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ORIGINAL RESEARCH

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High expression of SPINT2 promotes immune infiltration and tumor progression in ovarian cancer

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Abstract

This study aimed to identify the function and mechanism of Serine Peptidase Inhibitor, Kunitz Type 2 (SPINT2) in ovarian cancer (OC). The expression of SPINT2 was analyzed using the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) database. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed to identify enriched functional categories of SPINT2 and its correlated genes. Correlation of SPINT2 with OC immune infiltration level was analyzed using the Tumor IMmune Estimation Resource (TIMER) web server. The effect of SPINT2 on cell proliferation was detected using cell counting kit-8 (CCK-8) and colony formation assay. Its effects on cell migration and invasion were examined using the transwell assay. Function of SPINT2 in M2 macrophage recruitment was detected using the migration assay. The role of SPINT2 on M2 macrophage differentiation was evaluated using M2 macrophage markers and by detection of interleukin 10 (IL-10) release. An OC cohort study (GSE12470) showed that SPINT2 was highly expressed in OC. The high SPINT2 expression was related to shorter overall survival (OS) and poor recurrence-free survival (RFS). GO and KEGG analysis indicated that SPINT2 associated genes played roles in glycoprotein catabolism, cell adhesion and T cell differentiation. SPINT2 also played a key role in infiltration of macrophages in OC. shSPINT2 reduced viability and colony formation ability of ovarian cancer cell line SK-OV-3 cells. Moreover, shSPINT2 also inhibited the cell migration and invasion. Co-culture of shSPINT2 transfected SK-OV-3 cells with macrophages inhibited the migration of M2 macrophages, and inhibited macrophages polarization from M0 to M2. These results suggested that SPINT2 is involved in infiltration of tumor-associated macrophages (TAMs). SPINT2 also plays an important role in the polarization and migration of macrophages. These findings suggested that SPINT2 has the potential to be explored as a biomarker for OC and a potential target.

Keywords

SPINT2; TAMs; Ovarian cancer

1. Introduction

Ovarian cancer (OC) is a heterogeneous disease with multiple subtypes, which are part of the female reproductive system [1]. Due to its high mortality rate, it has become a leading cause of death among gynecological tumors [2, 3]. Most OC patients have no obvious symptoms in the early stages, and there's currently no reliable screening method available for OC [4]. Therefore, it's often difficult to diagnose in an early stage, and patients may have already reached a relatively advanced stage by the time of diagnosis. Based on data from SEER (Surveillance, Epidemiology and End Results) 2012–2018, the relative five-year survival rate for ovarian cancer is 49.7% and approximately 17% of OC patients are diagnosed with early stage disease. However, few patients can be cured and patients will often experience a relapse and require further treatments [5, 6]. Currently, the most effective treatment methods for OC are surgical resection of tumor tissue and combination with chemotherapy [7]. First-line maintenance therapy with poly ADP-ribose polymerase (PARP) inhibitors can further significantly improve outcomes, especially in Breast Cancer Susceptibility Genes (BRCA) mutation populations. Immunotherapy, such as checkpoint inhibitors, has revolutionized cancer treatment, but limited efficacy has been observed in OC [8]. The main reason is due to its cold tumor immune microenvironment with few tumor-infiltrating lymphocytes [9]. Therefore, developing new methods for early diagnosis of OC and individualized treatment for OC are the key for improving clinical efficacy and safety.

Tumor-associated macrophages (TAMs) are a type of immune cells that infiltrate into the tumor microenvironment [10]. TAMs are abundantly present at tumor sites and

can be categorized into two subsets: M1 and M2 [11]. Immunologically cold tumors are often infiltrated with abundant macrophages, and infiltration of M2 macrophages correlates with metastasis and poor prognosis [12, 13]. Tumor cells can induce macrophage infiltration and polarization towards M2 phenotype to promote their malignant progression [14]. The mechanisms of how tumor cells, especially OC cells control the recruitment of macrophages and regulation of M2 polarization remain unclear.

SPINT2 (Serine Peptidase Inhibitor, Kunitz Type 2) is expressed in various tissues and is involved in multiple biological processes including embryonic development, immune response, cell adhesion, cancer occurrence and metastasis [15–17]. Previous study showed that SPINT2 mRNA level was up-regulated in breast cancer tissues [18]. A recent study also reported that SPINT2 expression was significantly associated with poorer prognosis in breast cancer [19]. Furthermore, SPINT2 co-expressed genes mainly participated in regulating cell adhesion and migration [19]. Graumann *et al.* [20] identified that SPINT2 protein was upregulated in the plasma of OC patients and associated with poor prognosis. However, the detailed function and mechanism of SPINT2 in OC remain unclear.

In this study, the function of SPINT2 in OC was investigated. Results revealed that SPINT2 was highly expressed in OC. SPINT2 over-expression promoted the migration and invasion of SK-OV-3. SPINT2 also plays a role in mediating the recruitment and polarization of macrophages. Thus, these results demonstrated the complex function of SPINT2 in OC.

