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ORIGINAL RESEARCH

Crosstalk of *SPINK4* Expression With Patient Mortality, Immunotherapy and Metastasis in Pan-Cancer Based on Integrated Multi-Omics Analyses

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Background: Cancer remains a major global health challenge, with early detection and prompt treatment being crucial for reducing mortality rates. The *SPINK4* has been linked to the development of several tumors, and there is growing evidence of its involvement. However, its specific functions and effects in different cancer types remain unclear.

Methods: The association between *SPINK4* expression levels and tumor progression was investigated and confirmed using the TCGA dataset. Kaplan-Meier curves were utilized to examine the correlation between *SPINK4* expression with survival outcomes in pan-cancer patients. The Pearson method was employed to investigate the association of *SPINK4* expression with the tumor microenvironment, stemness score, immunoinfiltrating subtype, and chemotherapy sensitivity in human different cancer types. Wound healing and Transwell assays were performed to confirm the roles of the model gene in colon adenocarcinoma cells.

Results: The expression of *SPINK4* shows heterogeneity across pan-cancer tissues, and is closely associated with poor prognosis, immune cell invasion, tumor cell resistance, and tumor metastasis in a various human cancer. Mutation of *SPINK4* hold significant predictive value for poor prognosis of pan-cancer patients. In addition, *SPINK4* expression was significantly correlated with the tumor microenvironment (stromal cells and immune cells) and stemness score (DNAss and RNAss) in human pan-cancer tissues, particularly in BLCA and COAD. Single-cell sequencing analysis showed that SPINK4 is mainly expressed in endothelial cells in BLCA and in malignant cells in COAD. Drug resistance analysis showed a significant association between *SPINK4* expression and sensitivity to several cancer chemotherapy drugs. Importantly, overexpression of *SPINK4* promoted the metastasis of colon cancer cell lines (HCT116 and RKO), whereas *SPINK4* knockout markedly inhibited their metastasis.

Conclusion: These findings reveal the crucial role of *SPINK4* in the pan-cancer process and may have significant implications for the diagnosis and treatment of cancer in the future.

Keywords: pan-cancer analysis, immune infiltration, genetic alteration, single-cell sequencing, drug sensitivity

Introduction

Cancer is primarily driven by oncogenic and/or germline alterations in oncogenes and tumor suppressor genes. Oncogenic alterations play important roles in tumor development, including generating increased proliferative signals, conferring resistance to cell death, bypassing replication limits, and causing increased genomic instability.[1] The unlimited proliferation, invasion and metastasis, heterogeneity, and immune escape properties of tumors have led to the treatment of cancer becoming an insurmountable clinical problem and posing a significant threat to global public health security. In

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the beginning (before 1970), surgery was the main treatment modality for cancer. With the advancement of science and technology and the emergence and improvement of various treatment modalities for cancer, such as radiotherapy, chemotherapy, hematopoietic stem cell transplantation, and targeted therapy, the prognosis of cancer has improved compared to the previous period.[2] In addition, since cancer is a genetic disease driven by oncogenic alterations, targeted therapy targeting oncogenic altered genes and related signaling pathways will continue to be an important form of cancer treatment in the foreseeable future.[3]

Colorectal cancer (CRC) is a common malignancy and the fourth most deadly malignancy worldwide, and a major contributor to cancer-related incidence and case-fatality rates.[4,5] According to incomplete statistics, more than 900,000 individuals die from bowel cancer annually.[6] It is estimated that the global incidence of CRC could reach 2.5 million cases by 2035.[7] Currently, the primary treatment for early-stage CRC is still surgery, but most patients have already metastasized by the time of diagnosis, leading to a discouraging prognosis. With the advent of targeted therapies, treatment strategy for CRC are becoming more precise.[5] However, only a restricted proportion of tumors have specific targets, and treatment outcomes are far from optimal.[6] Therefore, there is an urgent need to identify additional diagnostic biomarkers and therapeutic targets to monitor and improve the long-term prognosis of CRC patients.

Serine proteases are an important family of proteinases in eukaryotic cells that serve a crucial role in the regulation of gene expression by cleaving peptide bonds in proteins.[8] They are divided into two main types (trypsin-like and subtilisin-like serine proteases) based on structural differences. Serine proteases have been implicated in several diseases, including aging, Alzheimer's disease and cancers.[9–11] More recently, whole genome analyses have identified serine proteases and regulators of the complement system as common dysfunctions in certain cancers.[12] These discoveries provide many potential targets for cancer treatment, including molecules that regulate serine proteases such as trypsin and the serine protease inhibitor Kazal-type families (SPINK).The SPINK family has several members that have been identified and characterized, including SPINK1 - SPINK12.[13] Some members of the SPINK family are involved in the pathogenesis of various tumours. For example, SPINK1 is synthesized and secreted by human pancreatic follicular cells. Studies have shown that mutations in the gene are closely related to autophagy, the epidermal growth factor, the pathogenesis of pancreatitis, and tumour development and prognosis.[14] SPINK2, which is mainly synthesized in the testis and seminal vesicles and is involved in the reproductive process, has its expression closely associated with lymphoma and hepatocellular carcinoma.[15] The SPINK5 gene, on the other hand, encodes a serine protease containing 15 potential inhibitory domains, which can exhibit unique activity and specificity.[16] Its expression is markedly elevated in oral squamous - cell carcinoma and correlates with the prognosis of this cancer.[17] However, knowledge about SPINK4 remains limited.

SPINK4, originally discovered in the pig intestine, is involved in the negative regulation of endopeptidase activity and the response to external stimuli.[18] This gene is critical for protecting against protein degradation in mucosal and epithelial tissues, contributing to processes such as embryonic development, cell growth and maintenance of internal balance.[19–21] Several studies have shown that SPINK4 expression is associated with tumor drug resistance and invasion. For instance, SPINK4 promotes cancer cell proliferation by inhibiting ferroptosis in rectal cancer, and its expression is related to tumor tissue classification, staging and poor prognosis.[22,23] This suggests that SPINK4 not only plays a key role in the regulation of proteasomes, but also has potential for clinical evaluation. Therefore, exploring the comprehensive role of SPINK4 in pan-cancer is both valuable and necessary to better understand its role in cancer development and its clinical therapeutic potential. In addition, there is an increasing focus on the role of SPINK4 in promoting tumor immune evasion and suppression.[24] It is vital to investigate the potential links between SPINK4 expression and the tumor immune infiltration.

In this study, a comprehensive analysis of SPINK4 expression patterns in pan-cancer was performed by integrating bioinformatics and experimental validation. We also investigated the potential correlation of SPINK4 expression with the tumor microenvironment (TME), stemness scores, immune infiltration subtypes and drug sensitivity in different human cancers. Additionally, we analyzed the potential significance of SPINK4 genetic alterations in assessing the poor prognosis of pan-cancer and evaluated the effect of its expression on the proliferation, invasion and migration of tumor cells, particularly in CRC. Our findings reveal the important role of SPINK4 in tumor biology and provide a valuable contribution to the future clinical treatment of cancer by targeting SPINK4.

Materials and Methods

Data Mining and SPINK4 Expression Analysis

Based on the Cancer Genome Atlas (TCGA) database (a public online tumor database, https://portal.gdc.cancer.gov/), we obtained data of 33 types of human cancers, including RNAseq (HTSeq - FPKM), clinical parameters, immune subtypes and stemness scores.[25] The main information of the different tumors is shown in Table S1. Additionally, using the online database (TIMER2, http://timer2.cistrome.org/), we investigated *SPINK4* expression in human pancreatic cancer compared to normal tissue. In addition, immunohistochemical images of SPINK4 were retrieved from the Human Protein Atlas database (https://www.proteinatlas.org/search/SPINK4). The GEPIA2 database (http://gepia2.cancer-pku.cn/#index) was used to determine the correlation between *SPINK4* expression and pathological tumor staging. Then, using the resources of the TNM plotter (https://tnmplot.com/analysis/), we also investigated SPINK4 expression differences among normal tissues, tumor tissues, and metastatic tissues.

