prethrombotic state (PTS). According to its aetiology and pathogenesis, RSA can be divided into nonimmune RSA (chromosomal abnormality type, reproductive tract abnormality type, endocrine abnormality type, and genital tract infection type) and immune RSA (autoimmune type and alloimmune type). Notably, over fifty percent of cases involving repeated miscarriages cannot be attributed to a specific underlying cause, leading to their classification as unexplained recurrent spontaneous abortion (URSA) [3,4].

RSAs remain a significant and complex concern within the field of reproductive health. Despite efforts in genetic research, the identification of specific genes associated with RSA has proven largely inconclusive. Consequently, RSA has garnered considerable attention and has become a focal point of investigation across various medical disciplines in recent years. Notably, several studies have established a noteworthy association between RSA and increased susceptibility to cancer [5,6].

A longitudinal cohort study conducted in Taiwan used 10-year follow-up data to compare women who had abortions with those who did not, specifically investigating the incidence of female cancers such as breast, cervical, uterine, and ovarian cancers [7]. The results of this study revealed a correlation between abortion and a reduced risk of uterine and ovarian cancers. Nevertheless, few studies have explored the molecular mechanisms underlying the association between RSA and cancer, particularly through the utilisation of bioinformatics analysis. The intricate pathogenetic mechanisms through which RSA and cancer interact remain largely unknown. The condition is distinguished by its occult onset, challenging early detection, and unfavourable prognosis in the context of cancer in RSA, resulting in high morbidity and mortality rates. Consequently, timely identification, diagnosis, and treatment of RSA and cancer patients are imperative for their optimal well-being. Identifying novel biomarkers that can accurately predict the prognosis of RSA and cancer patients, as well as elucidating the molecular mechanisms underlying their progression, is essential.

The advancement of next-generation high-throughput RNA sequencing technologies has significantly propelled genomic studies in recent years. Bulk RNA sequencing (RNA-seq) in the field of RSA research facilitates the acquisition of transcriptome data, thereby enabling further advancements in the study of RSA [8,9]. Additionally, the analysis of single cells through scRNA-seq has emerged as a feasible substitute for bulk RNA analysis. The introduction of scRNA-seq has made it feasible to unravel cellular heterogeneity, decipher cell states, and identify subpopulation structures across diverse cell types [10–12]. Zhu et al. conducted a comprehensive analysis of decidual tissue data obtained from individuals who underwent induced abortions and those with RSA. This analysis involved the use of bulk RNA-seq, reduced representation bisulfite sequencing (RRBS), and scRNA-seq [13]. The findings of this study revealed that a thorough understanding of the cellular and molecular mechanisms underlying RSA can be achieved, with a particular focus on the potential impact of IGF2BP1 promoter methylation on pregnancy loss. Furthermore, investigation of the hub genes associated with RSA in a pancancer context is necessary to evaluate their prognostic value for patients and to identify potential therapeutic targets. Several studies have explored potential biomarkers of RSA by integrating bulk RNA-seq analysis with single-cell RNA-seq analysis, aiming to improve the accurate stratification and prediction of RSA outcomes [14]. However, comprehensive analyses of RSA-related biomarkers across various cancer types are lacking.

Therefore, our study represents the first attempt to simultaneously compile RSA data from the scRNA-seq and bulk RNA-seq databases, employing machine learning algorithms to identify candidate hub gene biomarkers. Additionally, we investigated the functional role of these hub genes across cancers. A comprehensive examination of the clinical prognosis of cancer, the tumour microenvironment, immune checkpoints, gene mutations, and drug sensitivity prediction, among other factors, was conducted. The outcomes of this study potentially offer the opportunity to ascertain a prognostic predictor and novel therapeutic targets for patients with RSA.

2. Materials and methods

2.1. Sources and processing of data

The scRNA-seq datasets GSE164449 and GSE214607 and the transcriptome dataset GSE65099 of RSA were downloaded from the GEO (<https://www.ncbi.nlm.nih.gov/>) database. GSE164449 includes 3 normal patients and 3 RSA patients; GSE214607 includes 10 normal patients and 6 RSA patients; and GSE65099 includes 10 normal patients and 10 RSA patients. The TCGA data portal site (<http://tcga-data.nci.nih.gov/tcga/>) was used to obtain data on clinical information and SNP mutation sites.

R version 4.2.2 was used to perform differential expression gene (DEG) analysis of GSE65099 via the Limma package. For differential gene selection, P value < 0.05 and $|\log_2\text{FC}(\log_2\text{FoldChange})| > 1$ were applied. In addition, module analysis was carried out via the R package weighted gene coexpression network analysis (WGCNA) [15]. First, the function 'goodSamplesGenes' in the 'WGCNA' package was used to determine which input samples and genes were eligible for constructing coexpression networks. Additionally, coexpression modules were generated via dynamic tree cutting criteria by limiting the number of genes in a module to 100. The associations of module signature genes (MEs) with RSA were analyzed via Pearson correlation analysis.

A Seurat object was created by converting scRNA-seq data (GSE164449, GSE214607) via the R software "Seurat package". After postmapping quality control, we filtered cells with fewer than 2500 or more than 200 genes expressed and those expressing fewer than 5 % mitochondrial genes, as well as genes expressed in at least three cells. After normalisation with NormalizeData, on the basis of the scaled data with 2,000 highly variable genes, the top 20 principal components were extracted via principal component analysis (PCA). The cell populations in the 2D maps were visualized in an unbiased manner via uniform manifold approximation and projection (UMAP). Gene expression levels were compared between clusters via the "FindAllMarkers" function. Finally, for each cluster, we used a P value < 0.05 and $|\log_2\text{FC}| > 1$ [16,17]. Afterwards, marker genes were used to identify cell subpopulations via the "SingleR" package and manual annotation. The common hub genes were defined on the basis of the overlap among DEGs, WGCNA and scRNA-seq hub

genes.

2.2. Machine learning analysis of the hub genes

For the purpose of screening disease characteristic genes, we used four machine learning methods, XGBoost, RF, LASSO, and SVM. With the R software survival ROC package, the prognosis was verified by generating a ROC curve based on the GSE65099 dataset.

2.3. Coexpression analysis

The GeneCards database (<http://www.genecards.org>) was used to search for apoptosis-related and cell proliferation-related genes. The top 50 apoptosis- and cell proliferation-related genes were then sorted from top to bottom on the basis of their relevance score. Coexpression analysis was performed to determine the correlation between SLC8A1 and apoptosis and cell proliferation genes.

2.4. Pancancer analysis

TCGA (<http://cancergenome.nih.gov/>) contains gene expression information and clinical details on 33 types of cancer [18]. To identify the differentially expressed hub gene (SLC8A1) in each cancer type, we used the pancancer Deseq2 package in R to analyse the expression characteristics of the hub gene (SLC8A1). A receiver operating characteristic (ROC) curve was used to determine diagnostic efficacy.

2.5. Construction of prognostic signatures

In the UCEC TCGA Research Network, RNA-seq data and clinical information for 514 UCEC patients were downloaded. A LASSO regression model was used to construct the clinical prognosis model and compute the clinical prognostic risk score (CP risk score) [19]. To determine whether UCEC patient risk scores and clinical values could be used as independent prognostic factors, we conducted univariate and multivariate Cox regression analyses [20]. A survival curve was derived via the R packages 'survminer' and 'survival' according to high- and low-risk values.

2.6. Mutation analysis

Data on somatic mutations from patients with UCEC were downloaded from the TCGA database. The mutation frequency and exon length were calculated for each tumour sample, and the TMB determination was based on a division of the nonsynonymous mutation sites by the total size of the protein-coding region [21]. Using the R package maftools, we plotted the mutation landscapes of the 6 genes that presented the highest mutation frequencies.

2.7. Immune microenvironment analysis

Immune, stromal, and ESTIMATE scores were calculated for UCEC patients via expression data (ESTIMATE) algorithms to estimate stromal and immune cells [22]. The enrichment levels of 22 immune cell types were calculated via the Estimating Relative Subsets of RNA Transcripts (CIBERSORT) algorithm [23]. The immune activity of each sample was determined accurately via single-sample GSEA (ssGSEA) [24].

2.8. Correlation analysis between SLC8A1 and immune checkpoints

A Wilcoxon test was used to examine the correlation between immune clusters and immune checkpoints (ICPs) and SLC8A1. With the ggplot2 R package, immunological correlation scores and immune checkpoint analyses were plotted [25].

2.9. Drug sensitivity analysis

On the basis of the Genomics of Drug Sensitivity in Cancer (GDSC) database (<https://www.cancerrxgene.org>), drug sensitivity was evaluated, which predicts the chemotherapeutic response of UCEC patients [26]. Chemotherapeutic agents are calculated via the oncoPredict package.

2.10. Experimental verification in vitro

2.10.1. Cell culture

HTR-8/SVneo human extravillous trophoblast cells were purchased from iCell (Cat No. iCell-h390). The cells were cultured in DMEM at 37 °C in a humidified atmosphere containing 5 % CO₂ in DMEM.

2.10.2. Small interfering RNA (siRNA) transfection

siRNAs were designed by Yuanen Biological Technology Co., Ltd. (Hefei) and synthesised by GENERAL BIOL Company Co., Ltd.,

Anhui, China. HTR-8/SVneo cells were seeded in a 6-well plate, centrifuged at 1000 rpm for 1 min, and then the siRNA was dissolved in 125 μ L of DEPC-treated water. The siRNA and NC (5 μ L) were dissolved in 250 μ L of DMEM without serum, and the GP-transfect-Mate (10 μ L) was dissolved in 1000 μ L of DMEM without serum, mixed well, and incubated at room temperature for 15 min. The GP-transfect-Mate mixture was divided, added to the siRNA and NC, mixed well, and incubated at room temperature for 20 min. Five hundred microlitres of media were added to each well of a 6-well plate, which was shaken gently, and the cells were placed in the incubator for 4 h. The cells were harvested after 48 h. The efficiency of SLC8A1 knockdown was measured via real-time quantitative PCR (qRT-PCR), and cell lines with high knockdown efficiency were subsequently used. The sequences of primers used for qRT-PCR are listed in Table 1.

2.10.3. Cell cCounting Kit-8 (CCK-8) assays

A CCK-8 (Biosharp, Cat No. BL1055B, 23146974) was used for the cell proliferation assay. Cell transfections were performed for 48 h, after which the cells were incubated at 37 °C for 1 h after 10 μ L of CCK-8 solution was added. For each well, the absorbance at 450 nm was measured.

2.10.4. Plate cloning experiment

Cells were seeded in a 6-well plate. A humidified chamber (37 °C, 5 % CO₂) was used to maintain the cultures, which were allowed to grow to form colonies for 10–14 days. Afterwards, the cells were fixed at room temperature for 30 min in 4 % paraformaldehyde. The cells were stained for 20 min with 0.5 % crystal violet. The cells were counted after the chamber was gently washed twice with PBS. Three visual fields were randomly selected for cell counting under the microscope, and the results were tallied.

2.10.5. Flow cytometry

Flow cytometry was used to detect cell apoptosis. Adherent cells were digested with trypsin without EDTA. The cells were harvested and centrifuged at 1,500 rpm for 3 min at room temperature, after which the supernatant was discarded. The cells were washed with precooled PBS two times and centrifuged at 1500 rpm for 3 min. The reconstituted cells were suspended in 100 μ L of 1*binding buffer, 5 μ L of FITC was added, and the mixture was incubated in the dark for 15 min. Then, 5 μ L of PI was added, the mixture was mixed well, and the mixture was incubated for 5 min at room temperature in the dark. Data analysis was performed with NovoExpress software.

2.10.6. Live/dead staining

We used a Calcein/PI Live/Dead Viability/Cytotoxicity Assay Kit (Beyotime Biotechnology, Cat. No. C2015S, 091622230407) to perform the live/dead staining assay. Living cells fluoresced green under the microscope, whereas dead cells fluoresced red.

2.10.7. Calcium concentration analysis

The intracellular calcium concentration in HTR-8/SVneo cells was measured via flow cytometry. This calcium assay was conducted via a Fluo-4 Direct Calcium Assay Kit (Beyotime Biotechnology, Cat No. S1061, 040423230428). NovoExpress software was used to analyse the data.