2. Materials and methods

2.1 GEO and TCGA database analysis

The differentially expressed genes (DEGs) in OC were identified by GEO database (GSE12470). GSE12470 contained the gene expression signature of 43 serous OC samples and 10 normal peritoneum samples. The analysis was done by GEO2R on the GEO website (https://www.ncbi.nlm.nih.gov/geo/geo2r/). The Principal Component Analysis (PCA) was computed by the FactoMineR (version 2.8) package in R (version 4.2.1, Robert Gentleman, Auckland, New Zealand). The heatmap was created by the pheatmap package in R. A volcano plot was generated with ggplot2 (version 3.4.2) in R. The screening criteria for DEGs were FC > 2 and $p < 0.05$.

All available TCGA (The Cancer Genome Atlas) data on OC were obtained from the TCGA data portal, and the SPINT2 expression was investigated. The expression level of SPINT2 was analyzed using UALCAN (The University of ALabama at Birmingham CANcer data analysis Portal, https://ualcan.path.uab.edu/analysis.html), as well as their association with individual cancer stages, tumor grade, patient's race, patient's age and tumor protein p53 (TP53) mutation status [21]. OS and RFS were analyzed using KMplot (https://kmplot.com/analysis/index.php?p=service) [22].

2.2 Functional analysis of SPINT2 and its correlated genes

SPINT2 correlated genes were identified from UALCAN with Pearson-CC ≥ 0.3. Metascape (Genomics Institute of the Novartis Research Foundation, San Diego, CA, USA, http://metascape.org) was used for GO (Gene Ontology) enrichment and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses to identify enriched functional categories of SPINT2 and its correlated genes.

2.3 TIMER database analysis

Gene module on TIMER web server was used to investigate correlation between SPINT2 expression and abundance of immune infiltrates in OC [23]. Gene expression level was displayed with log2 transcript per million (TPM).

2.4 Reverse transcription quantitative PCR (RT-qPCR) assays

Total RNAs were extracted from cells with TRIzol RNA Isolation Reagents (Invitrogen, Code No. 15596026, Carlsbad, CA, USA). cDNAs were synthesized from total RNAs. qPCR was conducted through SYBR® Green Realtime PCR Master Mix (TOYOBO, Code No. QPK-201, Tokyo, Japan). Primers used for reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and SPINT2 were referred from the primer sequences validated by ORIGENE and shown in Table 1.

2.5 Western-blotting assay

Total proteins were extracted from human normal ovarian epithelial cell (IOSE80) and ovarian cancer cell (SK-OV-3, A2780 and OVCAR4) cell pellets with radio immunoprecipitation assay (RIPA) Lysis Buffer (Beyotime, Code No. P0013B, Shanghai, China). The RIPA Lysis Buffer contain multiple inhibitors to inhibit protein degradation during sample lysis. Total protein samples were separated by electrophoresis through 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Western-blotting were performed according to standard procedures. The results from western-blotting were analyzed by ImageJ for semi-quantitative analysis. The primary antibodies and the corresponding dilution ratio used were shown as follows: β-actin (Abcam, Code No. ab8226, 1:5000, Cambridge, UK), SPINT2 (Invitrogen, Code No. PA5-121929, 1:2000, Carlsbad, CA, USA), CD163 (Abcam, Code No. ab182422, 1:1000, Cambridge, UK), CD206 (Abcam, Code No. ab300621, 1:1000, Cambridge, UK).

2.6 Cell counting kit-8 cell proliferation/cytotoxicity assay

5000 SK-OV-3 cells per well were seeded into 96-well plates. Cell viability was measured by CCK-8 kit (Beyotime, Code No. C0038, Shanghai, China).

2.7 Crystal violet assay

SK-OV-3 (100 cells/well) was seeded into 6-well plates, and transfected with shSPINT2-1#, shSPINT2-2# or negative control (shNC) for 7 days. Colonies were fixed with ice-cold

TABLE 1. The primers used for RT-qPCR.

GAPDH forward	5′-GTCTCCTCTGACTTCAACAGCG-3′
GAPDH reverse	5′-ACCACCCTGTTGCTGTAGCCAA-3′
SPINT2 forward	5′-GTGCCTCAAGAAATGTGCCACTG-3′
SPINT2 reverse	5′-GGAGTGGTCTTCAGAATCCTGC-3′

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SPINT2: Serine Peptidase Inhibitor, Kunitz Type 2.

methanol for 10 min. Expanded colonies were stained by 0.5% crystal violet (Sigma) for 15 min and washed for 3 times. Images were acquired and the number was counted.

2.8 Transwell assay for cell migration and invasion detection

SK-OV-3 cells were transfected with shSPINT2-1#, shSPINT2-2# or shNC for 24 h, and detached cells with 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA) solution. Centrifuged and resuspended cells in serum-free cell culture media. Matrigel® Matrix (Corning) was thawed on ice. 350 μL of Matrigel was added to the upper chamber of 24-well transwell plate and solidified for 30 min. 100 μL cell suspension (100,000 cells) was added into the upper chamber, and 600 μL dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) was added to the bottom. After overnight incubation, removed the transwell insert from the plate, and cells remaining on the upper chamber were carefully removed with a cotton-tipped applicator. Place the transwell insert into 4% paraformaldehyde for 30 min to allow cell fixation. Cells were stained with 0.2% crystal violet for 20 min. Gently removed the crystal violet from the top of the membrane, and washed with phosphate buffer saline (PBS). Images were captured.