Survival and COX Regression Analysis

Using GEPIA2, we obtained the overall survival (OS) and disease-free survival (DFS) curves for patients with different tumors.[26] The median expression level of *SPINK4* was then selected as the intergroup threshold. The survival curve was compared with the log-rank and the hazard ratio (HR) was displayed. The COX univariate regression module was used to investigate the relationship between gene expression and clinical prognosis of human malignant tumors (including OS, DSS, DFI, PFI). The R package "forestplot" was used to construct the forest plot, and the relationship between *SPINK4* expression and tumor patient survival was evaluated using HR, with HR <1 representing low risk and HR >1 representing high risk. A log-rank p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

Tumor Microenvironment (TME) Analysis

The "ESTIMATE algorithm" was used to analyze the correlation between gene expression and TME in human cancers by calculating stromal score and immunescore. The Pearson correlation coefficient was then used to determine the association between TME and gene expression. The R-packages "ggpubr" and "limma" were used to visualize the analysis results. R represents the correlation coefficient, where $R > 0$ indicates a positive correlation and $R < 0$ indicates a negative correlation. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Immunoinfiltration Subtypes and Stemness Score Analysis

To evaluate the correlation of *SPINK4* expression with patients' immunoinfiltration subtypes and stemness score, TCGA clinical data were employed. This included downloading and utilizing the latest software package updates for clinical data corresponding to each cancer sample. The immunoinfiltration subtypes included six categories, labeled C1 through C6.[27] The R-packages "limma" and "reshape2" were used for data analysis after eliminating missing values (NA). The visualization of results presented that the R packages "ggplot2" and "Wilcox.test" were use to identify significant differences. During the stemness score analysis, the Pearson method was applied to investigate the correlation between *SPINK4* expression and DNA stemness scores (RNAss) as well as RNA stemness scores (DNAss). R represents the correlation coefficient, with $R > 0$ representing a positive correlation and $R < 0$ representing a negative correlation. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Immune Cell and Single-Cell RNA (scRNA) Analysis

The R packages "ggplot2" and "ggpubr" were used to investigate the correlation between *SPINK4* expression and immune cell infiltration in tumor tissues. The immune cells analyzed mainly included B cells, CD4 T cells, CD8 T cells, macrophages, neutrophils and dendritic cells (DC). The Pearson correlation coefficient was employed to determine the association between TME and gene expression. R represents the correlation coefficient, with $R > 0$ representing a positive correlation and $R < 0$ representing a negative correlation. A p-value of less than 0.05 ($p<0.05$) was considered statistically significant. For single-cell RNA analysis, we used interactive single-cell transcriptome visualization to explore the association between gene expression and TME through a comprehensive network resource, the Center for

Single-Cell Tumor Immunity (TISCH2, http://tisch.comp-genomics.org/). The Datasets module was used to visualize *SPINK4* expression at the single-cell level in the BLCA_GSE130001 and COAD_GSE146771 datasets. These datasets are publicly available online.

Genetic Alterations Analysis in Pan-Cancer

To integrate genetic alterations data across different cancer types, we investigated the mutation patterns of *SPINK4* in human pan-cancer based on the online database (https://www.cbioportal.org/). Patients were subsequently divided into SPINK4 altered and unaltered groups through data filtering. The association between *SPINK4* mutations and clinical outcomes of patients was then explored by constructing Kaplan-Meier survival curves. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Drug Sensitivity Analysis

To evaluate the correlation between gene expression and drug sensitivity, CellMiner™ datasets were employed.[28] This involved downloading and utilizing the latest package updates for drug sensitivity data corresponding to the RNAseq expression profiles of 60 different human cancer cell lines. The R packages "limma", "ggpubr", and "ggplot" were employed to investigate the association between *SPINK4* expression with drug sensitivity analysis. Correlation analysis was carried out by the Pearson method. R represents the correlation coefficient, with $R > 0$ representing a positive correlation and $R < 0$ representing a negative correlation. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Cell Culture, Plasmid and Transfection Assay

The human COAD cell lines RKO and HCT116 were obtained from the research group of Junjiang Fu, at the Center for Basic Medical Research, Southwest Medical University (Luzhou, China). The human cells lines and the specimens of COAD were collected with the permission of the Ethics Committee of Jilin province people's hospital, Jilin, China (approval no. 2024129). Under 37°C and 5% CO_2, these COAD cells were maintained in Dulbecco's modified eagle medium (DMEN) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin. The overexpression plasmid and knockdown plasmid against *SPINK4* were synthesized and purchased from the Public Protein/Plasmid Library (Nanjing, China). The plasmid sequences are provided in Table S2. Lipofectamine 3000 (Invitrogen, L3000015) was used for cell transfection.

RNA Extraction and Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from cells using the MolPure® Cell/Tissue Total RNA kit according to the manufacturer's instructions. cDNA for RT-PCR was reverse transcribed by Hifair®III 1st Strand cDNA Synthesis Kit (gDNA digester plus) according to the supplier's guidelines with ACTIN as a control. The primer sequences are provided in Table S3.

Wound Healing Assay

The cells were inoculated into 6-well plates with a density of 6×10^5 cells/well and were transfected with 6 cells/well, empty plasmid and shSPINK4 plasmid,6 cells/well, respectively the next day. Transfected RKO and HCT116 cells were then scraped, and the suspensions were rinsed with PBS. Cell migration images were obtained from an inverted microscope at 0, 6, 12 and 24 hours after incubation. Monolayer cells were scraped with the tip of a 200 μL straw before rinsing the suspension with phosphate buffered saline (PBS). Cell migration images were obtained from an inverted microscope at 0, 6, 12 and 24 hours after incubation.

Transwell Migration and Invasion Assay

For the migration experiment, the cells were suspended in 200 μL serum-free medium and inoculated in the upper chamber of the transposition pore. Subsequently, 500 μL DMEM and RPMI-1640 medium containing 20% serum were added to the lower chamber, respectively. For invasion assay, the matrix glue was diluted with serum-free medium 1:8

before inoculation, then coated with the upper chamber. After 48 hours, the cells were first fixed and stained through the bottom of the lower chamber, and then photographed and counted.

Statistical Analysis

The experimental data were analyzed with GraphPad Prism 8.0 software. Data were expressed as the mean ± standard deviation (mean ± SD) of at least three independent experiments. The Student's *T*-test was used to analyze the paired samples, and ANOVA was performed to detect the unpaired samples. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant. *p< 0.05, **p< 0.01, ***p< 0.0001.

Results

Expression Patterns of SPINK4 in Common Cancers

To investigate the expression of *SPINK4* in different human tissues, we observed that it was predominantly expressed in the human digestive tract (especially in female), indicating tissue-specific expression (Figure 1A). Analysis of its structure and subcellular localization showed that SPINK4 was mainly expressed in the cytoplasm (Figure 1B). Subsequently, we used RNA-seq data obtained from the TCGA database to evaluate *SPINK4* expression in cancer samples compared to normal groups. The results revealed a significant increase in *SPINK4* expression levels in several