2.10.8. Western blot (WB) analysis

We determined the expression levels of proliferation and apoptosis proteins, including Caspase-3, PCNA, Bax, and Bcl-2, via Western blotting. Total protein extraction was performed after siRNA transfection of HTR-8/SVneo cells. Protein loading buffer for SDS-PAGE was then used to separate the proteins (80 V for 3 h). Each membrane was blocked with 5 % BSA in TBS for 120 min at room temperature. The primary antibodies used were anti- β -actin (1:1000), anti-PCNA (1:500), anti-Bcl-2 (1:1000), anti-Bax (1:500), and anti-Caspase-3 (1:500) antibodies. The samples were incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary antibodies (1:10000) at room temperature for 2.5 h. Finally, the membranes were washed twice in TBST (10 min each) and detected by electrochemiluminescence (ECL). A flow chart of the experimental design is shown in Fig. 1.

2.11. Statistical analysis

For all the statistical analyses, R software (version 4.2.2) was used. Significance was determined by $P < 0.05$ for all analyses.

Table 1
Primer sequence.

Gene	Amplicon Size (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
β -actin	96	CCCTGGAGAAGAGCTACGAG	GGAAGGAAGGCTGGAAGAGT
SLC8A1	83	CTGCTTTGTGCTTCCACAG	GAAAATACGGCGCACTCCCT
SLC8A1-Homo(NC)		UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
SLC8A1-Homo-969(Si1)		GGACCAAGAUGAUGAAGAATT	UUCUUAUCAUCUUGGUCCTT
SLC8A1-Homo-1026(Si2)		GCAGAAGCAUCCAGAUAAATT	UUUAUCUGGAUGCUUCUGCTT
SLC8A1-Homo-1429(Si3)		GGUGAUACCCAGAGGGAAATT	UUUCCUUCUGGUAUACCTT

Note: We used β -actin as an internal control.

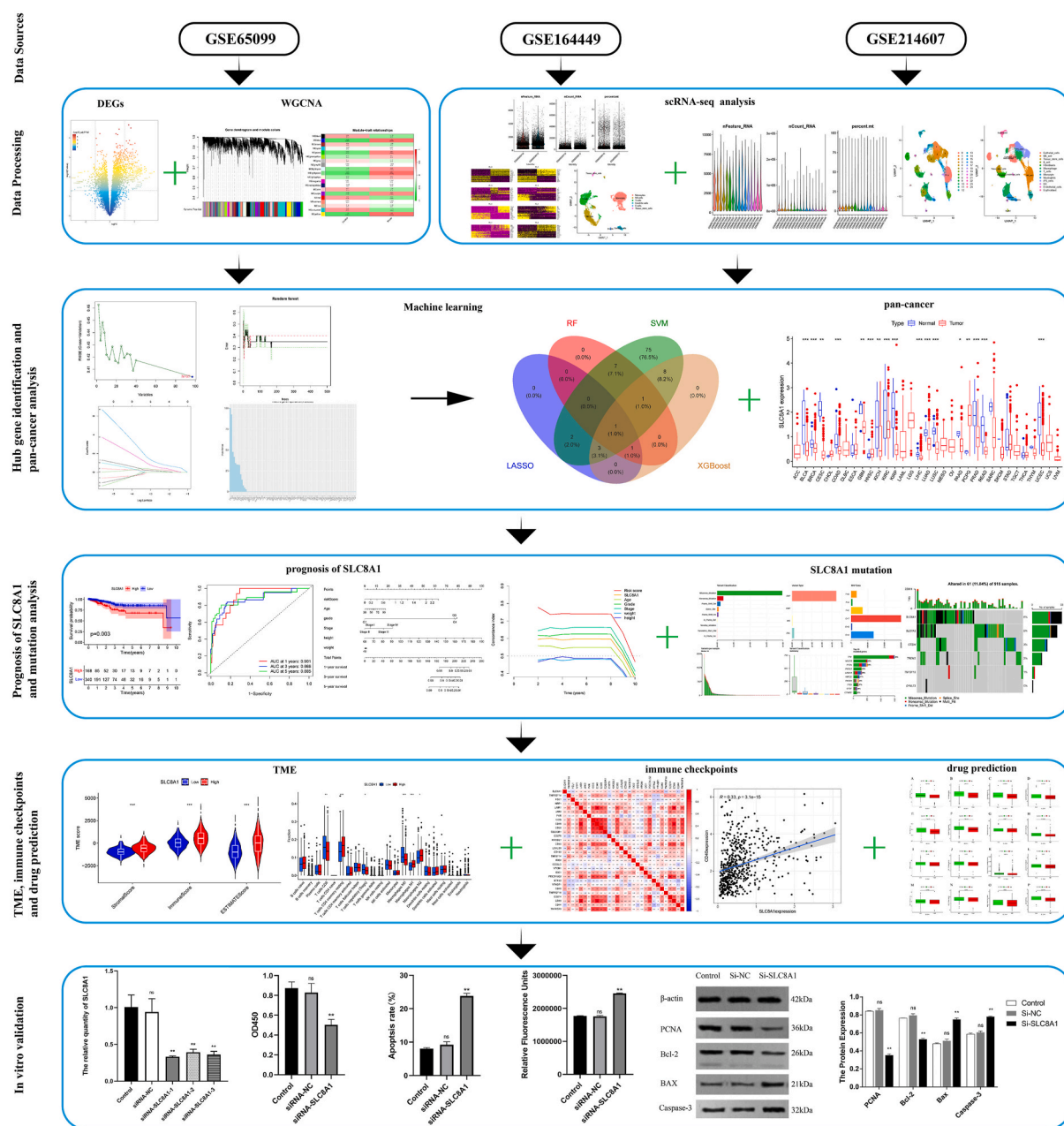


Fig. 1. A flow chart of the experimental design.

Statistical analysis was conducted with SPSS version 21.0. The data are expressed as the ($\bar{x} \pm s$) deviation. Comparisons between unpaired sample groups were performed via two independent sample t tests, whereas several one-way single-factor ANOVAs were applied to compare multiple groups.

3. Results

3.1. Identification of DEGs in GSE65099

The criteria for screening the hub DEGs included a $|\log_2FC| > 1$ and a P value < 0.05 . A total of 395 DEGs were identified, with 256 upregulated and 139 downregulated. The volcano plots in Fig. 2A visually represent these DEGs.

3.2. WGCNA identification of RSA-related key modules in GSE65099

To construct the WGCNA network, a soft-thresholding power of 11 was employed, resulting in an achieved R2 value of 0.85. The adjacency matrix and topological overlap matrix were then calculated, as shown in Fig. 2B. The module network dendrogram was generated on the basis of clustering module eigengene distances, followed by the correlation heatmaps depicted in Fig. 2C and D. Fig. 2C illustrates the presence of 20 distinct gene expression patterns, referred to as coexpression networks. Among these networks,

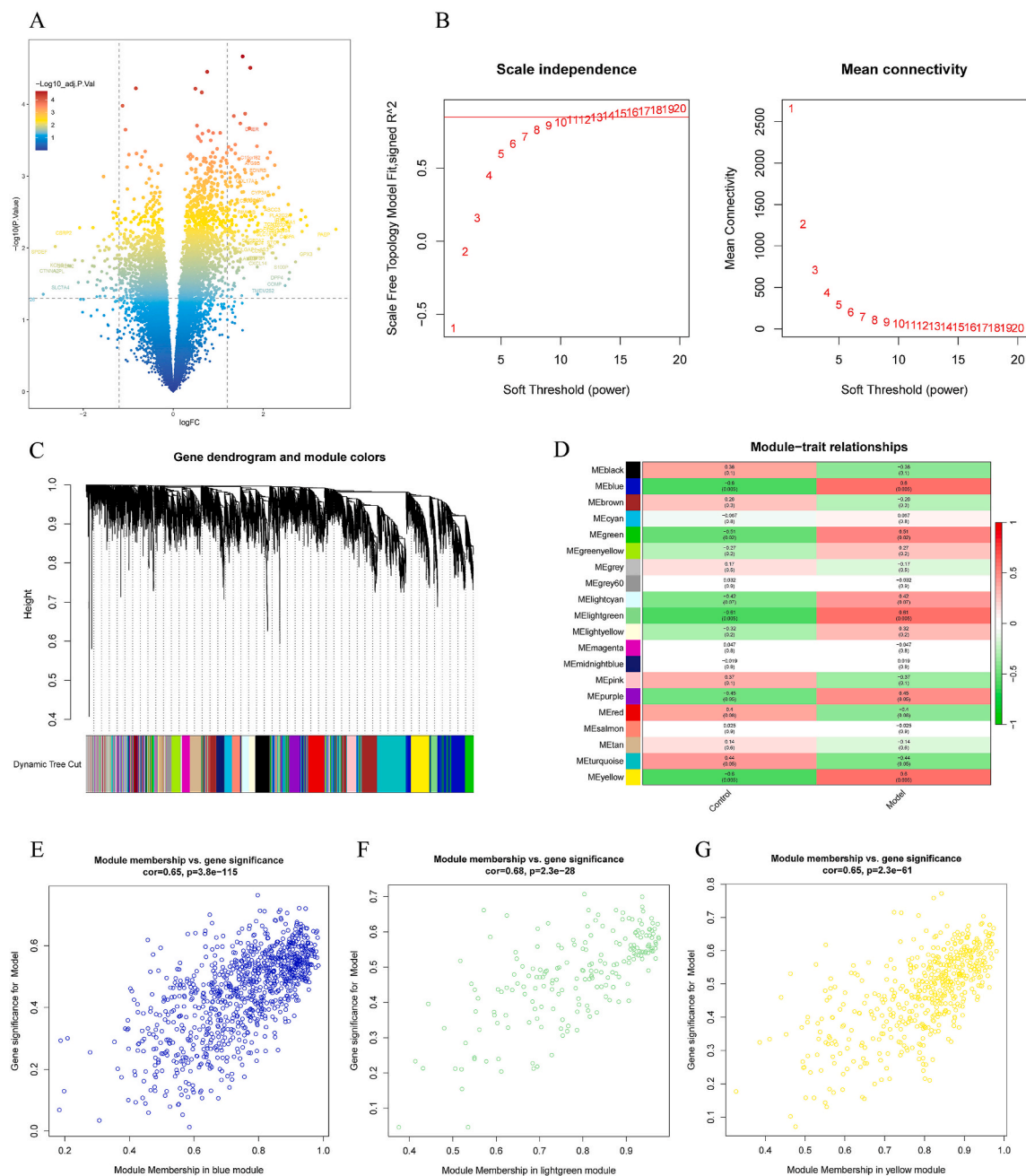


Fig. 2. Identification of DEGs in GSE65099 by R and WGCNA analysis. **A** The volcano plots visually represent DEGs. **B** An analysis of the fit of the scale-free topology model compared to the candidate soft threshold powers. Soft threshold power = 11 and scale-free topological fit index (R^2) = 0.85. **C** The original and combined modules under the clustering tree. Hierarchical clustering groups genes into different modules, represented by different colors. **D** Heat map of module-trait correlations. Red represents positive correlations and green represent negative correlations. **E** Module Membership vs Gene Significance scatter plot of blue module. **F** Module Membership vs Gene Significance scatter plot of lightgreen module. **G** Module Membership vs Gene Significance scatter plot of yellow module.

three modules, namely, the blue module, light green module, and yellow module, exhibited significant correlations with phenotypic traits. A total of 1650 genes were identified, and their correlations with gene significance (GS) and module membership (MM) were examined for the blue, light green, and yellow modules in Fig. 2E, F, and G, respectively.

3.3. Identification of RSA cell subtypes in GSE164449 and GSE214607

The data from GSE164449 were normalised via the LogNormalize method, and high-variability genes in the decidua are identified in Fig. 3A and B. Through the utilisation of PCA and UMAP techniques, an impartial examination of cell composition clustering was conducted, resulting in the identification of 14 distinct clusters. The first eight principal components of the PCA are visually represented in Fig. 3C. Additionally, Fig. 3D presents a heatmap showing the relative expression of marker genes within each cluster. By cross-referencing markers associated with cell types through SingleR annotation and manual annotation, six cell types were accurately annotated: monocytes, NK cells, dendritic cells, B cells, and tissue stem cells, as depicted in Fig. 3E and F. The bubble plots in Fig. 3G display the percentage expression of marker genes categorised by major cell types. Through analysis, we identified 1922 marker genes that exhibited differential expression, as determined by a $|\log_2FC| > 1$ and a P value < 0.05 .