2.9 Macrophage polarization and migration

Human monocytic leukemia cells (THP-1) cells were differentiated from monocytes into M0 macrophages upon stimulation with phorbol-12-myristate-13-acetate (PMA). M0 macrophages were incubated with IL-4 and IL-13 to further polarize into M2 macrophages.

Macrophage migration was assayed by transwell assay. Cell culture inserts with membrane pore sizes of 5 μm were used in this assay. M2 polarized macrophages were seeded into the upper chamber. SK-OV-3 in cultured condition medium was added to the bottom of the plate. After overnight incubation, the transwell insert was removed from the plate, and cells remaining on the upper chamber were carefully removed with a cotton-tipped applicator. Stained the cells with 0.2% crystal violet. Images were captured.

2.10 M0 macrophages co-cultured with SK-OV-3 cells

Indirect co-culture assay was performed using 3.0 μm cell culture inserts. M0-polarized THP-1 cells were seeded in the upper chamber and SK-OV-3 cells were seeded into the bottom of transwell chamber in the presence of PMA. Macrophages were collected and M2 macrophage markers (CD163 and CD206) were detected by western-blotting. The cell culture supernatants were collected for cytokine measurement. Human IL-10 ELISA Kits (Invitrogen, Code No. 88-7106-77, Carlsbad, CA, USA) were purchased from Absin.

3. Results

3.1 Expression level of SPINT2 in OC

The DEGs in OC and corresponding normal controls were investigated in the GSE12470 database. 5703 up-regulated genes and 2346 down-regulated genes were identified in Fig. 1A–C. The results in Fig. 1D illustrated that SPINT2 expression was significantly higher in OC compared to the normal controls. To further explore the SPINT2 expression in OC, SPINT2 expression was investigated using the RNA-seq data of OC in TCGA. Compared to the normal tissue, the expression level of SPINT2 was also significantly increased in OC tissues (Fig. 1E). The expression levels of SPINT2 based on individual cancer stages, tumor grade, patient's race, patient's age and TP53 mutation status were further investigated using the data of OC in UALCAN. As shown in Fig. 1F,G, SPINT2 expression was significantly higher in patients above 40 years old. In patients with OC above grade 2, SPINT2 expression was higher than patients with grade 1. However, SPINT2 expression was not significantly correlated with individual cancer stages, patient's race and TP53 mutation status. The correlation between SPINT2 expression and patient survival was analyzed using an online Kaplan Meier plot. Patients with high SPINT2 expression had shorter OS ($p = 0.022$). As shown in Fig. 1H, elevated SPINT2 expression was significantly associated with poor RFS ($p = 0.077$), which indicated a poor prognosis.

3.2 Gene ontology (GO) classifications and KEGG mapping

SPINT2 correlated genes were identified using the UALCAN. Then, the enriched pathways of SPINT2 and its correlated genes involved in these pathways were also identified. The GO analysis in Fig. 2 showed that the genes were enriched in glycoprotein catabolic process and protein catabolic process. KEGG analysis showed that the genes were enriched in herpes simplex virus infection, cell adhesion and T cell receptor signaling pathway. These results suggested that SPINT2 associated genes may play roles in glycoprotein catabolism, cell adhesion and T cell differentiation.