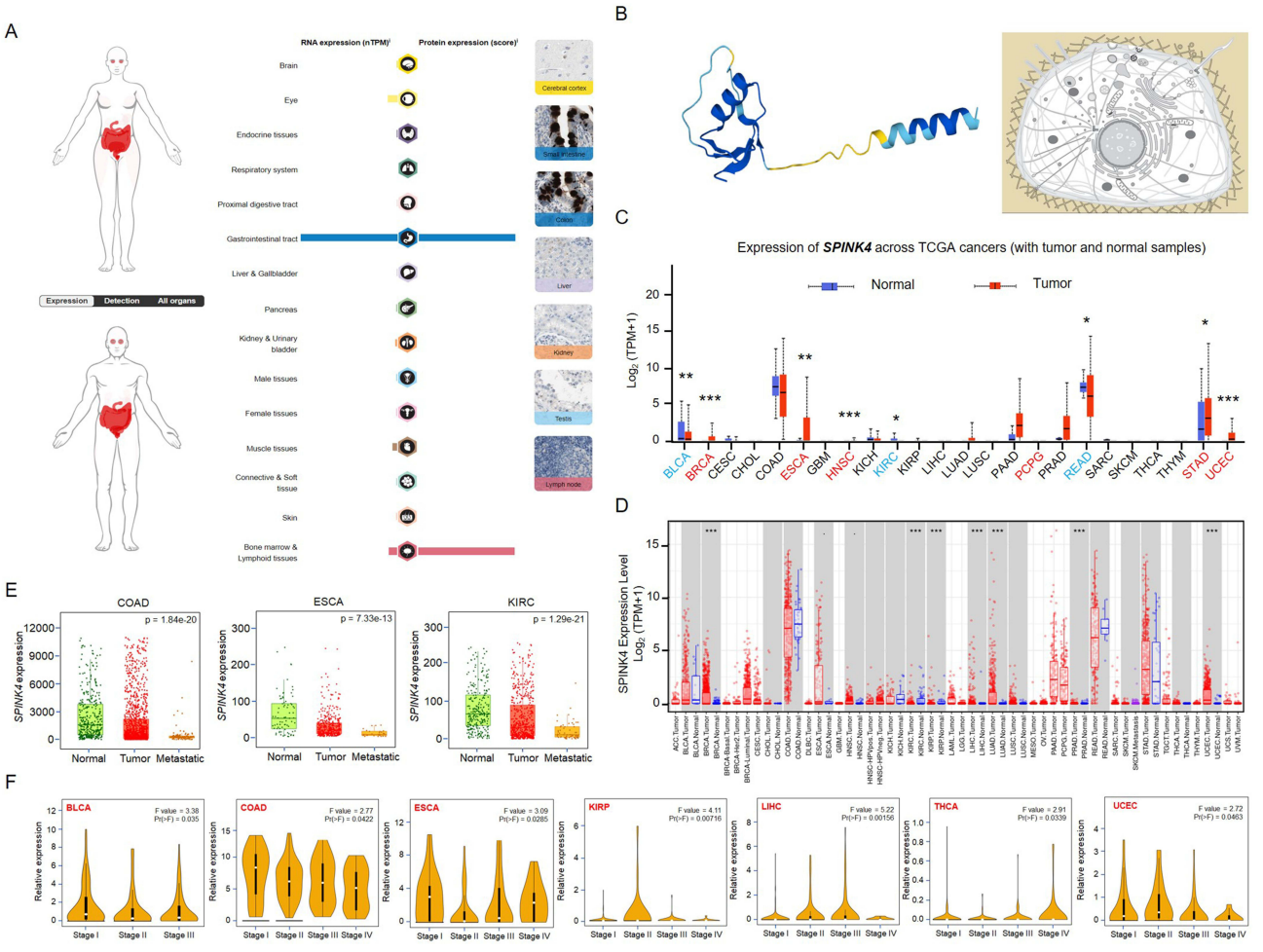

Figure 1 Expression level of *SPINK4* in pan-cancer. (**A**) Summary of mRNA and protein expression. (**B**) The protein structure and subcellular localization of SPINK4 (**C**) *SPINK4* expression status in different types of cancers from TCGA (**D**) The expression level of *SPINK4* in tumor and normal tissues via TIMER 2.0. (**E**) Differences in *SPINK4* expression between normal, tumor, and metastatic tissues among COAD, ESCA, and KIRC. (**F**) The expression of *SPINK4* in different types of cancers (BLCA, COAD, ESCA, KIRP, LIHC, THCA, and UCEC) were analyzed according to pathological stage (stage I, stage II, stage III, and stage IV). The $p < 0.05$ was considered to be statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001.

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common cancers, including BRCA, ESCA, HNSC, PCPG, STAD and UCEC (Figure 1C). To validate these findings, we re-examined *SPINK4* expression using the TIMER database. The expression of *SPINK4* was significantly upregulated in LIHC, LUAD and UCEC, while it was down-regulated in BLCA and COAD (Figure 1D). In addition, we observed that *SPINK4* expression was low in some common metastatic tumors, such as COAD, ESCA and KIRC (Figure 1E). Interestingly, we also found a significant correlation between SPINK4 expression and the pathological stage of various tumors such as BLCA, COAD, ESCA, KIRP, LIHC, THCA and UCEC (Figure 1F). These findings emphasize substantial expression changes of *SPINK4* in common cancers, suggesting its possible oncogenic role in various cancers.

Effect of SPINK4 Expression on Unfavorable Prognosis of Pan-Cancer

To assess the association between *SPINK4* expression and cancer prognosis, Cox regression analysis was performed. The results revealed a correlation between *SPINK4* expression and the prognosis of various tumors. For overall survival (OS), it was a protective prognosis factor in BLCA and COAD (HR<1, LogRank<0.05), but an adverse risk in LIHC (HR>1, LogRank<0.05) (Figure 2A). Additionally, in disease-specific survival (DSS), *SPINK4* was a low-risk gene for prognosis in BLCA, but unfavorable prognosis factor in LGG and LUSC (Figure 2B). And in disease-free interval (DFI), *SPINK4* was only a detrimental prognostic factor for LIHC (Figure 2C). Conversely, in progression-free interval (PFI), *SPINK4* acted as a low-risk gene affecting the prognosis of LIHC, but a protective factor affecting the prognosis of BLCA and BRCA (Figure 2D). We also explored the correlation between *SPINK4* expression with OS in patients with different cancer types (Figure 2E). The results showed that lower *SPINK4* expression was associated with poorer OS in BLCA (Figure 2F) and COAD (Figure 2G). Similar results were validated in the Kaplan-Meier database (Figure 2H and I) The acquired findings indicated that *SPINK4* expression levels could be considered as a useful prognostic biomarker in some cancers, particularly in BLCA and COAD.

The Significance of SPINK4 Genetic Alteration in Pan-Cancer

To further explore the mutation pattern of *SPINK4*, we investigated its genetic alteration across different cancers using the cBioPortal database. The results indicated that the amplification pattern of *SPINK4* was present in most common cancer types, particularly in LUSC (Figure 3A). By analyzing the comprehensive mutation data related to important domains of *SPINK4* in different cancer backgrounds, we found a higher frequency of relative missense mutations (Figure 3B). Subsequently, we analyzed the effect of *SPINK4* genetic alteration on the prognosis of different malignant tumors. Acquired results suggested that *SPINK4* genetic alterations were significantly associated with OS (p=0.00846, Figure 3C) and DSS (p=0.0124, Figure 3D), but not with PFS (p=0.112, Figure 3E) and DFS (p=0.636, Figure 3F). These findings indicate that *SPINK4* genetic alterations are linked to poor outcomes in various human cancers.

Correlation of SPINK4 Expression and Immune and Stemness Score in Pan-Cancer

To further explore the correlation between *SPINK4* expression and the TME, we utilized the ESTIMATE algorithm to calculate stromal and immune scores. The analysis showed that *SPINK4* expression was significantly positively or negatively correlated with stromal score, immune score and ESTIMATE score in pan-cancer (Figure 4A), as well as with DNAss and RNAss (Figure 4B). After that, we focused on the association of *SPINK4* expression with these tumor microenvironments. The observations revealed a significant negative correlation of *SPINK4* expression with stromal score, immune score, and ESTMATE score in BLCA (Figure 4C). Conversely, its expression was positively correlated with DNAss and RNAss in BLCA (Figure 4D). However, we did not observe a significant correlation of *SPINK4* expression with stromal score, immune score, and ESTMATE score in COAD (Figure 4E), as well as DNAss, and RNAss (Figure 4F).

Effect of SPINK4 Expression on Immune Cell Infiltration and Clinical Diagnosis

We previously estimated the correlation between *SPINK4* expression and immune score in BLCA and COAD. After that, we further investigated the effect of its expression on immune subtype. The results showed that *SPINK4* expression was significantly correlated with the immunological subtypes of BLCA and COAD (Figure 5A and B). In addition, we explored the diagnostic value of *SIPNK4* expression. Compared to BLCA (AUC=0.505, Figure 5C), *SIPNK4* expression had a higher