The data from GSE214607 were normalised via the LogNormalize method, and the identification of high-variability genes in decidua is depicted in Fig. 4A, B and C. By using PCA and UMAP, an unbiased clustering approach was employed to explore the composition of cells, resulting in the identification of 25 distinct clusters. An analysis was conducted via the first eight principal

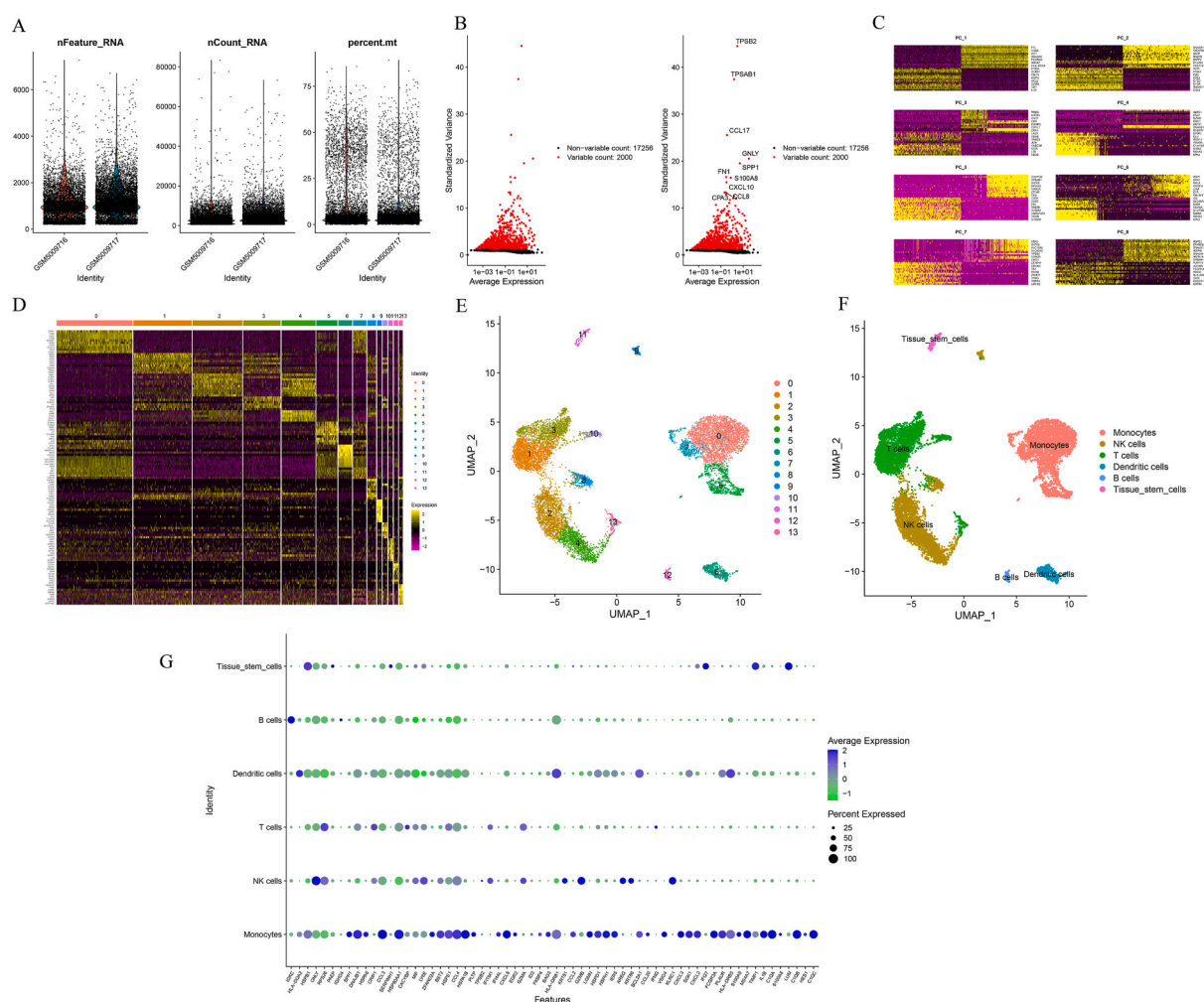


Fig. 3. Identification of RSA cell subtypes in GSE164449 by scRNA-seq analysis. **A** Quality control of scRNA-seq data from RSA samples. **B** The variance plot showed 17256 genes in all cells, red dots represent the top 2000 highly variable genes. **C** The first eight principal components of the PCA analysis. **D** The heatmap showed the relative expression of genes in 14 clusters. Yellow represents high expressed genes and purple represents low expressed genes. **E** 14 clusters were visualized based on the UMAP algorithm. **F** Cell subsets identified by marker genes. **G** The bubble plots displayed the percentage expression of marker genes, categorised by major cell types. X-axis, mean expression level of marker genes; Y-axis, each cell type.

components of PCA to examine the relative expression of marker genes in each cluster (Fig. 4D). By comparing markers associated with cell types expressed through SingleR annotation and manual annotation, 13 cell names were identified: epithelial cells, NK cells, tissue stem cells, B cells, fibroblasts, macrophages, T cells, monocytes, neutrophils, iPS cells, DCs, endothelial cells, and erythroblasts (Fig. 4E)

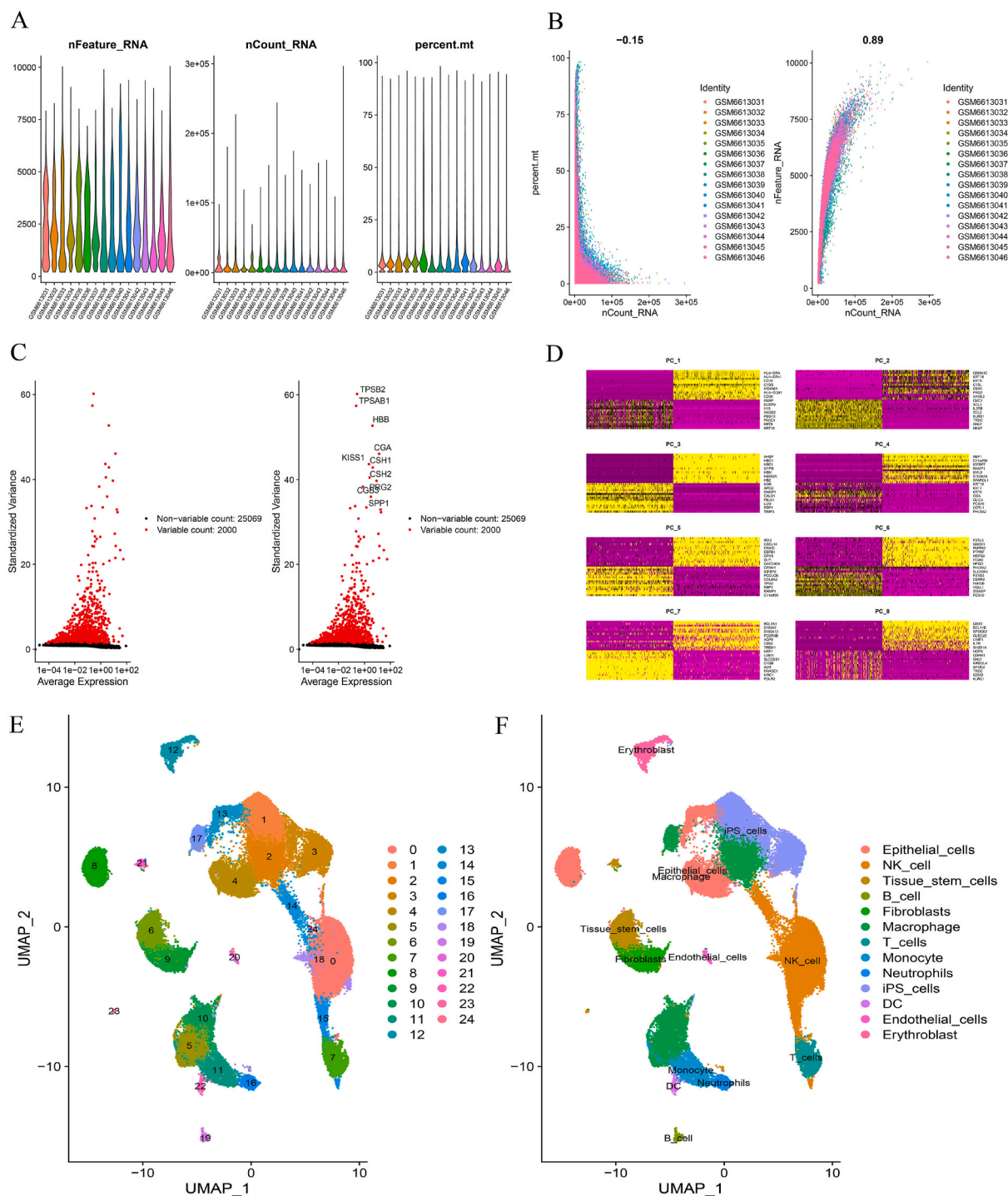


Fig. 4. Identification of RSA cell subtypes in GSE214607 by scRNA-seq analysis. **A** Quality control of scRNA-seq data from RSA samples. **B** Relationship between the Count RNA and mitochondrial ratio, pearson correlation coefficient = -0.15 ; relationship between the Count RNA and Feature, pearson correlation coefficient = 0.89 . **C** The variance plot showed 25069 genes in all cells, red dots represent the top 2000 highly variable genes. **D** The first eight principal components of the PCA analysis. **E** 25 clusters were visualized based on the UMAP algorithm. **F** Cell subsets identified by marker genes.

and F). Furthermore, 3791 marker genes were identified as having differential expression on the basis of a $|\log_2FC| > 1$ and a P value < 0.05 .

The DEGs obtained from the datasets GSE164449 and GSE214607 were combined, resulting in the identification of 4108 DEGs.