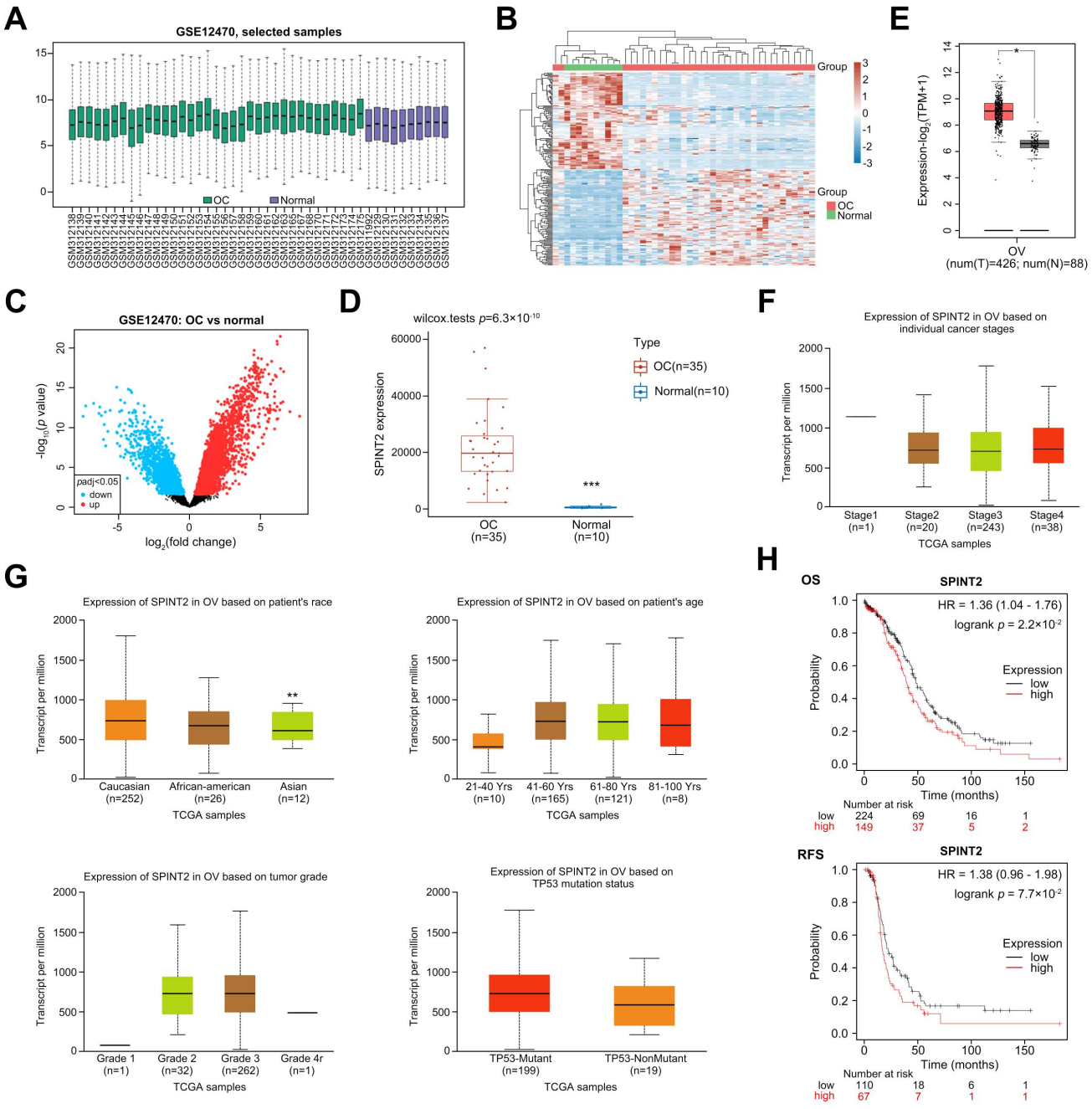

FIGURE 1. Expression level of SPINT2 in ovarian cancer. (A) Batch-effect correction of RNA-seq data in GSE12470. (B) Heatmap created by the pheatmap package in R. (C) Volcano plot generated with ggplot2 in R. (D) SPINT2 expression in ovarian cancer (GSE12470). (E) SPINT2 expression was investigated using the RNA-seq data of OC in TCGA. (F) The expression level of SPINT2 based on individual cancer stages. (G) The expression levels of SPINT2 based on tumor grade, patient's race, patient's age and TP53 mutation status. (H) The correlation between SPINT2 expression and patient survival (OS and RFS). **, $p < 0.01$ *vs.* OC. OC: ovarian cancer.

3.3 Correlation of SPINT2 expression with immune infiltration level in OC

The relationships between SPINT2 and immune cell infiltration in OC was investigated by TIMER. A scatterplot displaying SPINT2 expression levels against tumor purity was displayed in Fig. 3. Six panels, showing the purity-corrected partial Spearman's rho value and statistical significance between SPINT2 expression and each cancer immune infiltrates subset, were also displayed in Fig. 3. The results indicated that SPINT2 expression was correlated with the infiltration status of macrophages ($r = 0.219$, $p = 1.29 \times 10^{-6}$). A similar correlation was also observed for infiltrating level of neutrophils ($r = 0.137$, $p = 2.57 \times 10^{-3}$). These findings strongly demonstrated that SPINT2 played a specific role in the infiltration of macrophages in OC.