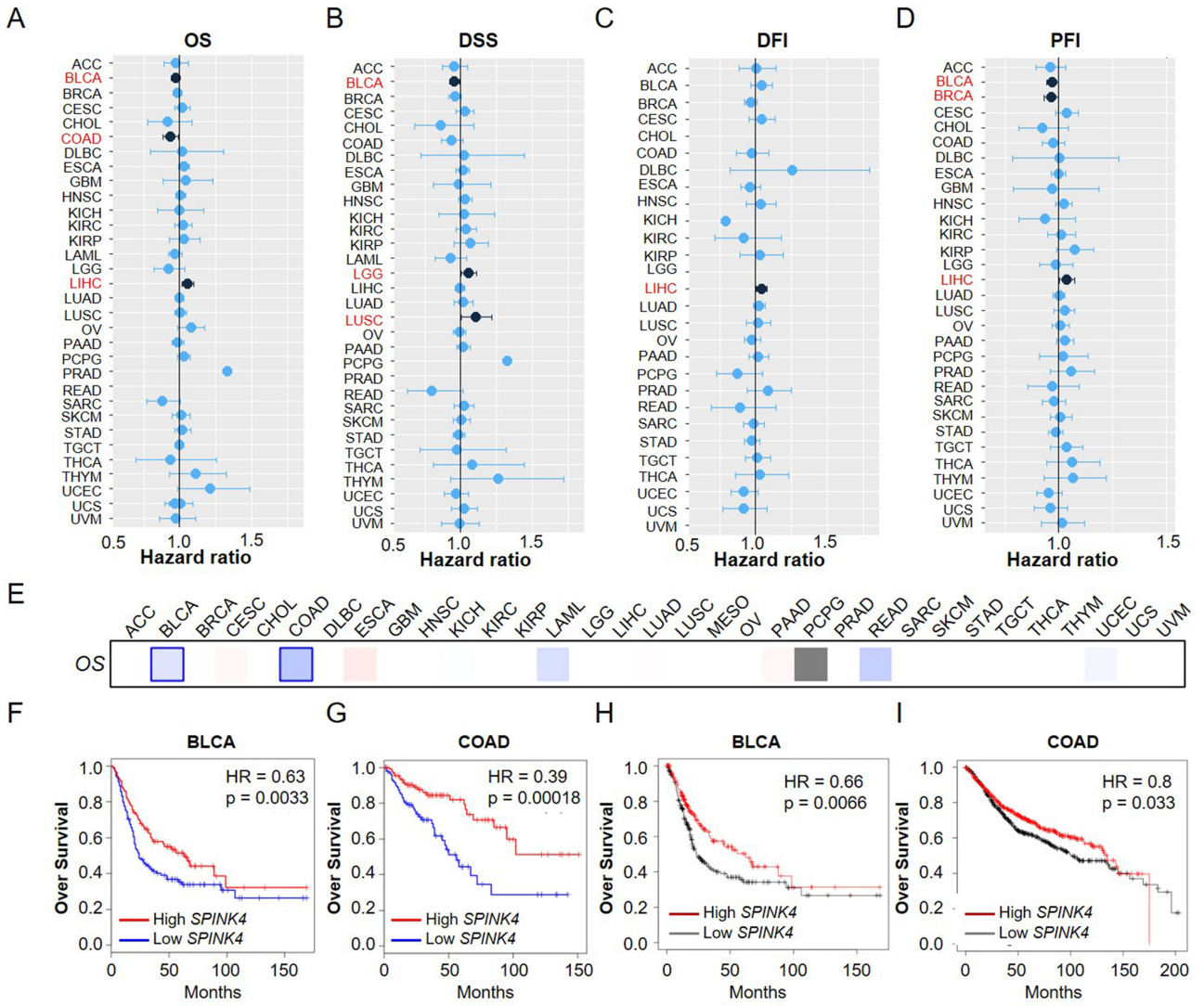

Figure 2 Correlation between *SPINK4* expression and overall survival in patients with different cancer types. (**A**) Cox analysis of the correlation between *SPINK4* expression and OS in different cancer types. (**B**) The correlation of *SPINK4* expression with DSS in different cancer types. (**C**) The correlation between *SPINK4* expression and DFI in different cancer types. (**D**) Cox analysis of the correlation of *SPINK4* expression with PFI in different cancer types. (**E**) Using GEPIA2 to construct an overall survival (OS) map of *SPINK4* expression. (**F**) Kaplan–Meier survival curves (OS) of patients with high and low *LDHA* expression in BLCA. (**G**) Kaplan–Meier survival curves (OS) of patients with high and low *LDHA* expression in COAD. (**H**) Overall survival curves comparison of high or low expression of *SPINK4* in BLCA by using Kaplan-Meier Plotter database. (**I**) Overall survival curves comparison of high or low expression of *SPINK4* in COAD by using Kaplan-Meier Plotter database.

diagnostic value in COAD (AUC=0.615, Figure 5D). Considering the association between immune cell infiltration and tumor progression, we performed a correlation analysis of different immune cells. The results showed that in BLCA, the expression of *SPINK4* was negatively correlated with the presence of CD4 T-cells, CD8 T-cells, neutrophils, macrophages and dendritic cells, with the exception of B-cells (Figure 5E). However, in COAD, its expression was significantly positively correlated with B cells, CD8 T cells, neutrophils and dendritic cells, except with CD4 T cells and macrophages (Figure 5F). However, single-cell RNA-seq (scRNA) sequencing revealed that *SPINK4* was mainly expressed in endothelial cells in BLCA (Figure 5G), while it was mainly expressed in malignant cells in COAD (Figure 5H). This finding suggests that *SPINK4* plays an important role in regulating immune cell infiltration, contributing to the malignant progression of tumors.

Effect of SPINK4 Expression on Tumor Sensitivity to Chemotherapy

Drug resistance to chemotherapy poses a significant challenge in cancer therapy.[29] Improving drug sensitivity is an crucial strategy to prevent tumor cells from developing drug tolerance.[30] Therefore, we further investigated the

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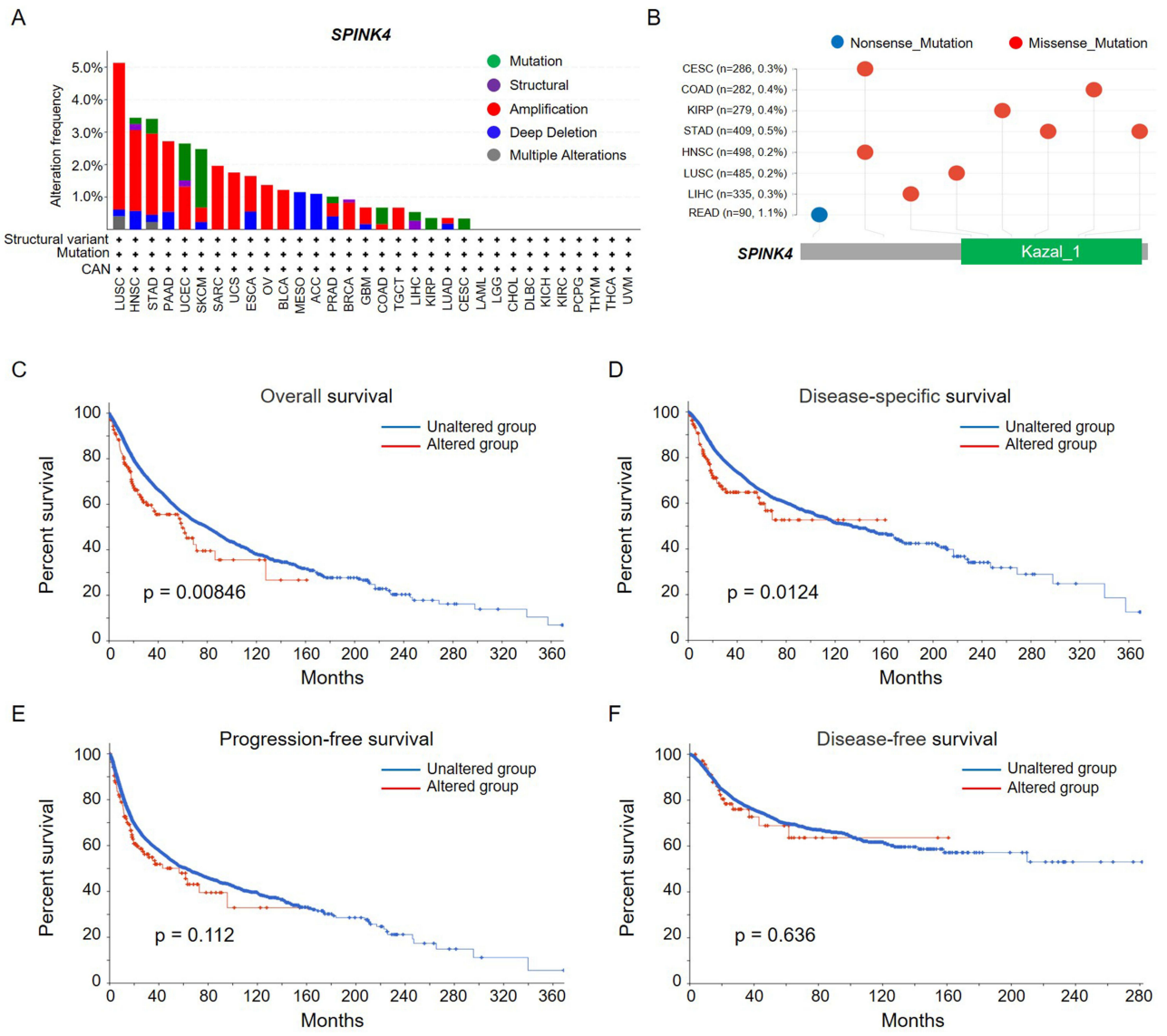

Figure 3 Analysis of *SPINK4* genetic alterations in different types of cancers. (**A**) The frequency of *SPINK4* mutations with mutation type across TCGA cancers by cBioPortal. (**B**) Oncoprint of *SPINK4* alterations in cancer cohorts. (**C-F**) The associations of pan-cancer *SPINK4* mutation status with OS, DSS, DFS and PFS by cBioPortal.

correlation between *SPINK4* expression and the susceptibility of 60 human cancer cell lines (NCI-60) to more than 200 chemotherapeutic agents. The results revealed that *SPINK4* expression was significantly positively correlated with the sensitivity to multiple drugs, including TYROTHRICIN, TAK-901, Perifosine, sitravatinib, PF-562271, Altiratinib, KHK-Lndazole, Crizotinib, Rebastinib, Foretinib, and SNS-314 (Figure 6A–K). In contrast, *SPINK4* expression was significantly negatively associated with the sensitivity to Mitomycin, CAMPTOTHEN, Camptothecin, Topotecan, Floxuridine, Cisclatin, Bleomycin, and Gemcitabine (Figure 6L–S). These findings suggest that *SPINK4* expression is closely related to the sensitivity of certain chemotherapy drugs, providing important reference value for future cancer treatment strategies.