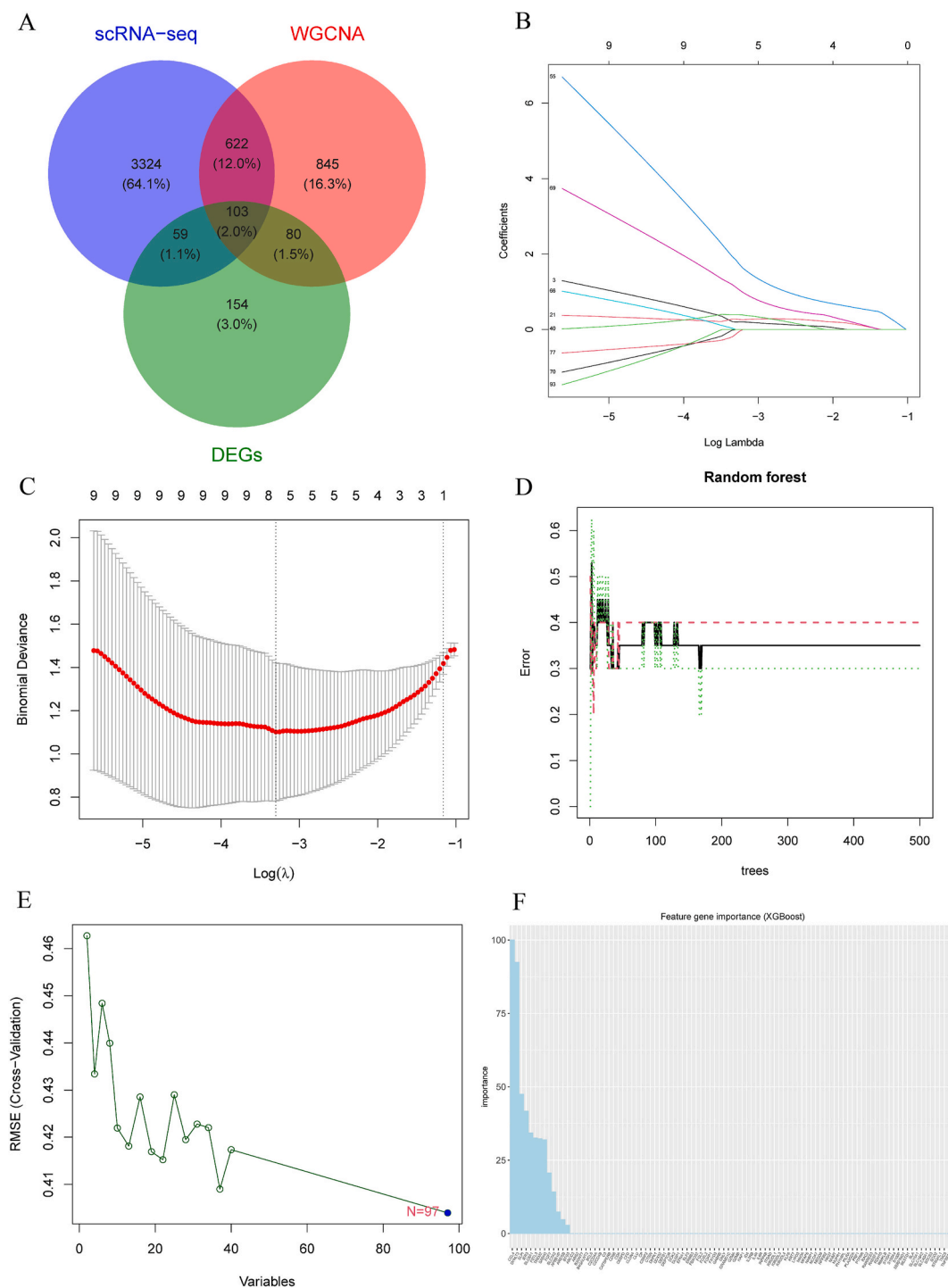


Fig. 5. Machine learning screening for hub gene. **A** The Venn diagram of scRNA-seq, WGCNA and DEGs. **B, C** LASSO analysis. **D** random forest (RF) analysis. **E** SVM analysis. **F** XGBoost analysis.

3.4. Acquisition of candidate genes

From the combined differential expression genes of GSE65099, WGCNA of GSE65099, scRNA-seq of GSE164449, and GSE214607, 103 candidate genes were obtained. A Venn diagram illustrating the overlap of these genes is presented in Fig. 5A.

3.5. Machine learning screening for hub genes

Through LASSO analysis, 7 DEGs were selected (Fig. 5B and C), whereas random forest (RF) analysis identified 10 DEGs (Fig. 5D). Additionally, SVM analysis identified 97 DEGs (Fig. 5E), and XGBoost analysis identified 14 DEGs (Fig. 5F). By identifying the overlapping DEGs identified by the four machine learning analyses, a central gene, SLC8A1, emerged as a hub gene. A visual representation of this overlap can be observed in Fig. 6A. To assess the diagnostic accuracy of the model, ROC curve analysis was conducted on the GSE65099 data, which resulted in highly accurate predictions. Notably, SLC8A1 demonstrated its significance as a prognostic gene for RSA, as indicated by its impressive AUC value of 0.930, as depicted in Fig. 6B.

3.6. SLC8A1-apoptosis and cell proliferation-related gene coexpression analysis

According to GeneCards, 21868 target genes are associated with apoptosis, and 21330 target genes are associated with cell proliferation. The top 50 apoptosis- and cell proliferation-related genes were then sorted from top to bottom on the basis of their relevance score. SLC8A1 and the top 50 apoptosis- and cell proliferation-related genes were used for coexpression analysis. The results revealed a significant association between SLC8A1 and 28 apoptosis-related genes and 24 cell proliferation-related genes. The partial results are shown in Figs. 7 and 8.

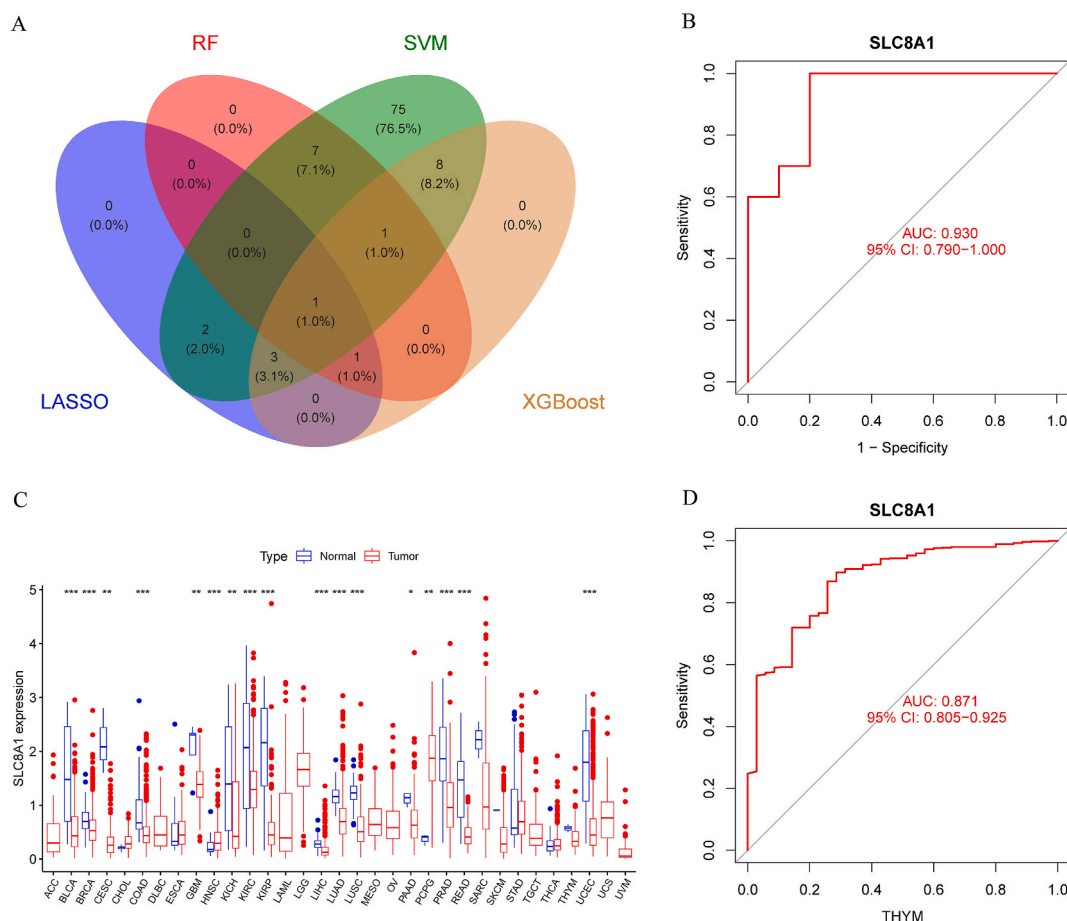


Fig. 6. Pancancer analysis of SLC8A1. **A** The Venn diagram of four machine learning analysis. **B** ROC curve analysis. **C** The expression levels of SLC8A1 in different cancer types from the TCGA database (* $P < 0.05$, *** $P < 0.001$). **D** ROC curve for UCEC patients to assess the predictive efficacy of SLC8A1.

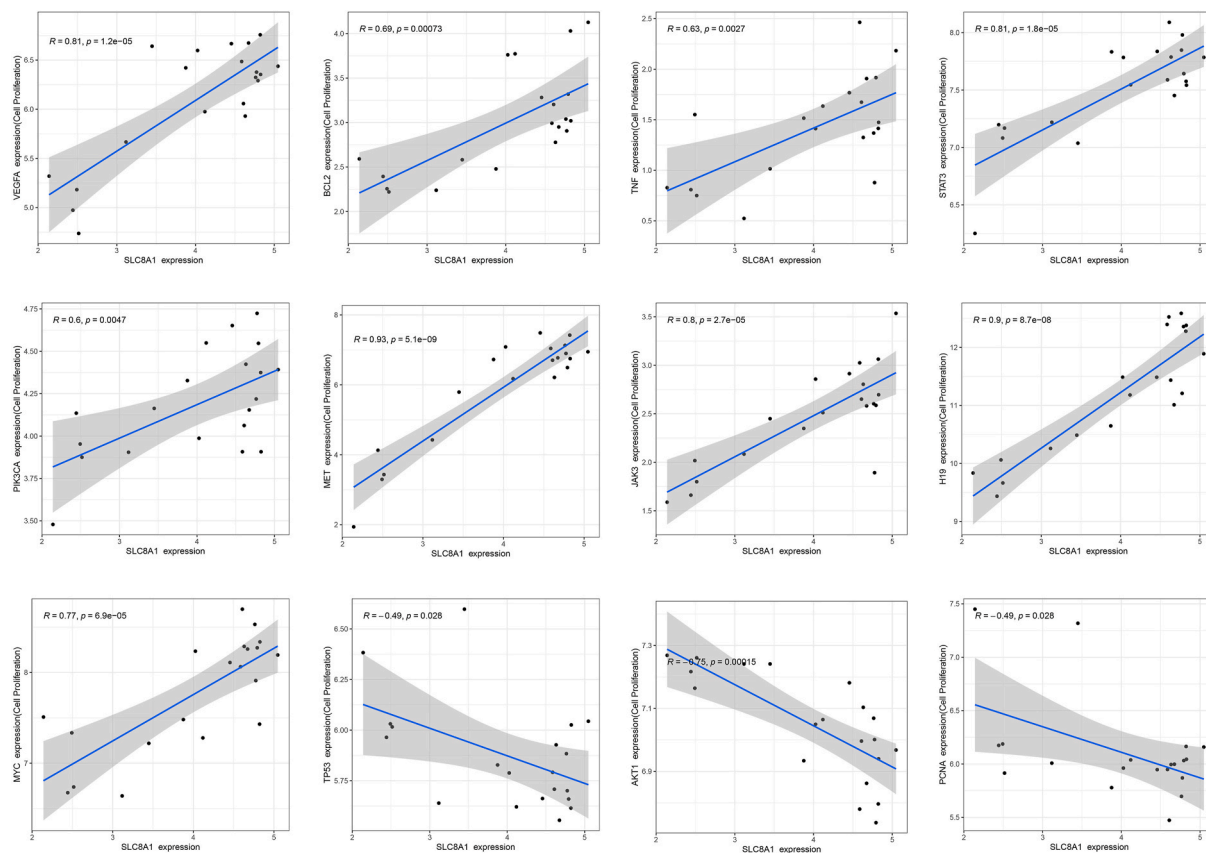


Fig. 7. Coexpression analysis of SLC8A1-cell proliferation-related genes. These include VEGFA, BCL2, TNF, STAT3, PIK3CA, MET, JAK3, H19, MYC, TP53, AKT1, PCNA.

3.7. Pancancer analysis of SLC8A1

Through an analysis of the TCGA database, we conducted calculations on the expression levels of SLC8A1 in various cancer types. The visualisation outcome, as depicted in Fig. 6C, revealed remarkable findings. Notably, lower expression of SLC8A1 was observed in UCEC tumour tissues, suggesting that SLC8A1 could offer valuable insights into the clinical prognosis of UCEC patients. To validate this hypothesis, we generated an ROC curve for UCEC patients to assess the predictive efficacy of SLC8A1, which yielded an AUC value of 0.871 (Fig. 6D).

3.8. Assessment of the prognostic value of SLC8A1 in UCEC

The UCEC samples were stratified into high-risk and low-risk groups on the basis of the median risk score. A novel risk score model was constructed via univariate Cox, LASSO, and multivariate Cox regression analyses. As depicted in Fig. 9A and B, the risk score and stage were identified as independent prognostic factors. Fig. 9C presents the survival curves for the high-risk and low-risk groups according to their respective risk scores, revealing that patients classified as high risk experienced poorer outcomes than those classified as low risk. Furthermore, in addition to assessing this risk signature, we evaluated the predictive capacity of the model for determining the survival status of UCEC patients at 1, 3, and 5 years via receiver operating characteristic (ROC) curves. As depicted in Fig. 9D, the AUC surpassed 0.85, indicating the robust predictive ability of the risk model.