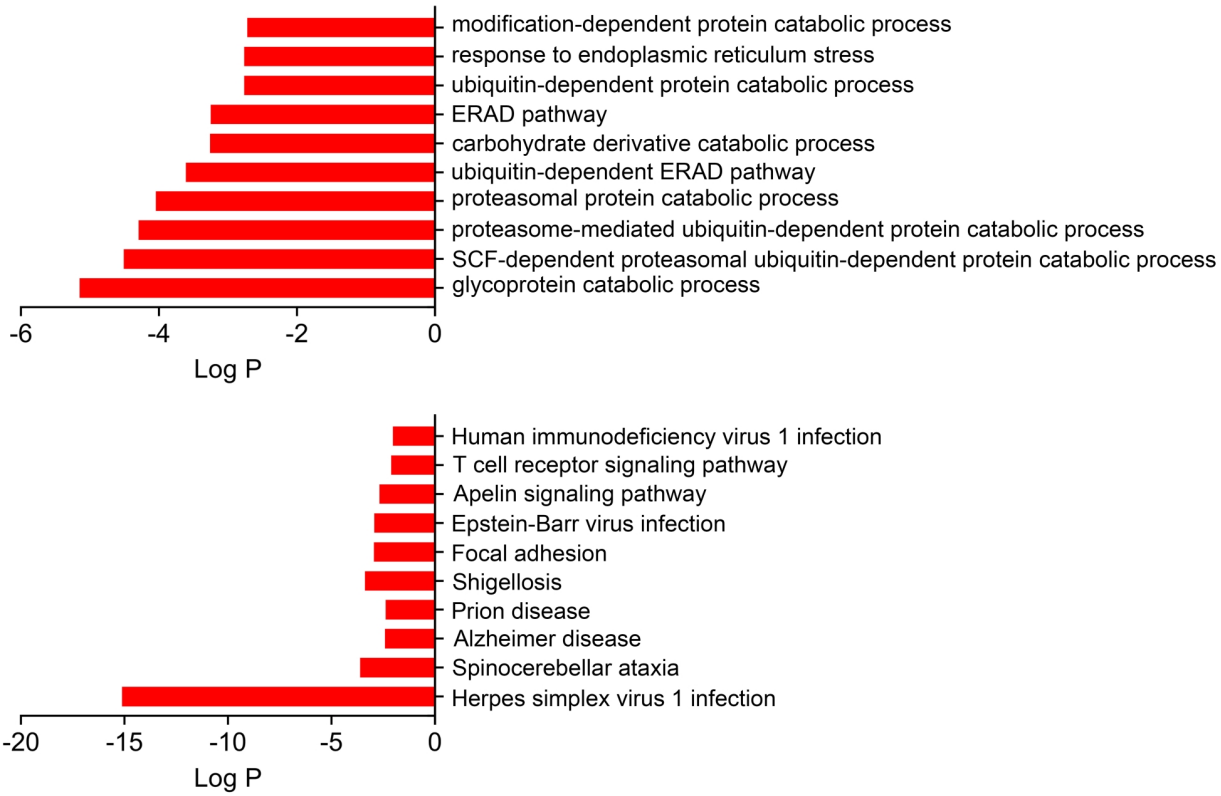

FIGURE 2. Gene ontology (GO) classifications and KEGG mapping. The upper bar graph represented the GO analysis of the SPINT2 and its correlated genes. The lower bar graph represented the KEGG analysis of the SPINT2 and its correlated genes. ERAD: endoplasmic reticulum associated degradation.

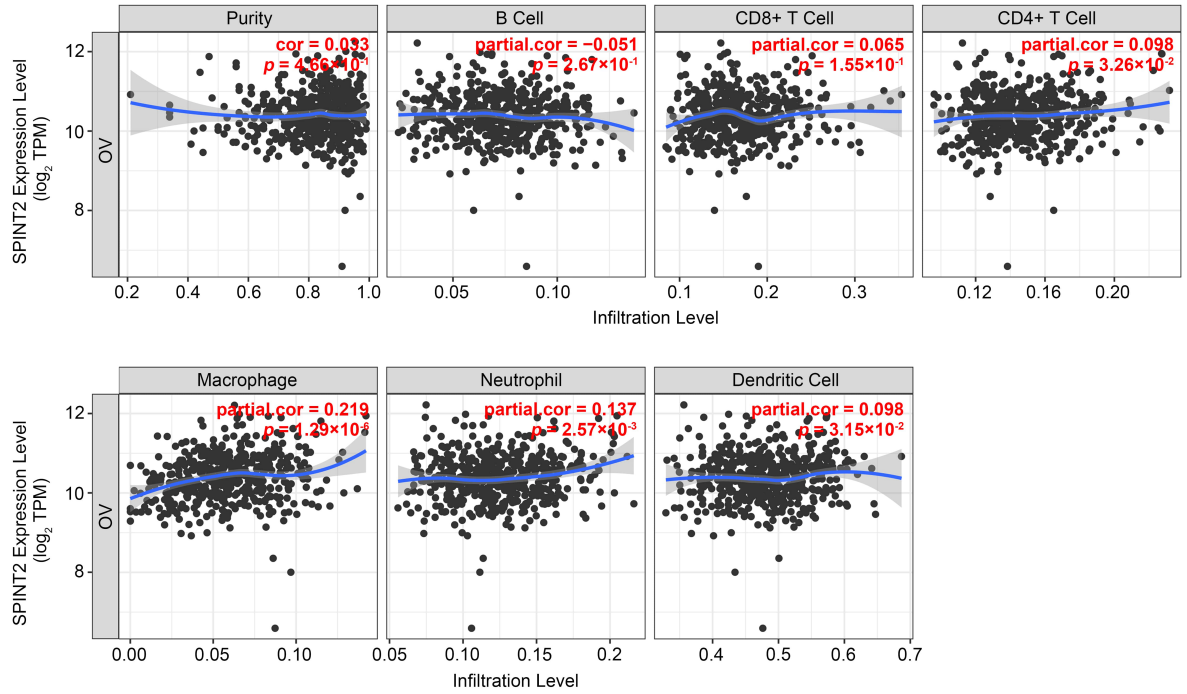

FIGURE 3. Correlation of SPINT2 expression with immune infiltration level in ovarian cancer. SPINT2 expression levels against tumor purity were displayed on the first panel. The correlations between SPINT2 expression and abundance of six immune infiltrates (B cells, CD4+ T cells, CD8+ T cells, Neutrophils, Macrophages and Dendritic cells) were displayed. The purity-corrected partial Spearman's rho value and statistical significance were included in the individual panels. SPINT2: Serine Peptidase Inhibitor, Kunitz Type 2; TPM: transcript per million; CD: cluster of differentiation.

3.4 SPINT2 in the proliferation and migration of ovarian cancer cells

SPINT2 expression was validated in three ovarian cancer cell lines SK-OV-3, A2780 and OVCAR4 cells, and the normal ovarian epithelial cell line IOSE80 cells. As shown in Fig. 4A,B, the mRNA level and protein levels were in consistent from the RT-qPCR and western-blotting results. Compared to the normal ovarian epithelial cell, all three ovarian cancer cells had significantly higher SPINT2 expression.