Effect of SPINK4 Expression on Epithelial-Mesenchymal Transformation (EMT) of Colon Cancer

We have previously demonstrated a correlation between *SPINK4* expression and different tumors in humans. Given that *SPINK4* has a higher diagnostic value in COAD, we selected the colon cancer cell lines (RKO and HCT116) for

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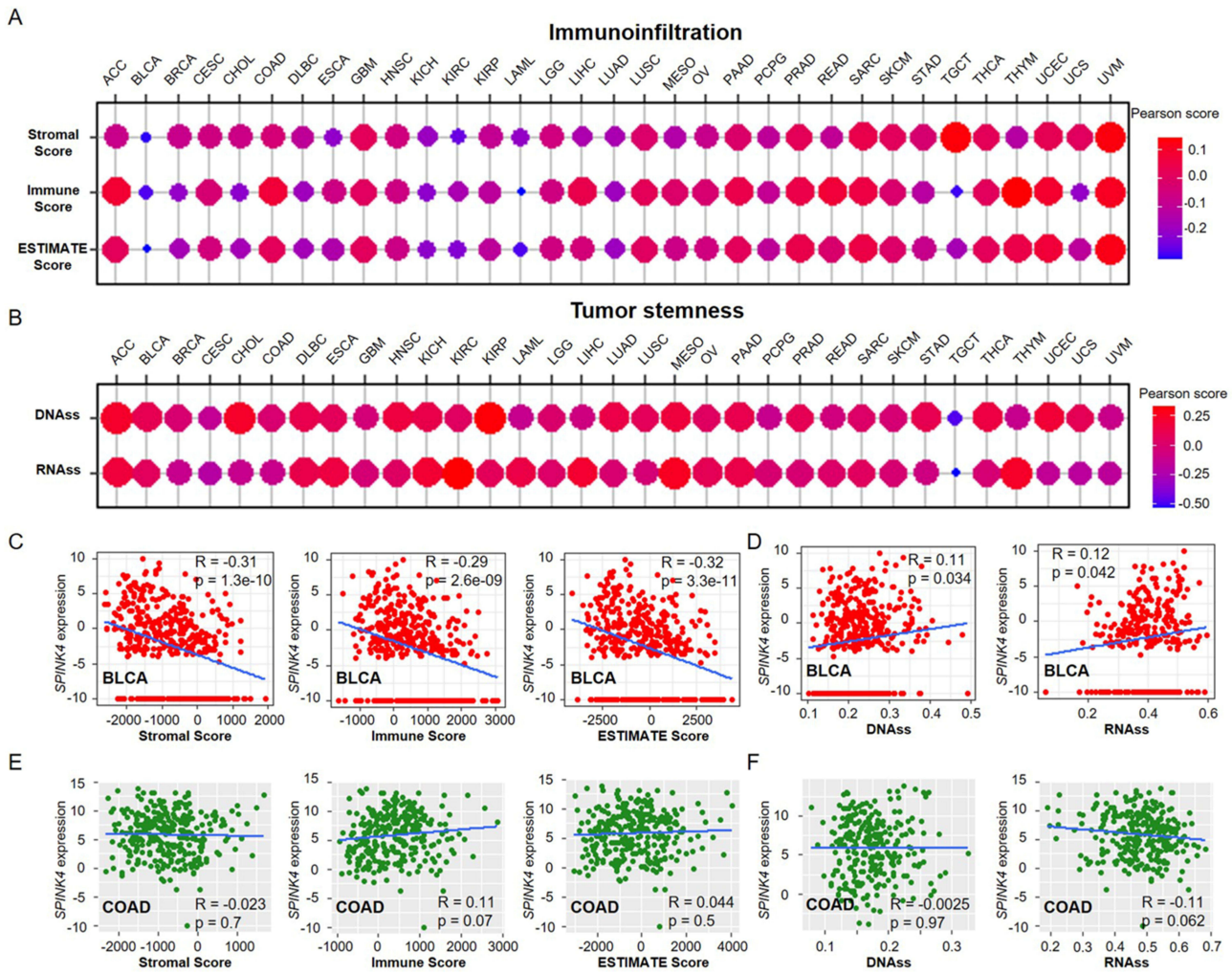

Figure 4 Correlation of *SPINK4* expression with tumor microenvironment and stemness score in different types of cancers. (**A**) The correlation of *SPINK4* expression with stromalscore, immunscore and ESTIMATEscore. (**B**) The correlation of *SPINK4* expression with DNAss and RNAss. (**C**) The expression of *SPINK4* correlated with stromal score, immune scores, and ESTIMATE scores in BLCA. (**D**) The expression of *SPINK4* correlated with DNAss and RNAss in BLCA. (**E**) The expression of *SPINK4* correlated with stromal score, immune scores, and ESTIMATE scores in COAD. (**F**) The expression of *SPINK4* correlated with DNAss and RNAss in COAD. Gray brown background indicates no correlation between the gene expression and the corresponding index ($p > 0.05$). Light background indicates that the gene is significantly correlated with the corresponding index ($p < 0.05$). R represents correlation value, $R > 0$ means positive correlation, $R < 0$ means negative correlation.

experimental verification. With the help of the HPA database, we found that *SPINK4* protein was down-regulated in COAD tissues compared with the normal tissues (Figure 7A). Subsequently, the knock-out efficiency of *SPINK4* in cells was verified by RT-PCR assay (Figure 7B and C). Among them, we found that plasmid $2^{\#}$ demonstrated effective knockdown and was selected for subsequent experiments. Wound-healing experiments revealed that SPINK4-silencing significantly promoted cell migration in both RKO and HCT116 cells (Figure 7D–F). To further investigate the effect of *SPINK4* expression on cell invasion and migration, a transwell assay was performed (Figure 7G). Quantitative analysis showed that down-regulation of *SPINK4* promoted the invasion and migration of RKO (Figure 7H and I) and HCT116 cells (Figure 7J and K). Conversely, *SPINK4* overexpression significantly inhibited the wound-healing rate of RKO cells (Figure 8A and B) and HCT116 cells (Figure 8A and C) compared to the control group. After that, a transwell assay was performed to observe the invasion ability of the cells (Figure 8D). The results showed that *SPINK4* up-regulation inhibited migration and invasion of RKO and HCT116 cells compared to the control group.

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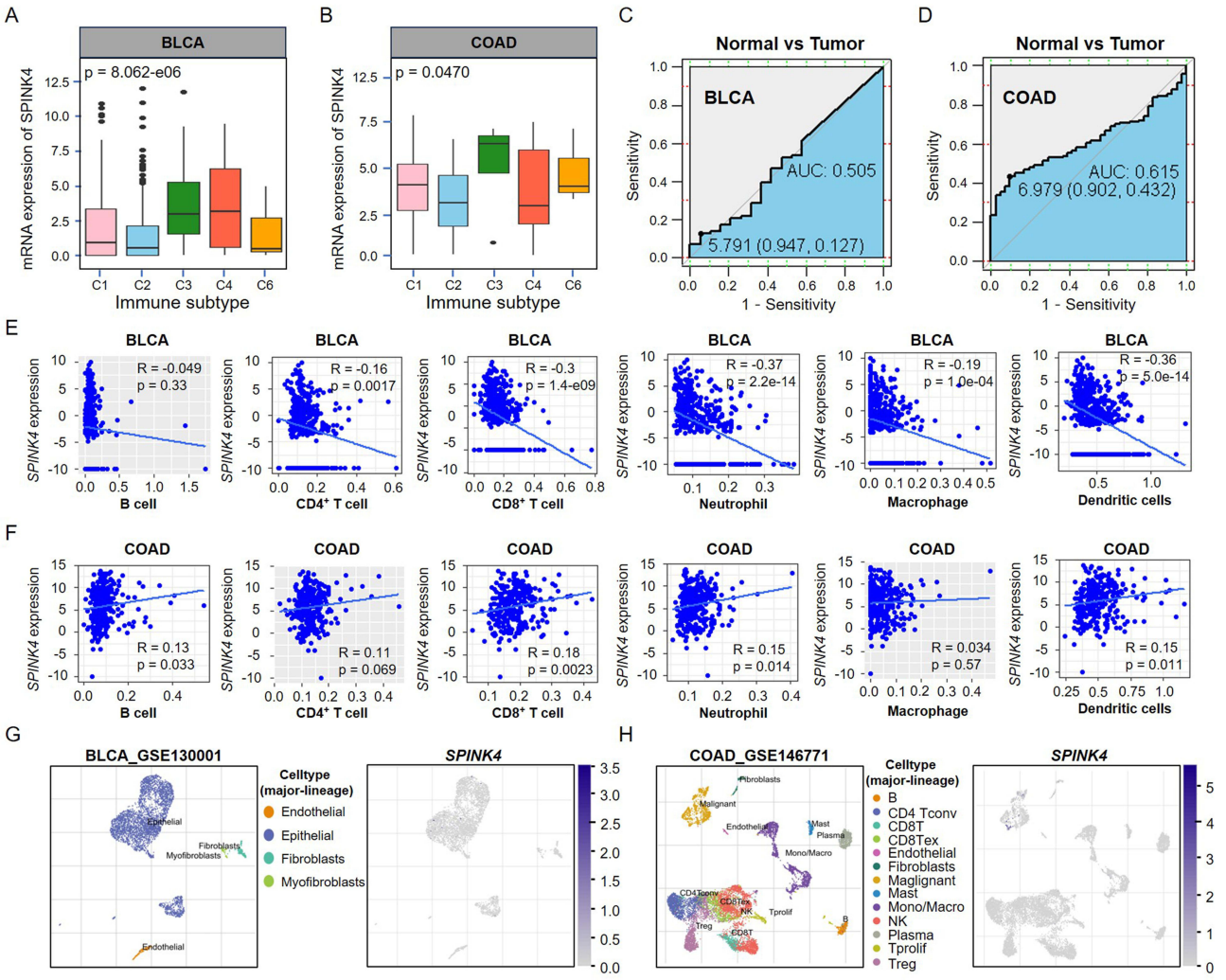