Additionally, we performed an additional analysis to ascertain the associations between risk scores and clinical characteristics (grade, age, weight, height, and stage) within distinct subgroups categorised by clinical characteristics. The findings revealed significant correlations between risk score and grade, age, and stage. Fig. 9E presents the expression heatmap, which was constructed using clinical factors and risk scores. Multiple indicator ROC curves can be generated for grade, stage, and risk score, as depicted in Fig. 9F. The AUC values surpassing 0.6 indicate a high level of accuracy in predicting overall survival (OS) when these indicators are used. The ranked risk scores and patient survival curves are illustrated in Fig. 10A and B, respectively, and revealed an increasing trend in the mortality rate with increasing risk score. All the significant independent factors were incorporated into the prognostic nomogram, as shown in Fig. 10C. A calibration curve was subsequently generated to assess the predictive ability of SLC8A1 via a nomogram, and the concordance index (C-index) was computed. The calibration curve confirmed the strong predictive value of SLC8A1, as

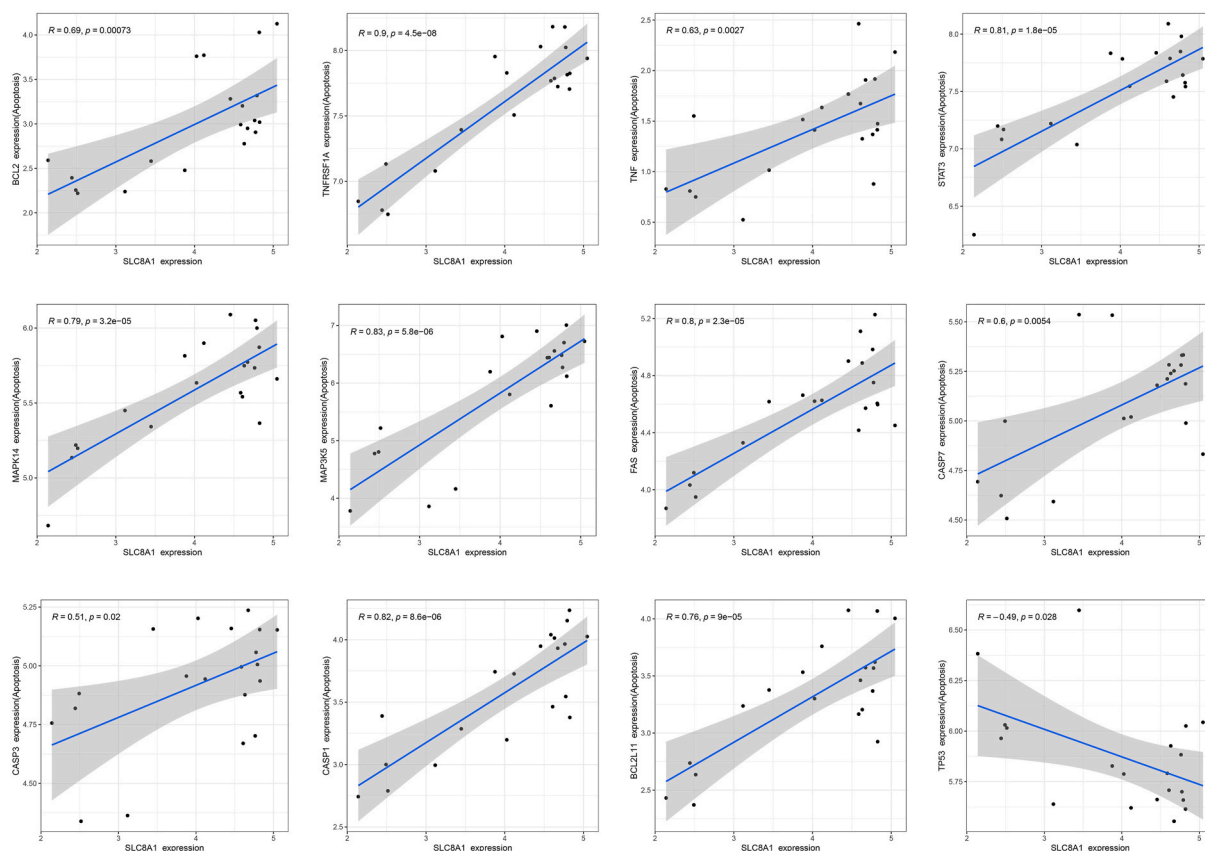


Fig. 8. Coexpression analysis of SLC8A1-apoptosis-related genes. These include BCL2, TNFRSF1A, TNF, STAT3, MAPK14, MAP3K5, FAS, CASP7, CASP3, CASP1, BCL2L11, TP53.

indicated by previous findings (Fig. 10D and E).

3.9. SLC8A1 mutation analysis

Missense single nucleotide polymorphisms (SNPs), particularly C > T mutations, account for the majority of overall mutation events. The comprehensive mutation profile of UCEC is depicted in Fig. 11A and B. The waterfall plot illustrates the somatic mutation rate of 6 NRmRNAs, with SLC8A1 exhibiting a mutation level of 6 % (Fig. 11C).

3.10. UCEC immune microenvironment estimation of SLC8A1

The ESTIMATE algorithm was employed to estimate the immune score, stromal score, and ESTIMATE score for each patient. The findings revealed that, compared with the low-risk group, the high-risk group presented significantly elevated stromal, immune, and estimated factor scores ($P < 0.05$) (Fig. 12A).

To assess the extent of immune infiltration, ssGSEA was used, and the immune infiltration in each UCEC sample is depicted in Fig. 12B. Furthermore, in addition to gene expression data, CIBERSORT was used to analyse the levels of immune cell infiltration. To examine the correlation between the SLC8A1 signature score and immune infiltration level, CIBERSORT online tools were used to calculate the infiltration level of 22 immune cell types on the basis of the expression data of SLC8A1. The samples were divided into two groups, namely, high and low, on the basis of the median level of SLC8A1 expression. Notably, the fractions of CD8 T cells, resting memory CD4 T cells, regulatory T cells, M0 macrophages, M01 macrophages, and M2 macrophages varied between the high and low groups. Box plots, as depicted in Fig. 12C, were employed to illustrate these differences. Furthermore, the correlation between SLC8A1 and immune cells is shown in Fig. 12D.

3.11. Immune checkpoints and SLC8A1: a correlation analysis

Additional investigations were conducted to examine the associations between SLC8A1 and immune checkpoint-related genes. A comprehensive correlation analysis revealed significant correlations between the SLC8A1 gene and 29 immune checkpoint-related

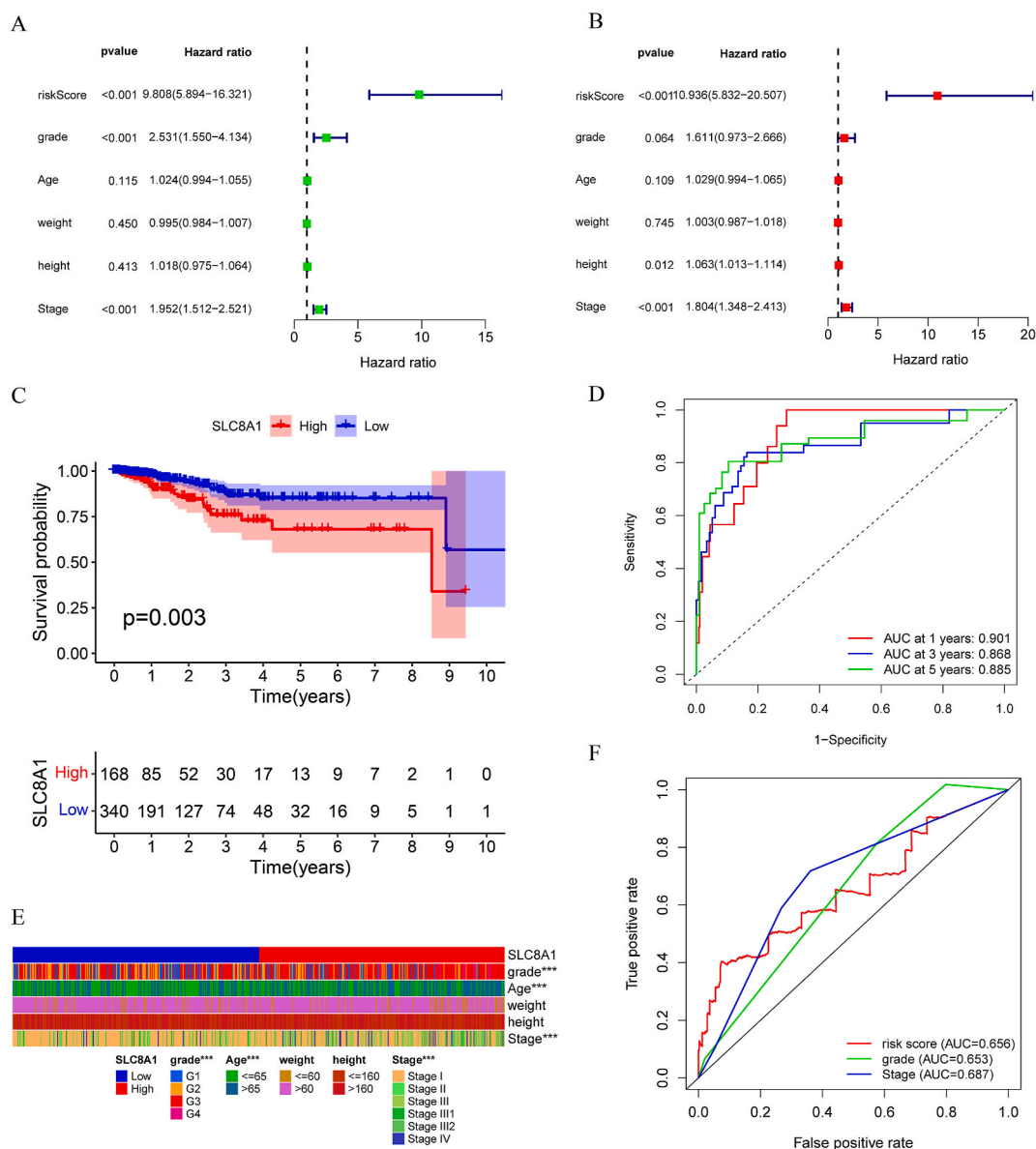


Fig. 9. An assessment of the prognosis of SLC8A1 in UCEC. **A** Univariate Cox analysis. **B** Multivariate Cox analysis. **C** The overall survival (OS) probability of the patients in high-risk and low-risk groups. **D** The AUC at 1 years, 3 years, and 5 years of prognostic models in the UCEC patients. **E** Patient distribution of clinical characteristics, including grade, age, weight, height, stage of the high-risk and low-risk groups (** $P < 0.001$). **F** Multi-indicator ROC curves of the nomogram (grade, stage, and risk score).

genes, particularly with CD40-positive immune checkpoint genes. This correlation is visually depicted in Fig. 12E. Furthermore, a scatter plot analysis was carried out, specifically focusing on the relationship between CD40 and SLC8A1, as demonstrated in Fig. 12F.

3.12. Indications for sensitivity to chemotherapy drugs

Furthermore, a comparison was conducted between the sensitivity of 198 anticancer drugs and SLC8A1 in the low-risk and high-risk groups, aiming to offer potential treatment recommendations for UCEC patients. Notably, patients in the low-risk group exhibited heightened sensitivity to osimertinib, dasatinib, sepantronium bromide, ibrutinib, JQ1, MN-64, BMS-754807, LY2109761, Navitoclax, PD173074, PFI3, SB216763, UMI-77, WEHI-539, WIKI4, and AZD1208. These findings suggest that SLC8A1 could be employed for screening or developing anticancer medications (Fig. 13).