The knockdown efficiency of shSPINT2-1# and shSPINT2-2# were evaluated in SK-OV-3 cells. According to the results in Fig. 4C, the western-blotting results showed that both shRNA could effectively down-regulate SPINT2 expression, and shSPINT2-2# displayed higher efficiency than shSPINT2-1# with the expression level decreased by nearly 90%. With the inhibition of SPINT2 expression by shRNA, the SK-OV-3 cell viability was significantly decreased (Fig. 4D). The effect of shSPINT2 on colony formation of SK-OV-3 cells was evaluated by the colony formation assay, and the result was shown in Fig. 4E. Compared to shNC, shSPINT2-1# and shSPINT2-2# transfection reduced the number of colonies by 50% and 75%, respectively. This result indicated that shSPINT2 transfection significantly reduced the colony formation ability of SK-OV-3 cells. The effect of shSPINT2 on the migration and invasion of SK-OV-3 cells was further investigated by transwell assay and the results were shown in Fig. 4F. The number of migration and invasion cells were significantly reduced by shSPINT2 transfection, and shSPINT2-2# showed higher inhibition activity. This result suggested that downregulation of SPINT2 could inhibit the migration and invasion of SK-OV-3.

3.5 SPINT2 in macrophage recruitment and polarization

THP-1 cells were first polarized into M2 macrophages, and then a migration assay was carried out to investigate the function of SPINT2 in M2 macrophage recruitment. The results in Fig. 5A showed that when induced with SK-OV-3 cells, the number of migrated M2 macrophages were significantly increased. Furthermore, the number of migrated M2 macrophages were reduced when induced with shSPINT2-2# transfected SK-OV-3 cells. This result indicated that SPINT2 promoted the migration of M2 macrophages.

THP-1 cells were first polarized into M0 macrophages, and the M0 polarized macrophages were co-cultured with SK-OV-3 cells. The western-blotting results in Fig. 5B showed that CD163 expression was significantly increased when co-cultured with SK-OV-3 cells. Moreover, when the M0 polarized macrophages were co-cultured with shSPINT2-2# transfected SK-OV-3 cells, the CD163 expression was reduced (Fig. 5C). A similar result was also observed in CD206 expression. ELISA result further found that SK-OV-3 co-cultured M2 macrophages expressed higher levels of IL10, and this increase could be reduced by transfection of shSPINT2-2# in SK-OV-3 cells. These results indicated that SPINT2 promoted the polarization of macrophages from M0 to M2.

4. Discussion

SPINT2 is involved in a variety of biological processes, including embryonic development, immune response, cell adhesion, cancer occurrence and metastasis. According to the data on The Human Protein Atlas, most cancer tissues displayed weak to moderate SPINT2 expression [24]. However, upregulated expression of SPINT2 was observed in ovarian, prostate, pancreatic, breast, colorectal and testis cancers. Limited studies also reported that SPINT2 was over-expressed in breast cancer samples compared to control samples of normal breast tissue [18, 19]. Elevated SPINT2 expression was significantly correlated with a poorer prognosis of breast cancer patients [19]. There is no research on the expression and role of SPINT2 in OC. OC is the fifth leading cause of cancer-related deaths among women, and has become a major threat to women's health. Therefore, it is urgent to develop new methods for diagnosis of OC and individualized treatment for OC. Hence, this study provides a novel insight for understanding the potential function of SPINT2 in tumor immunology and its use as a diagnosis biomarker or therapy target.

In the present study, the expression level and prognostic values of SPINT2 in OC were examined using an OC cohort study GSE12470 and TCGA database. SPINT2 was significantly up-regulated in OC and the high expression was associated with shorter OS and poorer prognosis. GO and KEGG analysis further indicated that SPINT2 associated genes played roles in glycoprotein catabolism, cell adhesion and T cell differentiation. All these results suggested that SPINT2 may play an important role in the development and progression of OC. The SPINT2 expression were validated in three ovarian cancer cell lines SK-OV-3, A2780 and OVCAR4 cells, and both the mRNA level and protein levels were up-regulated in all OC cells. Knockdown of SPINT2 significantly reduced the viability and colony formation ability of SK-OV-3 cells. Moreover, SPINT2 knockdown also inhibited the migration and invasion of SK-OV-3 cells. These results supported the hypothesis that SPINT2 play an important role in tumor cell proliferation, migration and invasion which contributed to the OC development and progression.

Further analysis using TIMER identified that SPINT2 also played a specific role in immune cells infiltration in OC, especially infiltration of macrophages. This correlation suggested a potential mechanism by which SPINT2 regulated the macrophage function and infiltration in OC. Based on this hypothesis, the macrophage recruitment and polarization were investigated using THP-1 cells as in-vitro cell model. THP-1 monocytes can be differentiated into macrophage-like cells that resemble functional mature macrophages *in vitro* using PMA as a stimulant. The results supported the hypothesis that SPINT2 played an important role in mediating the migration of M2 macrophages, and the polarization from M0 to M2 macrophages.