Figure 5 Association of *SPINK4* expression with immune infiltration subtypes in BLCA and COAD. (**A**). One-way analysis of variance was performed to test the correlation between *SPINK4* expression and BLCA immune infiltration subtypes. (**B**) One-way analysis of variance was performed to test the correlation between *SPINK4* expression and COAD immune infiltration subtypes. (**C**) Diagnostic value of *SPINK4* expression in BLCA. (**D**) Diagnostic value of *SPINK4* expression in COAD. (**E**) Association of *SPINK4* expression with different immune cell in BLCA, including B cells, CD4 T-cells, CD8 T-cells, neutrophil, macrophage and dendritic cells. (**F**) Association of *SPINK4* expression with different immune cell in COAD, including B cells, CD4 T-cells, CD8 T-cells, neutrophil, macrophage and dendritic cells. (**G**) UMAP plots showing cell clusters and *SPINK4* expression levels in different immune cell types in BLCA. (**H**) UMAP plots showing cell clusters and *SPINK4* expression levels in different immune cell types in COAD. Gray brown background indicates no correlation between the gene expression and the corresponding index ($p > 0.05$). Light background indicates that the gene is significantly correlated with the corresponding index ($p < 0.05$). R represents correlation value, $R > 0$ means positive correlation, $R < 0$ means negative correlation.

Discussion

Extensive research has shown that cancer is a complex disease charactered by heterogeneity at the molecular level, which poses significant challenges to its treatment.[31] Targeted therapy, which focuses on specific molecular signatures, has proven to be an effective cancer treatment.[32] Therefore, it is crucial to identify the genes that play key roles in the development of cancer. For this reason, exploring the similarities and differences between various tumor types (pan-cancers) at the cellular and genomic level has become a relevant therapeutic goal.[33] By analysing the TCGA database, we found that *SPINK4* expression patterns in different tumors differed from those in paired adjacent normal tissues. The expression of *SPINK4* was significantly up-regulated in LIHC, LUAD and UCEC, but down-regulated in BLCA and COAD. In addition, *SPINK4* expression is lower in some metastatic tumors, including COAD, ESCA and KIRC. These results suggest that the level of *SPINK4* expression can be considered a good prognostic biomarker associated with malignancy, especially in COAD. Moreover, immune subtype analysis revealed that *SPINK4* expression was significantly correlated with immune cell infiltration in BLCA and COAD. Previous studies have shown that elevated SPINK4

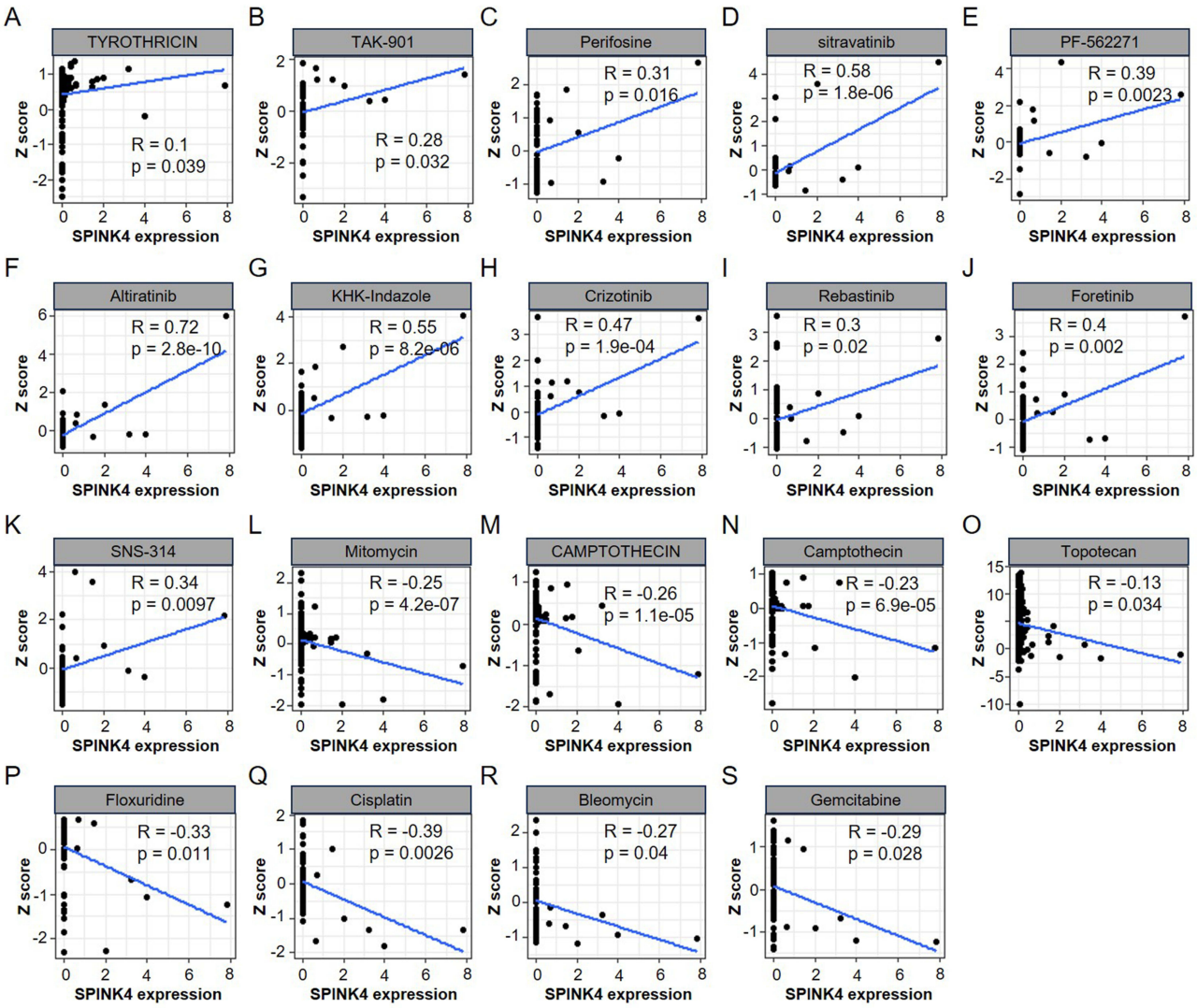

Figure 6 Relationship between *SPINK4* expression and drug sensitivity. *SPINK4* expression was positively correlated with drug sensitivity of TYROTHRICIN (**A**), TAK-901 (**B**), Perifosine (**C**), sitravatinib (**D**), PF-562271 (**E**), Altiratinib (**F**), KHK-Lndazole (**G**), Crizotinib (**H**), Rebastinib (**I**), Foretinib (**J**), and SNS-314 (**K**). However, its expression was negatively correlated with the drug sensitivity of Mitomycin (**L**), CAMPTOTHEN (**M**), Camptothecin (**N**), Topotecan (**O**), Floxuridine (**P**), Cisclatin (**Q**), Bleomycin (**R**), and Gemcitabine (**S**). R represents correlation value, R >0 means positive correlation, R <0 means negative correlation. The $p < 0.05$ was considered to be statistically significant.

expression predicts poor treatment response in CRC patients receiving concomitant radiotherapy.[22] These studies also suggest a strong correlation between SPINK4 and treatment response in CRC patients. Unexpectedly, our findings also suggest that SPINK4 expression has a greater diagnostic value in COAD. This suggests that further study of the biological significance of SPINK4 expression in different tumors may have a guiding role in the diagnosis and treatment of cancer in the future.