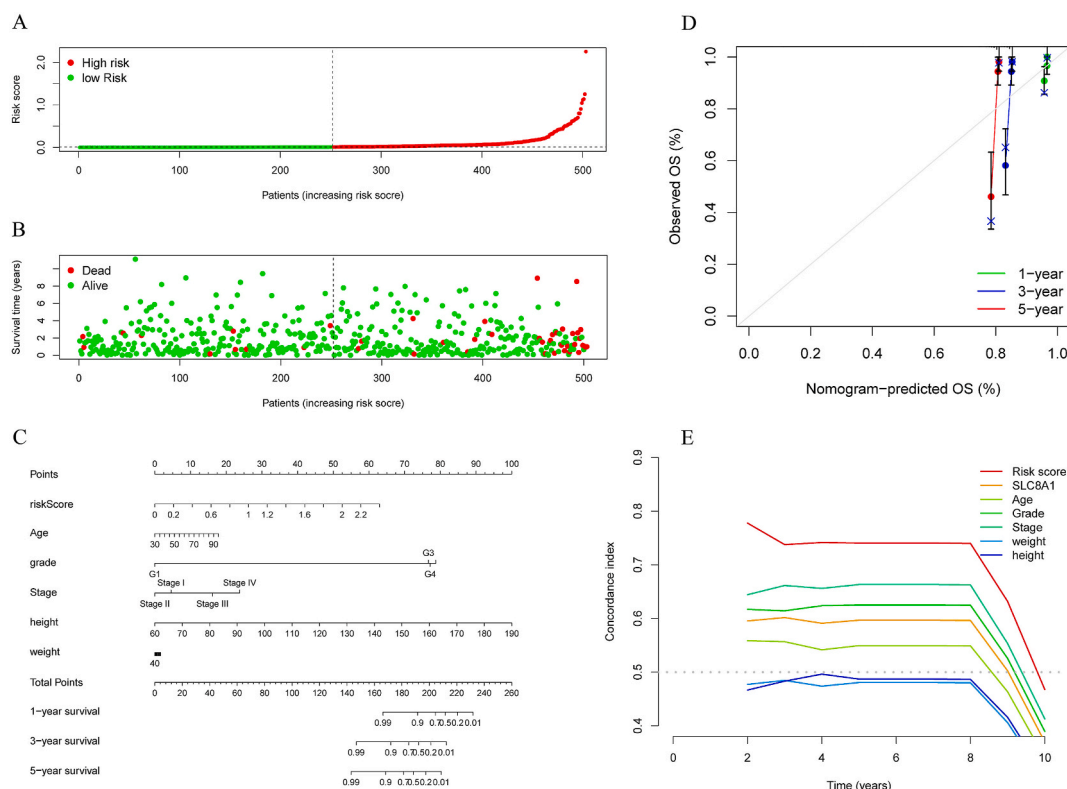


Fig. 10. An evaluation and construction of the nomogram of patients with UCEC in the TCGA cohort. **A** Distribution of risk score in UCEC cohort. **B** Scatter plot of the OS of each patient in the UCEC cohort. **C** The construction of the nomogram using risk scores and clinical features. **D** The calibration curves of the nomogram for the 1-year, 3-year, and 5-year OS. **E** The concordance index (C-index) analysis to verify the nomogram performance.

3.13. In vitro model validation

3.13.1. qRT-PCR analysis of si-SLC8A1

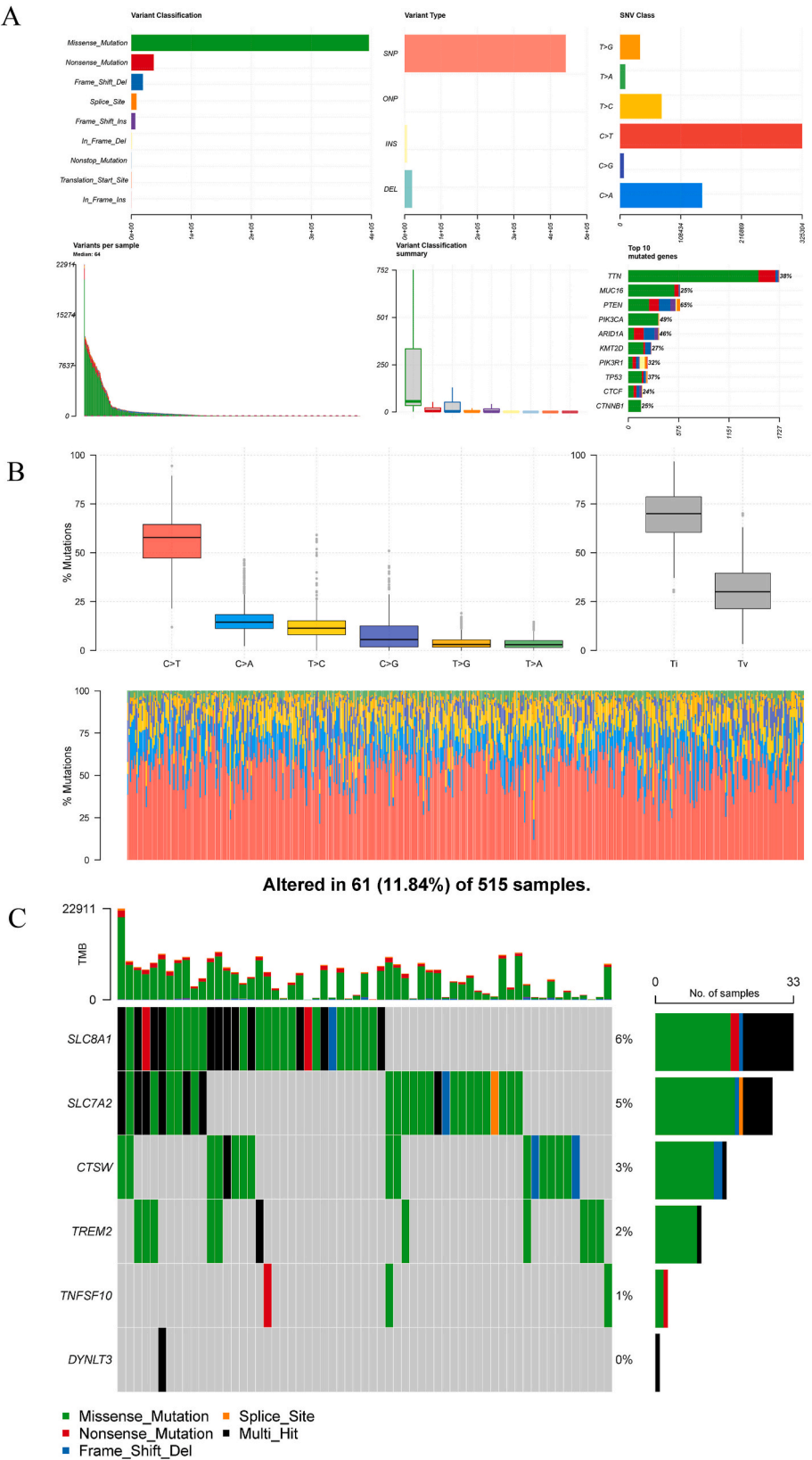
As a result of the increased expression of SLC8A1 in the RSA model group, siRNAs targeting SLC8A1, specifically si-SLC8A1, were developed. The efficacy of si-SLC8A1 in HTR-8/SVneo cells was assessed via qRT-PCR, which revealed that all three sequences of si-SLC8A1 significantly reduced SLC8A1 mRNA levels (Fig. 14A). Among these sequences, si-SLC8A1-969 (siRNA-SLC8A1-1) demonstrated the most pronounced effect and was therefore chosen for subsequent experiments.

3.13.2. Proliferation by CCK8 and cell cloning analysis

Cell proliferation was assessed through CCK-8 experiments and cell cloning techniques. The results of the CCK-8 assay revealed a significant decrease in optical density in the si-SLC8A1 group compared with the control group ($P < 0.01$) (Fig. 14B). Additionally, the plate cloning assay revealed a significant reduction in HTR-8/SVneo cell colonies in the si-SLC8A1 group compared with those in the control group (Fig. 14C). This study revealed a correlation between cell proliferation and SLC8A1 expression, with upregulation of SLC8A1 leading to increased proliferation and downregulation leading to decreased proliferation. Our hypothesis is that SLC8A1 may play a role in promoting proliferation in the context of RSA.

3.13.3. Cell apoptosis assessment by flow cytometry and live/dead stain analysis

Apoptosis in HTR-8/SVneo cells transfected with si-SLC8A1 was assessed via flow cytometry and live/dead staining. Flow cytometry analysis revealed a significant increase in the rate of apoptosis compared with that in the control group ($P < 0.01$) (Fig. 14D–G). Confocal fluorescence microscopy was employed to visualise live/dead cell staining, with live cells exhibiting green fluorescence when stained with calcein and dead cells displaying red fluorescence when stained with propidium iodide (PI). The live/dead staining analysis indicated a notable increase in the number of dead cells following si-SLC8A1 treatment compared with that in the control group (Fig. 15A). Both flow cytometric and live/dead staining findings consistently demonstrated a downregulation of SLC8A1 expression, leading to increased apoptotic capacity in cells. SLC8A1 may play a role in inhibiting apoptosis in the context of RSA.



(caption on next page)

Fig. 11. Somatic mutations analysis. **A** The overall mutation profile of UCEC. **B** Base substitution mutations analysis. **C** The waterfall plots of the 6 mutated genes (SLC8A1, SLC7A2, CTSW, TREM2, TNFSF10, DYNLT3) in the 515 samples.

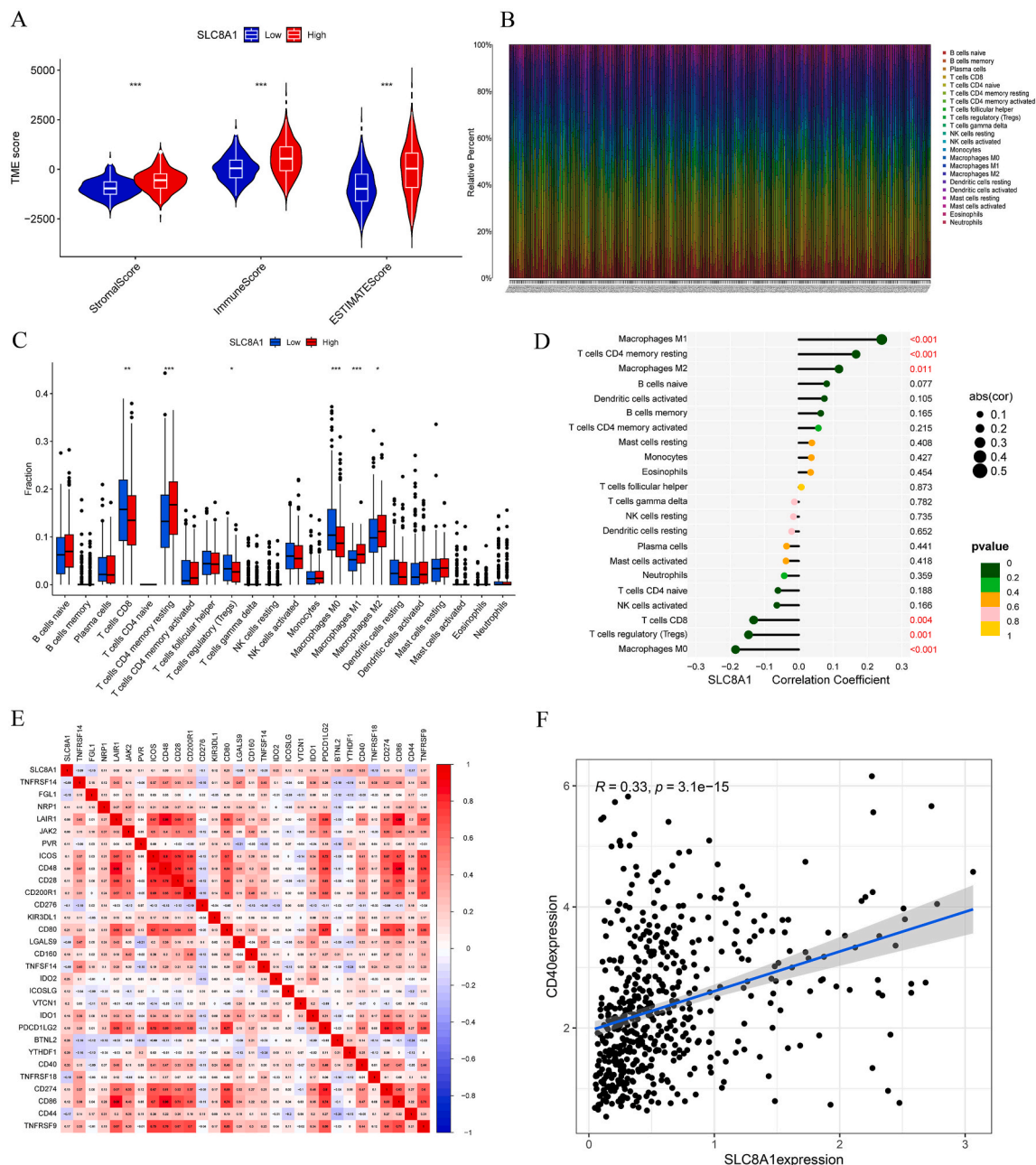


Fig. 12. Tumor immune microenvironment analysis. **A** Differences expression levels of stromal, immune, and ESTIMATE scores between low-risk and high-risk groups (***) ($P < 0.001$). **B** The stacked bar chart shows the immune infiltration in each UCEC sample. **C** Difference expression levels of 22 types of tumor-infiltrating immune cells between low-risk and high-risk groups. **D** Lollipop plot for the correlation between SLC8A1 and immune cells. **E** Heat map display a correlation between SLC8A1 expression and immune checkpoints. **F** A scatter plot analysis for the relationship between CD40 and SLC8A1.