5. Conclusions

In summary, the results in this study explained the underlying mechanism how SPINT2 expression was associated with immune cells infiltration and poor prognosis. On the one hand,

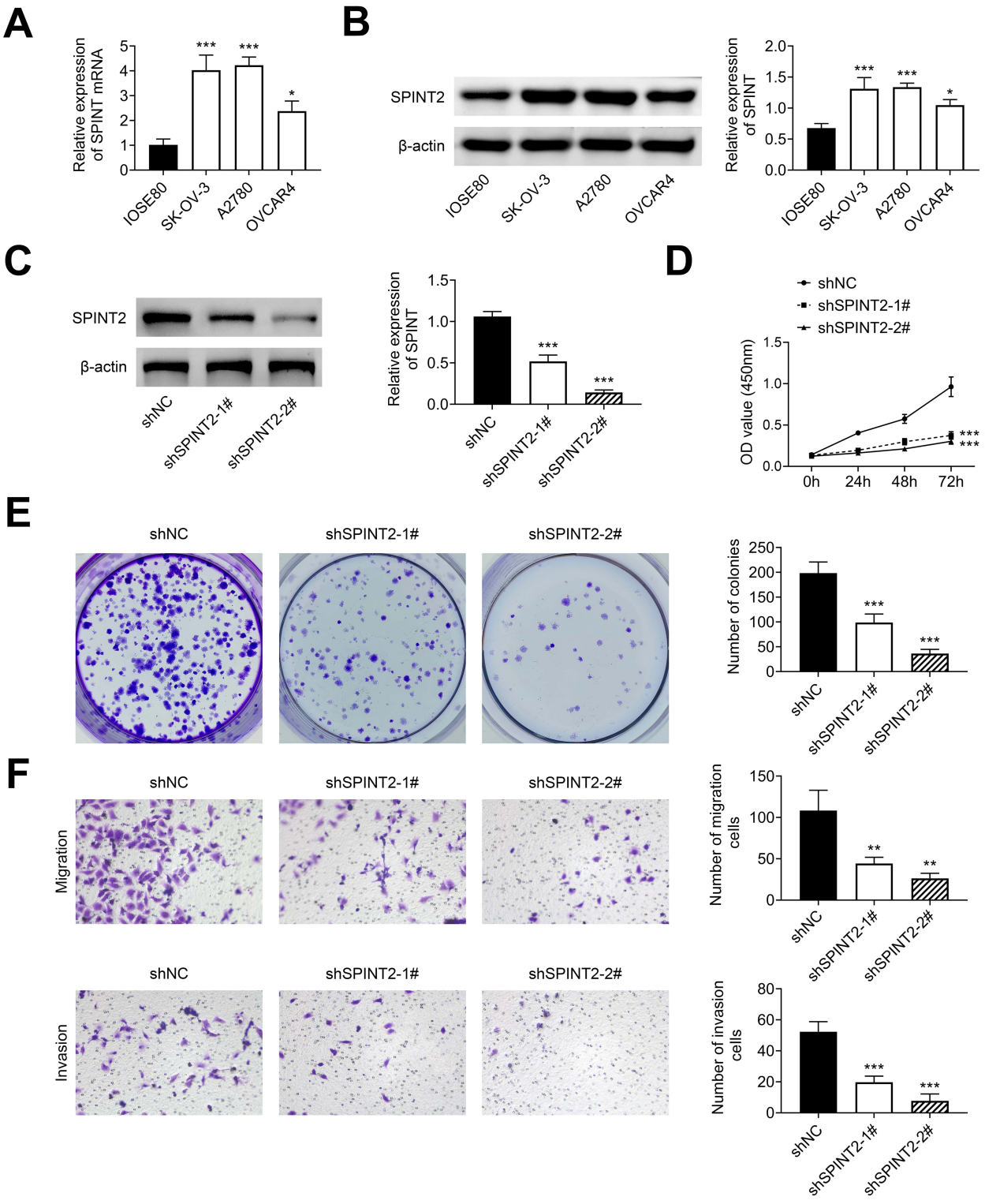

FIGURE 4. SPINT2 in the proliferation and migration of ovarian cancer cells. (A) Expression of SPINT2 in IOSE80, SK-OV-3, A2780 and OVCAR4 cells detected by RT-qPCR. (B) Expression of SPINT2 in IOSE80, SK-OV-3, A2780 and OVCAR4 cells detected by western-blotting. β-actin was selected as reference protein. Semi-quantitative analysis was performed through image analysis software to evaluate the relative level of SPINT2. (C) Expression of SPINT2 in SK-OV-3 cells with or without shSPINT2 treatment. (D) The cell viability of SK-OV-3 cells measured with the CCK-8 assay. (E) Crystal violet assay for determining viability of cultured SK-OV-3 cells, and colonies consisting of 50 or more cells were manually counted. (F) The migration and invasion ability detected by transwell assay. *, $p < 0.05$ *vs.* IOSE80, shNC; **, $p < 0.01$ *vs.* IOSE80, shNC; ***, $p < 0.001$ *vs.* IOSE80, shNC. RT-qPCR: reverse transcription quantitative PCR; CCK-8: cell counting kit-8; IOSE80: human normal ovarian epithelial cell; SK-OV-3, A2780, OVCAR4: ovarian cancer cell.