The TME is a complex multicellular environment necessary for tumor development and plays a key role in the pathogenesis of cancer.[34,35] The cellular composition and functional status of TME can vary widely depending on the organ in which the tumor arises, the intrinsic characteristics of the cancer cells, the stage of the tumor and the characteristics of the patient.[36] Previous studies have shown that activated $CD8^+$ T cells can effectively inhibit COAD proliferation and metastasis.[37] In addition, $CD8^+$ T cells have been positively associated with a good prognosis in COAD patients.[38] COAD cells with elevated levels of *SPINK4* promote the growth of $CD8^+$ T cells, block apoptosis, and increase tumor lethality.[39] In this study, we investigated the correlation between *SPINK4* expression and matrix score and immune score, discovering a significant positive correlation between *SPINK4* expression and $CD8^+$ T cells in COAD. In

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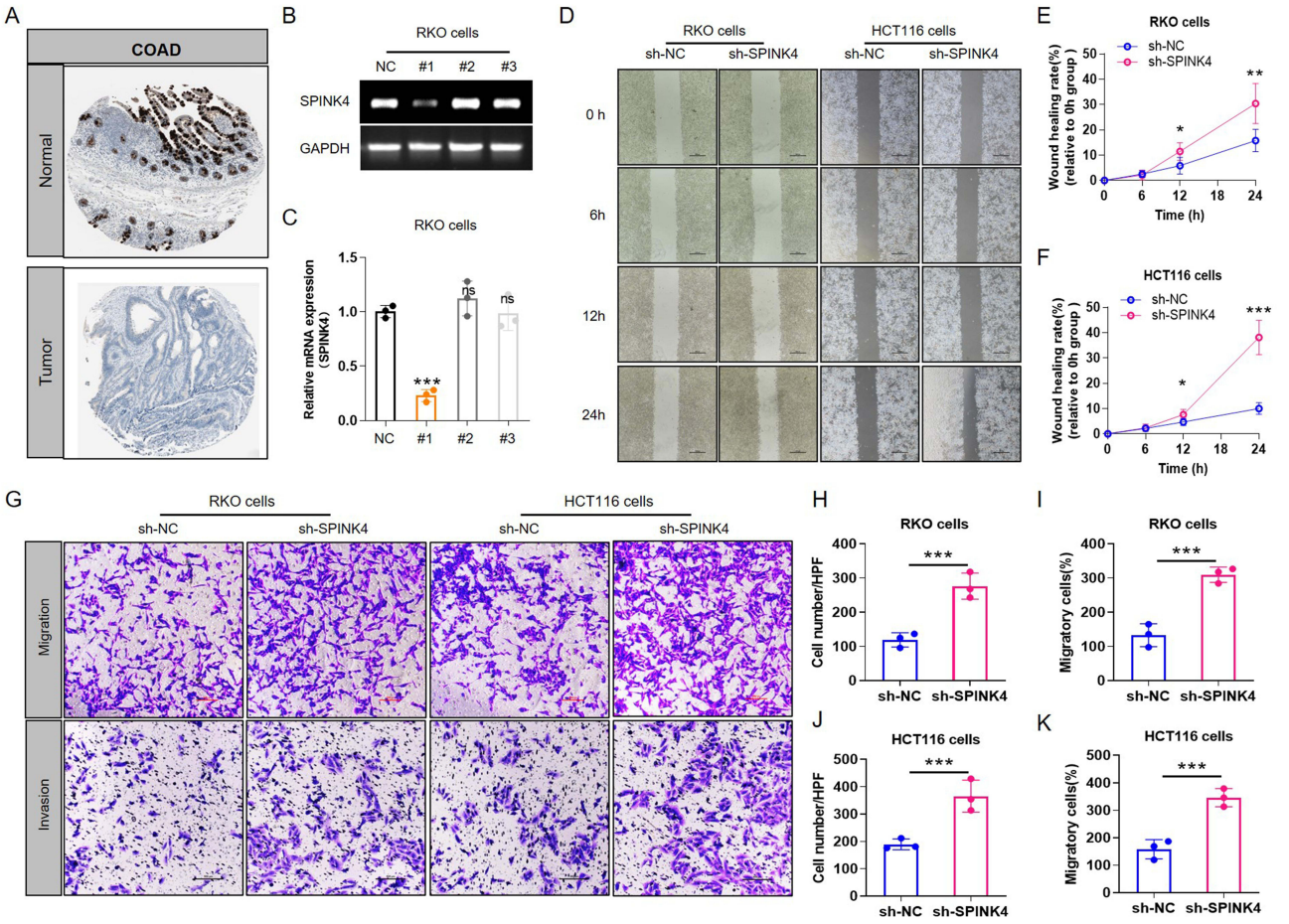

Figure 7 Knockdown of *SPINK4* promotes proliferation and migration of COAD. (**A**) Based on the HPA database, representative immunohistochemical staining of SPINK4 in normal and tumor tissues of COAD. (**B**) Verification of sh-SPINK4 using RT-PCR. (**C**) Verification of sh-SPINK4 using qRT-PCR. (**D**) Microscopic observations were recorded at 0, 12, and 24 h after scratching the surface of a confluent layer of the indicated RKO and HCT116 cells. (**E**) Quantitative analysis of wound healing percentage in RKO cells. (**F**) Quantitative analysis of wound healing percentage in HCT116 cells. (**G**) The effects of *SPINK4* on cell migration and invasion were examined by transwell assays in RKO and HCT116 cells. (**H, I**) Quantitative analysis of cell migration in RKO cells. (**J, K**) Quantitative analysis of cell migration in NCI-HCT116 cells. *p < 0.05, **p < 0.01, ***p < 0.001.

addition, we found that increased expression of SPINK4 significantly inhibited in vitro metastasis of the RKO and HCT116 colon cancer cell lines. Together with previous findings, these results further support the idea that SPINK4 inhibits COAD metastasis by regulating $CD8^+$ T cells. However, the specific molecular mechanisms underlying this regulation are still under investigation.

The assessment of prognosis is an important index of clinical therapeutic effect of the cancer. Although immunotherapy has made significant progress in a wide range of diseases, research into immunotherapy for cancer still faces significant limitations.[40] Drug resistance is also a major factor contributing to the poor prognosis of tumors. In recent years, the development of molecular biology technologies has provided new methods for assessing the prognosis of malignant tumors.[41] These include tumor-related gene detection, expression profile analysis, which can provide insight into the sensitivity of a tumor to a particular treatment and help predict treatment efficacy.[42] However, little research has been conducted on the SPINK4 gene in the context of tumour drug resistance. Epigenetic modifications are one of the important causes of drug resistance in tumour cells. Previous studies have shown that methylation modifications have a significant impact on the mechanism of tumour drug resistance. These studies have also shown that the expression of SPINK4 may be regulated by epigenetic modifications.[43] In this study, we explore the sensitivity analysis between *SPINK4* gene expression and currently used tumor chemotherapy drugs. The results suggested that *SPINK4* gene expression was related to TYROTHRICIN, TAK-901, Perifosine, sitravatinib, PF-562271, Altiratinib, KHK-Lndazole, Crizotinib, Rebastinib, Fore tinib is positively correlated with drug sensitivity of SNS-314. Conversely, there was