3.13.4. Calcium concentration levels by flow cytometry analysis

Flow cytometry analysis was conducted to measure the calcium concentration in HTR-8/SVneo cells following transfection with si-SLC8A1. The results revealed a significant increase in the calcium concentration compared with that in the control group ($P < 0.01$) (Fig. 15B–E). SLC8A1 may play a role in regulating calcium levels in RSA.

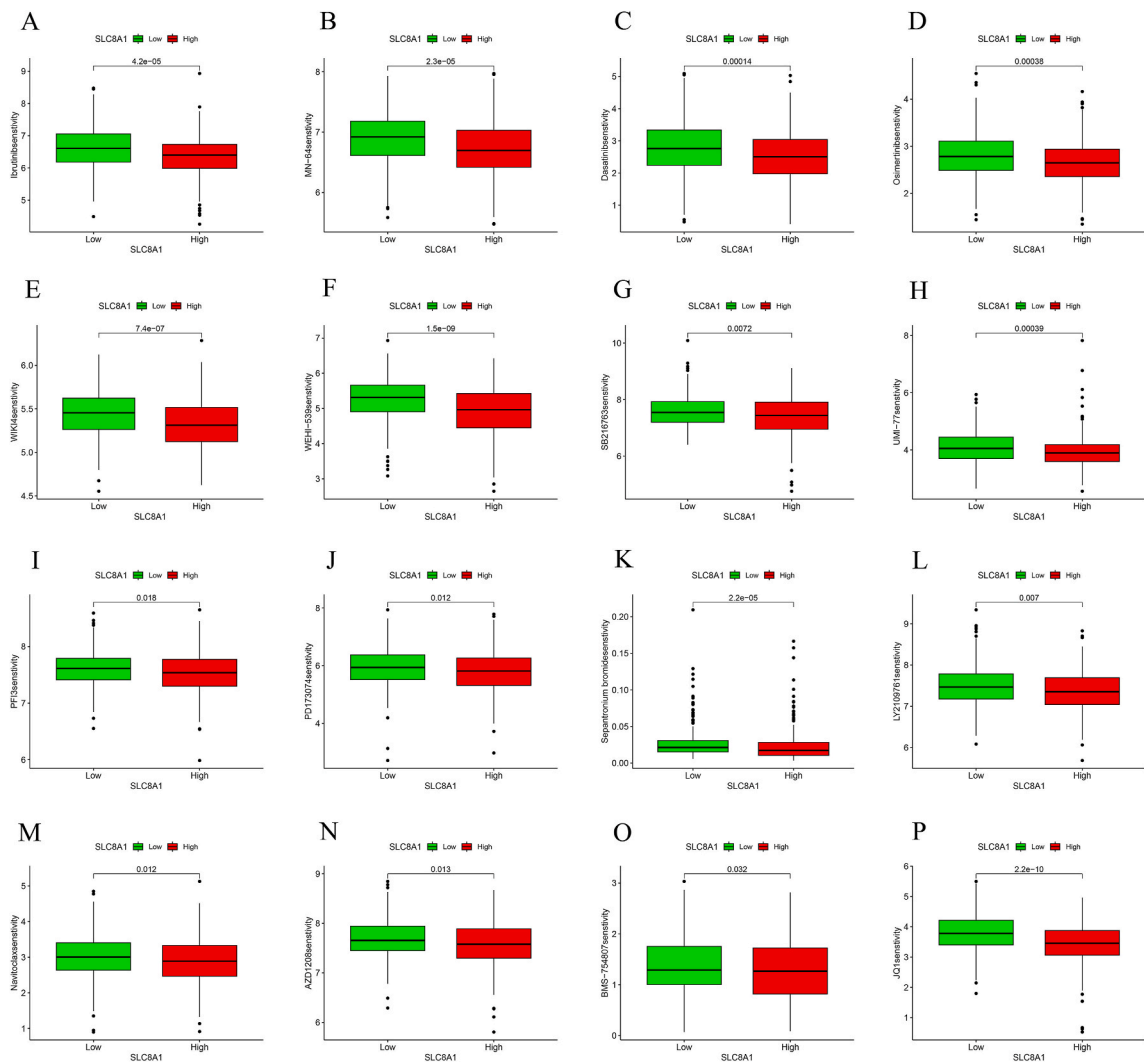


Fig. 13. Drug sensitivity assessment. **A** Ibrutinib. **B** MN-64. **C** Dasatinib. **D** Osimertinib. **E** WIK14. **F** WEHI-539. **G** SB216763. **H** UMI-77. **I** PFI3. **J** PD173074. **K** Sepantronium bromid. **L** LY2109761. **M** Navitoclax. **N** AZD1208. **O** BMS-754807. **P** JQ1.

3.13.5. WB analysis of the expression of apoptosis-related and proliferation-related proteins (caspase-3, PCNA, Bax, and Bcl-2)

The findings indicated that SLC8A1 was downregulated following transfection with siRNA, leading to a significant decrease in the expression of PCNA and Bcl-2 in the SLC8A1 siRNA group compared with the control group ($P < 0.01$). Conversely, the expression of caspase-3 and Bax was significantly elevated in the si-SLC8A1 group compared with the control group ($P < 0.01$) (Fig. 15F).

4. Discussion

During pregnancy, RSA is a prevalent complication that piques the interest of expectant couples and their healthcare providers [27]. The primary mechanism underlying RSA remains poorly understood. The literature suggests that repeated miscarriages, pregnancy terminations, diagnostic curettage procedures, and intrauterine infections may result in irreversible harm to the endometrium [28]. Such endometrial damage, along with its thinning, is a significant contributing factor to the development of endometrial carcinoma [29]. Additionally, fluctuations in hormone levels following miscarriages can potentially facilitate the onset of breast cancer [30,31]. The establishment of novel prognostic markers and therapeutic targets for RSA and associated cancer progression is highly important. This study aimed to investigate the characteristics and hub genes of RSA, analyse the underlying relationships between these hub genes and cancers, and predict the response of cancer patients to the immune landscape, immune checkpoints, and targeted therapy.

Initially, a comprehensive approach involving the integration of multiple datasets, differential expression analysis, WGCNA, and single-cell analysis was employed to identify key genes that may have substantial implications in the progression of RSA. Through the application of machine learning techniques such as LASSO, RF, SVM, and XGBoost analysis, our study identified SLC8A1 as a hub gene

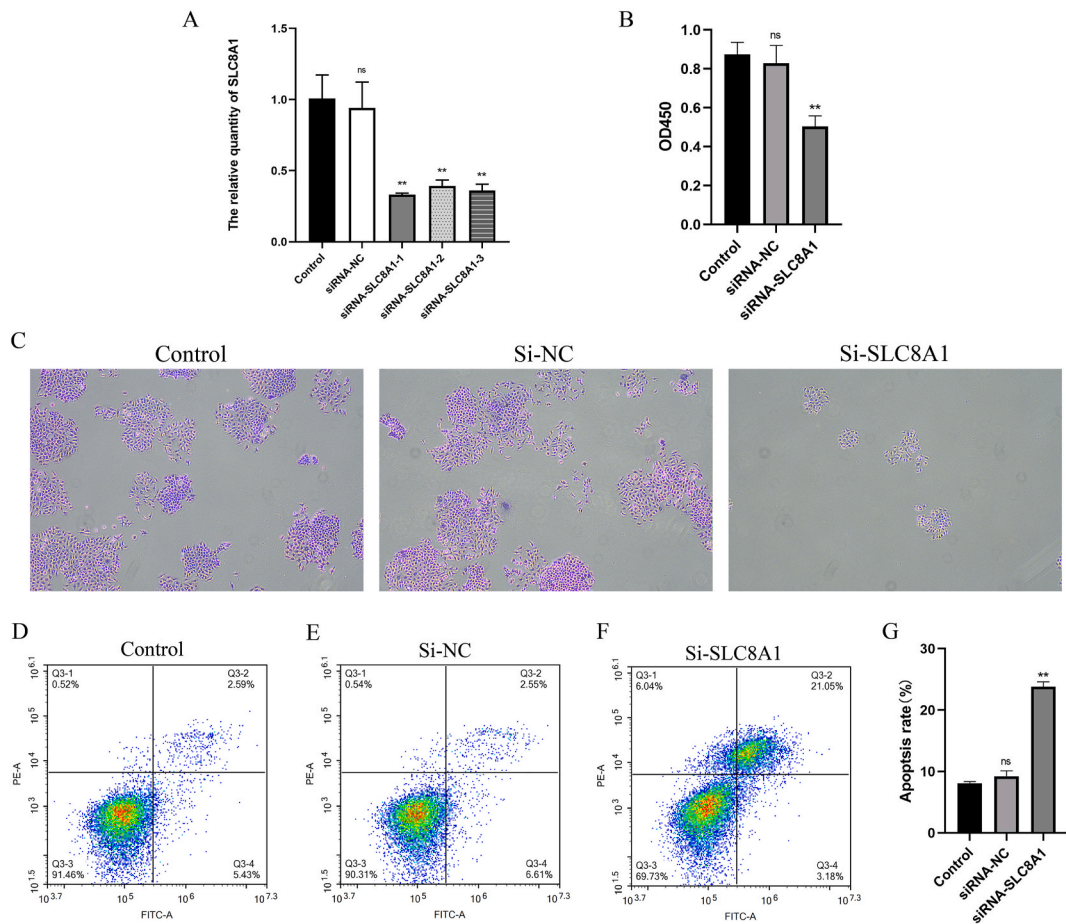


Fig. 14. Cell proliferation and apoptosis were investigated by *in vitro* studies using si-SLC8A1. **A** qRT-PCR analysis for si-SLC8A1. **B** Proliferation by CCK8 analysis. **C** cell cloning analysis (40X). **D–G** Cell apoptosis by flow cytometric analysis. siRNA-NC vs control; siRNA-SLC8A1 vs siRNA-NC (** $P < 0.01$).

associated with RSA. The understanding of the relationship between the SLC8A1 gene and cancer prognosis remains limited. However, recent research has acknowledged the increasing importance of genes in the development of cancer. Wang et al. proposed that NCAPG2 could function as a regulatory element and a biomarker for various malignancies [32]. Li et al. demonstrated that EIF4G1 may have the ability to modify the tumour microenvironment and inhibit the metastasis of breast cancer (BRCA), thus potentially serving as a prognostic biomarker and therapeutic target for BRCA [33]. Similarly, Zhao et al. revealed that CYFIP2 could be considered a potential therapeutic target for rheumatoid arthritis (RA) and various types of tumours, offering a new perspective on the treatment of immune-related RA diseases and cancer [34]. Consequently, to further explore the association between SLC8A1 expression and tumours, we conducted a comprehensive analysis across multiple cancer types via the TCGA database. The findings of our study indicate significant expression of SLC8A1 in patients diagnosed with UCEC, which was further validated through the use of ROC curve analysis.