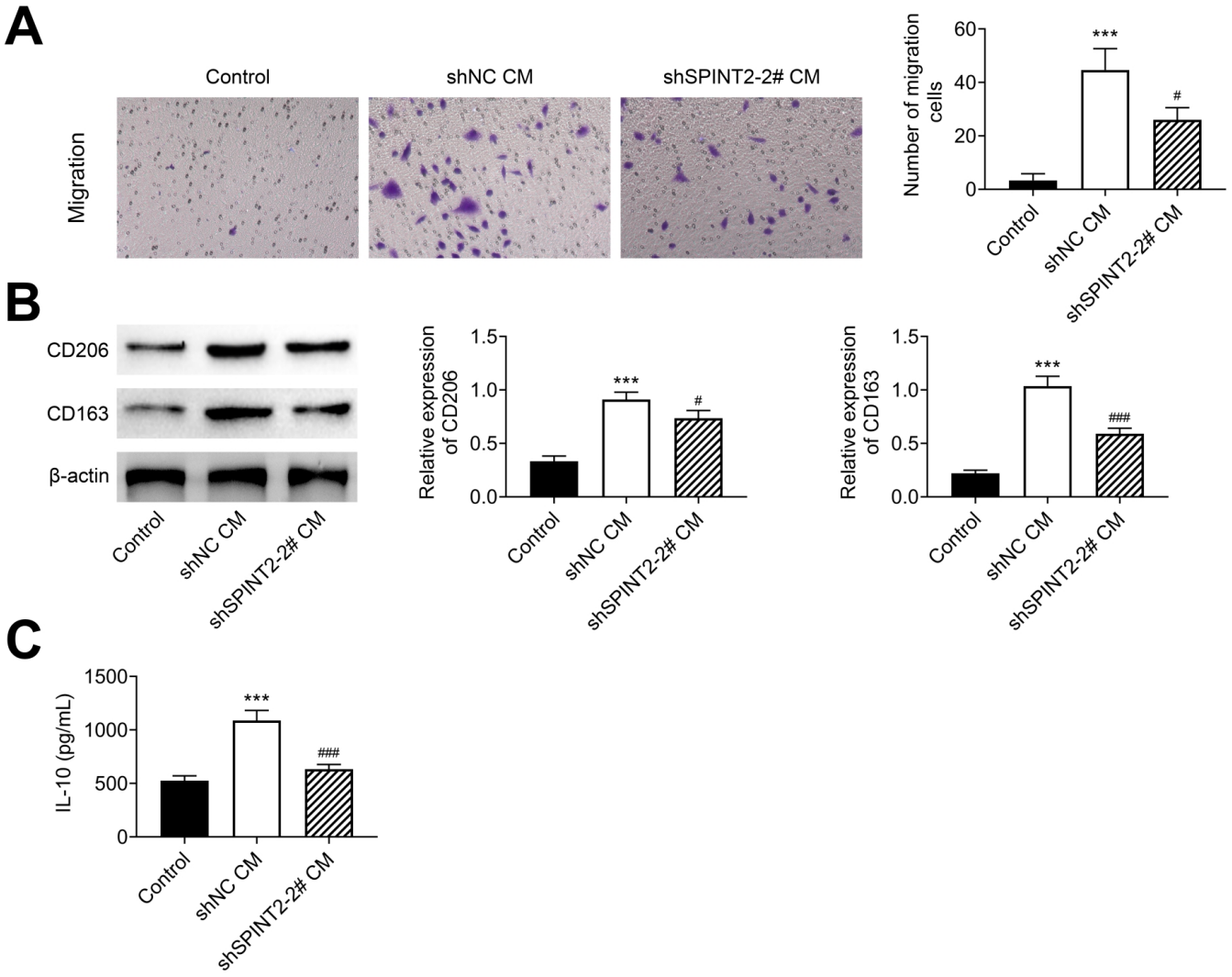

FIGURE 5. SPINT2 in macrophage recruitment and polarization. (A) The migration of M2 macrophages detected by transwell assay. Control: Medium without SK-OV-3 culture. shNC CM: Conditioned medium with shNC treated SK-VO-3 culture. shSPINT2-2# CM: Conditioned medium with shSPINT2-2# treated SK-VO-3 culture. (B) Western-blotting detection of M2 macrophage markers CD163 and CD206. (C) IL-10 secretion was detected with Human IL-10 ELISA Kits. ***, $p < 0.001$ *vs.* control; $^{\#}$, $p < 0.05$ *vs.* shNC CM; $^{\#\#}$, $p < 0.01$ *vs.* shNC CM; CD: cluster of differentiation; IL-10: interleukin-10.

SPINT2 overexpression in ovarian cancer cells could promote tumor cell proliferation, migration and invasion, thus contributing to the tumor development, progress and metastasis. On the other hand, overexpression of SPINT2 in ovarian cancer cells regulated the macrophage recruitment and polarization into M2 type in the microenvironment. Therefore, SPINT2 may play a prominent role in tumor cell proliferation and metastasis, as well as in macrophage infiltration and polarization. SPINT2 may have potential in serving as a prognosis biomarker or therapeutic target for OC.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

LL, WH and CYW—designed the study and carried them out, supervised the data collection, analyzed the data, interpreted the data, prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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