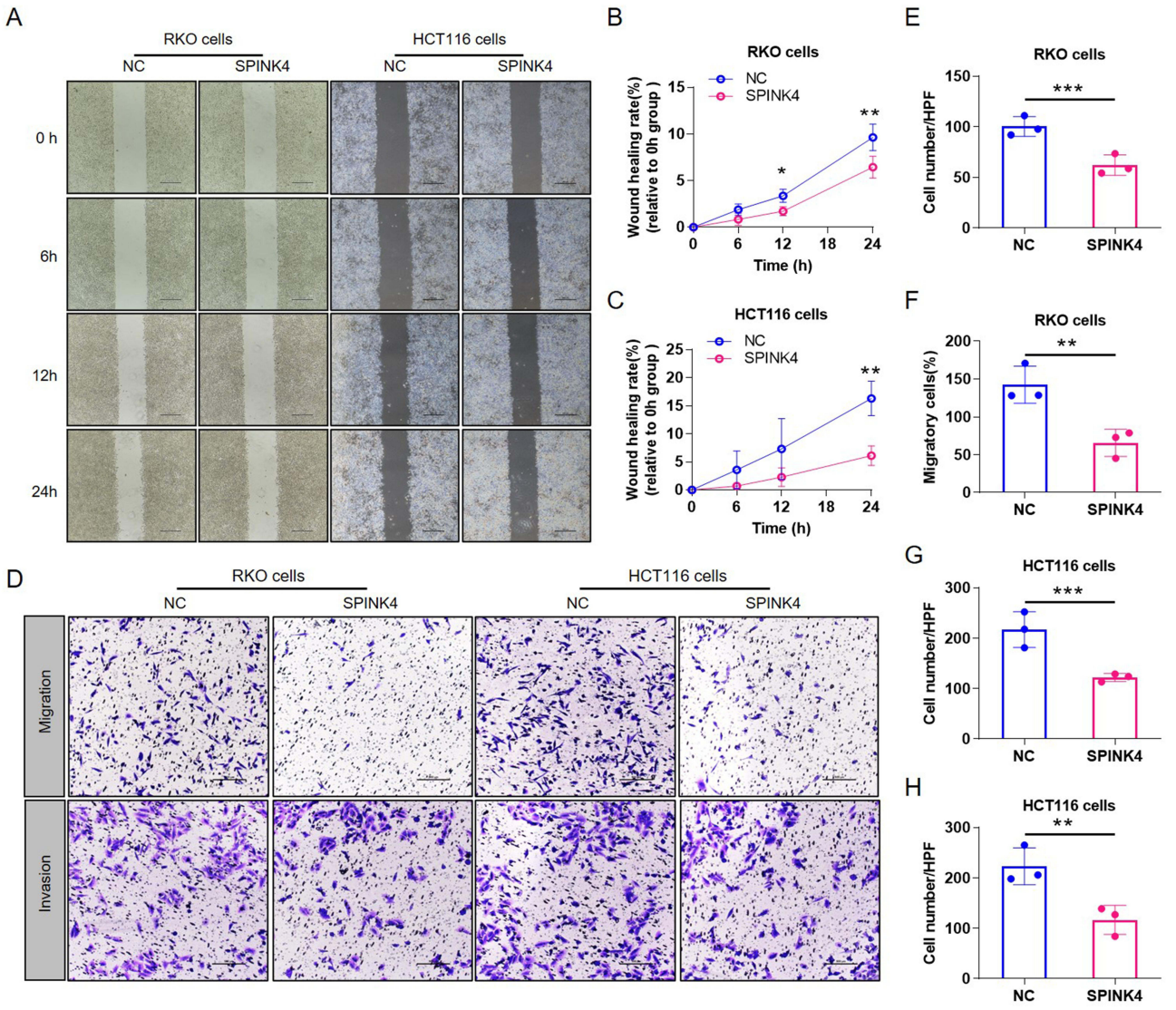

Figure 8 *SPINK4* overexpression inhibits the migration and invasion of liver cancer cells in vitro. (**A**) Microscopic observations were recorded at 0, 12, and 24 h after scratching the surface of a confluent layer of the indicated RKO and HCT116 cells. (**B**) Quantitative analysis of wound healing percentage in RKO cells. (**C**) Quantitative analysis of wound healing percentage in HCT116 cells. (**D**) The effects of *SPINK4* on cell migration and invasion were examined by transwell assays in RKO and HCT116 cells. (**E, F**) Quantitative analysis of cell migration in RKO cells. (**G, H**) Quantitative analysis of cell migration in NCI-HCT116 cells. *p < 0.05, **p < 0.01, ***p < 0.001.

a significant negative correlation with Mitomycin, CAMPTOTHEN, Camptothecin, Topotecan, Floxuridine, Cisclatin, Bleomycin and Gemcitabine. These results highlight the potential impact of SPINK4 transcript levels on drug resistance and sensitivity, providing a basis for individualized cancer treatment.

The application of multi-genomics to cancer has promoted the study of the functional molecules of tumor cells from the perspective of their inherent genetic characteristics.[44] Strengthening the link between genomic mutations and the metabolic dynamics of cancer may identify target for drug therapy in different types of cancer and improve the effectiveness of chemotherapy.[45] In this study, we revealed the mutational characteristics of *SPINK4* expression in different malignancies. We also found that *SPINK4* mutations had a significant negative impact on the OS and DSS in cancer patients. Furthermore, prognostic analysis revealed a significant correlation between reduced *SPINK4* expression and increased mortality in patients diagnosed with BLCA and COAD. These findings suggest that *SPINK4* may contribute to tumor progression through its genetic characteristics and phenotype, and its mutations could serve as important biomarkers for monitoring poor prognosis in cancer patients.

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Understanding the molecular mechanisms of tumor development is important for cancer treatment as well as for monitoring prognosis. *SPINK4*, a protein involved in a variety of physiological and pathological processes, plays a particularly prominent role in tumor progression and the immune response. *SPINK4* acts as a serine protease inhibitor that specifically inhibits the activity of a wide range of proteases including tryptic proteases. This inhibition may affect extracellular matrix remodeling and cell migration in the tumor microenvironment, thereby influencing the invasiveness and metastatic ability of tumor cells. Also, *SPINK4* expression may be associated with cell proliferation and apoptosis. By affecting relevant signaling pathways eg PI3K/Akt and MAPK pathways, *SPINK4* may promote the survival and proliferation of tumor cells. In addition, *SPINK4* expression can lead to resistance against chemotherapeutic agents in certain tumors, ultimately affecting treatment efficacy. The immune microenvironment is important role in regulating tumor growth, and *SPINK4* may facilitate tumor immune escape by regulating the function of tumor-associated macrophages and other immune cells. It may affect cytokine secretion and alter the activity of T and B cells, which in turn affects the anti-tumor immune response. In addition, *SPINK4* may affect the behavior of tumor cells by interacting with multiple cells signaling pathways. For example, it may regulate the inflammatory response and the formation of the tumor microenvironment through interference with the NF-κB or JAK/STAT signaling pathways. The role of *SPINK4* in tumor progression and immune response is complex and varied, and it has the potential to be a promising target for tumor therapy. Further studies will contribute to a deeper understanding of the function of *SPINK4* and provide new ideas for precision treatment of tumors.

As research advances, the significance of SPINK4 in cancer biology is gaining increasing attention. Studies have shown that, in addition to colorectal, SPINK4 is associated with the occurrence, progression and prognosis of various other cancer types. For example, SPINK4 expression is often upregulated in patients with prostate cancer, particularly those with high-risk features. There is a SPINK4 to other known biomarkers of prostate cancer (eg, PSA, TMPRSS2-ERG fusion genes, etc). High expression of SPINK4 can be combined with elevated PSA levels and may indicate disease progression or worsening. In pancreatic cancer, the expression of SPINK4 may work in conjunction with other biomarkers like CA19-9 to provide more comprehensive information for clinical diagnosis and treatment. Moreover, SPINK4 is implicated in other cancers, such as breast and gastric cancers, although the exact mechanisms and clinical relevance need further investigation. Combining SPINK4 with other biomarkers can improve the accuracy of cancer prognostic models. For example, multiple biomarker models, combining SPINK4 with traditional biomarkers (eg tumor size, grading, lymph node metastasis, etc) may aid in constructing a more accurate risk assessment model; or using machine learning algorithms to integrate SPINK4 with data from other biomarkers and search for potential prognostic models through big data analysis can reveal complex biological relationships. SPINK4 shows significant potential as an important biomarker in cancer research.

Based on a multi-database, we comprehensively explored the biological role of *SPINK4* in pan-cancer from multiple perspectives, including expression patterns, poor prognosis, tumor microenvironment, immune subtypes, genetic alterations and drug sensitivity. However, our current research still has some limitations. Firstly, our study mainly focused on bioinformatics analysis of *SPINK4* expression in tumor prognosis and immune cell infiltration, which lacked convincing experimental results. Secondly, the role of *SPINK4* in colorectal cancer has only been demonstrated in vitro and requires further confirmation through in vivo or molecular experiments. Lastly, the effect of genetic alterations of *SPINK4* on tumor prognosis needs to be further monitored, as this will help to overcome the shortcomings of this study and provide new strategies for clinical treatment of human cancer in the future.

Conclusion

In summary, we investigated potential associations between *SPINK4* expression and pan-cancer prognosis, the immune microenvironment, genetic alterations, and drug resistance using various databases. The expression of *SPINK4* exhibits a significant heterogeneity in different human cancers. At the same time, we also found that *SPINK4* expression is associated with tumor initiation and development, particularly in CRC. These findings provide an important basis for recognizing *SPINK4* as a novel pan-cancer biomarker and also contribute to the development of diverse strategies for cancer therapies targeting *SPINK4*.

Data Sharing Statement

Data supporting the conclusions of this article are included within the article. Please contact the corresponding author for data requests.

Ethical Approval and Consent to Participate

We are especially grateful to Dr. Zou Xiaopan for her support of the experiment, who works at the People's Hospital of Jilin Province. The human cells lines and the specimens of COAD were collected with the permission of the Ethics Committee of Jilin province people's hospital, Jilin, China (approval no. 2024129).

Consent for Publication

Not applicable.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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