We subsequently conducted an extensive analysis encompassing prognostic evaluation, mutational assessment, tumour microenvironment examination, immune checkpoint investigation, and drug sensitivity prediction of SLC8A1 in UCEC. The purpose of this comprehensive analysis was to ascertain the prognostic relevance of SLC8A1. Our study revealed a correlation between SLC8A1 expression and both clinical outcome and immune cell infiltration in UCEC, suggesting the potential involvement of this biomarker in immunoregulation and prognosis. The endometrium, which possesses glands identical to those of a typical endometrium, serves as the origin of UCEC, an epithelial malignancy. UCEC is the primary contributor to cancer among women globally, with increasing incidence and mortality rates [35–37]. Consequently, reliable biomarkers to address endometrial damage resulting from RSA, predict the progression of RSA to UCEC, and facilitate targeted immunotherapeutic interventions for UCEC are urgently needed.

The calcium extrusion regulatory molecule SLC8A1 encodes a member protein of the NCX1 antiporter that is activated by protein phosphatase 2A. This protein facilitates the transport of calcium out of cells and sodium into cells, thereby regulating calcium homeostasis [38,39]. Numerous studies have reported an association between SLC8A1 and various diseases. For example, deletion of the SLC8A1 gene or the presence of null mutations has been found to result in arrhythmia [40]. Furthermore, SLC8A1 plays a significant role in the pathogenesis of hypertension [41]. Additionally, SLC8A1 has been implicated in the development of diabetes and certain cancers, where it has the opposite function. Long et al. reported that the administration of KB-R7943, a reverse-mode SLC8A1

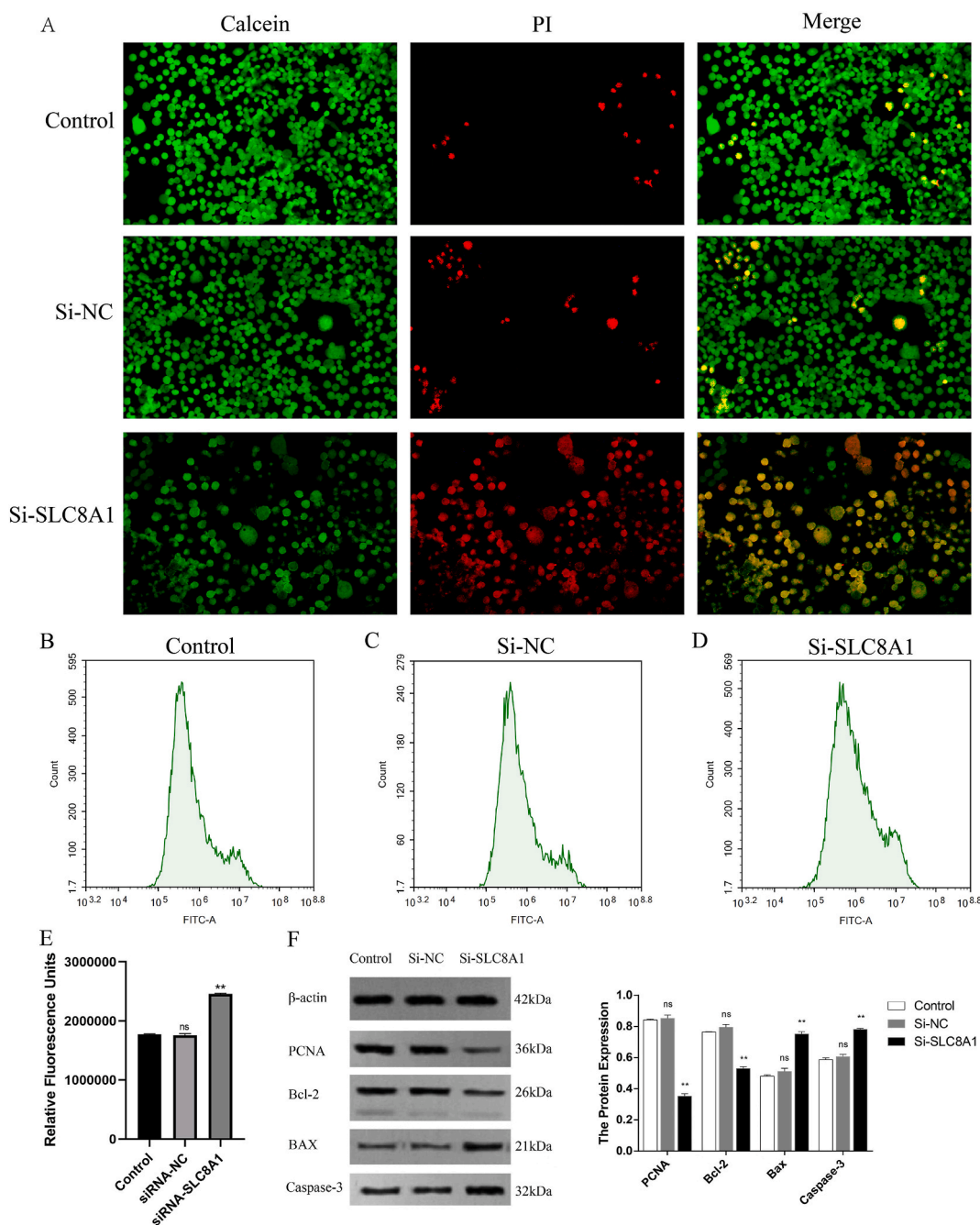


Fig. 15. Cell proliferation, apoptosis and calcium concentration levels were investigated by *in vitro* studies using si-SLC8A1. **A** live/dead stain analysis (200X). **B–E** Calcium concentration levels by flow cytometry analysis. **F** The expression of apoptosis-related and proliferation-related proteins (Caspase-3, PCNA, Bax, Bcl-2) by WB analysis. siRNA-NC vs control; siRNA-SLC8A1 vs siRNA-NC (** $P < 0.01$).

inhibitor, resulted in a notable increase in cell apoptosis in nude mice with PC3 tumours [42].

Multiple studies have demonstrated the abnormal expression of calcium regulatory molecules in patients with endometriosis, adenomyosis, and UCEC [43–45]. These calcium extrusion regulatory molecules in the endometrium include plasma membrane Ca^{2+} ATPases (PMCA) and sodium-calcium exchanger SLC8As, which are secreted into the uterine lumen. In a study conducted by Choi et al. the expression of SLC8A1 in the uterine endometrium of pregnant pigs suggested a potential association between calcium extrusion molecules and pregnancy [46]. In our study, we found that SLC8A1 expression is significantly greater in patients with RSA than in those with a normal endometrium. These findings lead us to speculate that dysregulation of Ca^{2+} homeostasis may contribute to the occurrence and progression of RSA and UCEC. Notably, SLC8A1 is implicated in sodium–calcium exchange and alterations in the

biological behaviour induced by Ca^{2+} under these conditions. The results were verified *in vitro* through experiments. Our experimental results indicated that SLC8A1 may play a role in regulating calcium levels in RSA. Furthermore, the results also indicated that SLC8A1 may play a role in inhibiting apoptosis and promoting proliferation in the context of RSA.

Additionally, our study conducted a comprehensive investigation into the association between SLC8A1 and prognosis through the use of Cox regression, survival curve analysis, and prognostic nomogram analysis. The results of our analysis revealed that SLC8A1 exhibited significant prognostic value. Furthermore, a comparative survival analysis demonstrated that the risk score and stage were independent prognostic factors. In the scope of our research, we also explored the potential correlation between SLC8A1 mutations in the endometrium and the development and progression of UCEC. Notably, an SLC8A1 mutation was identified in 6 % of the low-risk and high-risk groups.

The genetic heterogeneity of tumours contributes to their intricate structure, which uses environmental barriers to enhance their diverse properties and confines the tumour within its microenvironment [47,48]. In addition to tumour cells and immune cells, the tumour microenvironment (TME) comprises stroma and capillaries, forming a complex system that significantly influences the aggressive behaviour of cancerous tumours [49]. Extensive research on the mechanisms driving cancer progression has elucidated the pivotal role played by interactions between genes and immune cells within the TME. Chen et al. conducted a comprehensive analysis to elucidate the expression patterns of TRP family genes and their associations with the TME across different types of cancer [50]. In a separate study, Feng et al. demonstrated a significant positive correlation between CENPT expression and the infiltration of myeloid-derived suppressor cells (MDSCs), whereas a significant negative correlation was observed between CENPT expression and the infiltration of T-cell natural killer (NK) cells in the majority of cancer types [51].

The present investigation aimed to examine the relationship between SLC8A1 and immune cell infiltration, as well as the association between SLC8A1 and the immune microenvironment across cancers. We conducted an investigation to determine whether there were notable disparities in the stromal score, immune score, and ESTIMATE score between immune cell infiltrates categorised as high or low. Intriguingly, our analysis revealed a significant correlation between SLC8A1 and these three scores. Additionally, we proceeded to examine the association between SLC8A1 expression and immune infiltration. Our findings revealed a significant correlation between SLC8A1 expression and resting memory CD4^+ T cells, CD8^+ T cells, M0 macrophages, and M1 macrophages. These results provide a foundation for further exploration of the underlying mechanisms involved. The strong correlation between SLC8A1 expression and M1 macrophages, as well as its negative correlation with M2 macrophages, is of particular interest. The infiltration of macrophages is widely recognised to be associated with favourable survival outcomes and serves as a prognostic indicator. In addition to the aforementioned survival analysis, the potential utility of SLC8A1 agonists in the treatment of endometrial hyperplasia, endometrial cancer, endometriosis, and abnormal endometrium warrants consideration. Finally, an examination was conducted to determine whether a correlation exists between SLC8A1 expression and drug sensitivity. Low-risk patients exhibit increased sensitivity to osimertinib, dasatinib, sepantronium bromide, ibrutinib, and other drugs. Our study has demonstrated the promising prognostic potential of the SLC8A1-related prognostic signature in guiding clinical therapeutic decisions.

However, it is imperative to acknowledge the existing limitations of this study that necessitate further investigation. Additional experiments are indispensable to authenticate the exact involvement of SLC8A1 in RSA and elucidate its underlying mechanisms. Furthermore, more extensive research is warranted to obtain a comprehensive understanding of how SLC8A1 impacts the microenvironment of UCEC. Nonetheless, the outcomes of our study could aid in risk stratification and guide individual treatment options for RSA and UCEC in clinical settings.

5. Conclusions

In our study, we investigated the expression and function of SLC8A1 in RSA and UCEC while also exploring its associations with clinical characteristics. This analysis was approached from a bioinformatics perspective, marking the first instance of such an examination. Notably, our findings demonstrated the superior ability of SLC8A1 to predict survival, the TME, and the response to immunotherapy, chemotherapy, and targeted therapy in patients with UCEC compared with clinical applications.

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Data availability statement

The datasets generated for this study can be found in the GEO dataset: <https://www.ncbi.nlm.nih.gov/geo/>; <http://tcga-data.nci.nih.gov/tcga/>. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

Review and approval by an ethics committee was not needed for this study because [this study involves human data from public databases such as GEO and TCGA].

CRediT authorship contribution statement

Ji-jun Chu: Writing – original draft, Project administration, Funding acquisition. **Xiu-juan Qin:** Visualization, Methodology, Data curation. **Wenting Chen:** Validation, Resources. **Zhen Xu:** Software, Investigation, Formal analysis. **Xian-jin Xu:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

RSA	recurrent spontaneous abortion
GEO	Gene Expression Omnibus database
qRT-PCR	quantitative real-time PCR
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
PPI	protein protein interaction
scRNA-seq	single-cell RNA sequencing
UCEC	Uterine Corpus Endometrial Carcinoma
ssGSEA	single-sample gene set enrichment analysis
WGCNA	Weighted Gene Co-expression Network Analysis
PTS	prethrombotic state
URSA	unexplained recurrent spontaneous abortion
RNA-seq	RNA sequencing
RRBS	reduced representation bisulfite sequencing
ME	module signature genes
PCA	Principal Component Analysis
DEGs	differentially expressed genes
UMAP	uniform manifold approximation and projection
ICPs	immune checkpoints
GDSC	Genomics of Drug Sensitivity in Cancer
CCK-8	Cell Counting Kit-8
WB	Western Blot
ECL	electrochemiluminescence
GS	Gene Significance
MM	Module Membership
OS	overall survival
C-index	concordance index
SNPs	single nucleotide polymorphisms
TME	tumor microenvironment
MDSCs	myeloid-derived suppressor cells

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2024.e36899>.

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