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Original Article

GTSF1 promotes stemness in uterine carcinosarcoma through CCL1-mediated M1 macrophage aggregation

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Abstract: Uterine carcinosarcoma (UCS), a high-grade endometrial carcinoma, is a rare but increasingly prevalent malignant gynecologic neoplasm, now accounting for over 5% of endometrial cancers and associated with a characteristically poor prognosis. In this study, we demonstrate that elevated expression of GTSF1 is significantly correlated with reduced disease-free survival (DFS) in UCS patients and promotes enhanced invasive, migratory, and stem-like phenotypes in tumor cells. Mechanistically, we show that GTSF1 drives tumor progression via activation of CCL1, which induces chemotaxis of M1 macrophages toward malignant cells and subsequent IL-6 secretion, thereby amplifying cancer stemness. Multiplex immunohistochemical analysis revealed spatial co-localization and positive correlations among GTSF1, CCL1, and M1 macrophage infiltration in UCS tissue specimens. *In vitro* co-culture experiments further confirmed that GTSF1-mediated CCL1 expression promotes M1 macrophage recruitment and IL-6 production, shaping an immune-permissive microenvironment that supports metastatic progression and maintenance of tumor stemness. This comprehensive investigation highlights actionable therapeutic targets within both tumor cells and their immune niche, offering translational insights for the development of multimodal treatment strategies against this aggressive malignancy.

Keywords: Uterine carcinosarcoma, GTSF1, CCL1, M1-type macrophages, immune microenvironment

Introduction

Uterine carcinosarcoma (UCS), formerly known as mixed Mullerian tumor (MMMT), is now considered to be a metaplastic carcinoma with a sarcomatous component resulting from dedifferentiation of the cancerous component [1-3], and is a rare and biologically aggressive subtype of endometrial carcinoma that has a significantly lower 5-year survival than that of endometrial carcinoma [4, 5]. Paclitaxel-carboplatin is considered the standard chemotherapy for UCS [6], but resistance to standard chemotherapies as well as their extraordinarily high mortality suggest the need for screening and development of novel and more effective targeted therapies, especially for advanced and recurrent disease.

The gametocyte-specific factor 1 (GTSF1) gene is involved in DNA methylation and retrotransposon activation in germ cells [7], and acts as a proliferation factor in various physiological processes such as growth, development, differentiation and reproduction in plants and animals [8]. Studies have shown that GTSF1 is highly expressed in a variety of tumor tissues and may be a potential biomarker for the diagnosis of hepatocellular carcinoma [9-11]. We found that GTSF1 was highly expressed and associated with tumor stemness in recurrent patients by analysing clinical and sequencing information of UCS patients in the TCGA public database. Meanwhile, we found that GTSF1 has not been studied in UCS, and its mechanism of regulating tumor recurrence and metastasis is more worthy to be explored.

Here, we show for the first time that GTSF1 can modulate the cytokine-cytokine interaction pathway and then activate CCL1 to promote uterine sarcoma invasion, migration, and stem-

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ness phenotypes, which was validated in two uterine sarcoma cell lines. We speculate that this may be related to the fact that CCL1 induces M1-type macrophages cell aggregation and thus enhances tumor stemness and malignant progression. We therefore propose that GTSF1 may serve as a new predictive biomarker in UCS, and targeting GTSF1-driven CCL1-induced M1-type macrophages aggregation may be a promising therapeutic strategy for UCS.

Materials and methods

Analysis of GTSF1 gene in TGCA and GTEx

The Sangerbox platform (http://sangerbox.com/login.html) is utilized to analyze clinical and transcriptome data from TCGA and GTEx of UCS, enabling the examination of gene differences between the relapse group and recurrence group.

Analysis of survival prognosis

The Disease-free survival (DFS) map of GTSF1 in UCS was obtained from the GEPIA (http://gepia.cancer-pku.cn/) online platform, which utilizes data from the TCGA database. Special Survival plots with log-rank *P* values were generated using the "Survival Analysis" module within GEPIA.

Tumor stemness and gene expression analysis

The correlation between GTSF1 and tumor stemness was analyzed using the tumor stemness module in Sangerbox platform.

Pathway enrichment analysis

The DAVID software (https://david.ncifcrf.gov/) was utilized to conduct GO and KEGG enrichment analysis of the aforementioned differentially expressed genes.

Cell lines and cultures

The human uterine sarcoma cell lines (MES-SA and SK-UT-1) were procured from the American Type Culture Collection (ATCC) (Manassas, VA, USA), while the human THP-1 cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell line identification services were utilized, and routine mycoplasma testing was conducted. The sarcomatoid cells were cultured in DMEM (Gibco Laboratories, Gaithersburg, MD, USA) medium supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories, Gaithersburg, MD, USA), and incubated at 37°C in the presence of 5% CO_2 with 1% penicillin and streptomycin. THP1 was grown in 1640 medium (Gibco Laboratories, Gaithersburg, MD, USA).

Cell transfection

The lentiviral constructs for SH-GTSF1 and OV-CCL1 insertions were generated using the complementary DNA (cDNA) of MES-SA and SK-UT-1 cells as templates. PCR amplification was performed to obtain the fragments of SH-GTSF1 and CCL1. Subsequently, these amplified fragments were inserted into the Phblv-CMV-MCS-EF1-T2A-Puro lentiviral vector (gene Adv, Suzhou, China) (Table S1). Finally, the constructed positive plasmids were validated through DNA sequencing.

RNA extraction, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and Quantitative Real-Time PCR (qPCR) analysis

After cell lysis using TRIzol® reagent (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA), total RNA was extracted from the aqueous phase following chloroform mixing, precipitated with isopropanol, washed with 75% ethanol, and resuspended in nuclease-free water. Subsequently, cDNA synthesis was performed utilizing a cDNA Reverse Transcription kit (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR analysis was conducted employing SYBR Green premix (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA) on an ABI 7500 real-time PCR machine (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA). Data acquisition was carried out using ABI SDS 2.0.1 software package and the 2-ΔΔct method was employed for data analysis. See Table S2 for details.

Western blot

The BCA Protein Assay kit (Solarbio, Beijing, China) was employed for protein quantification. Proteins (20 μg) were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane (PVDF). Following

Table 1. The basic clinical pathological information of all patients

Clinical pathological parameters	Number of patients
Total	14
Gender	
Male	0
Female	14
Age	
<60	12
≥60	2
Stage	
I-II	9
III-IV	5
Metastasis	
Negative	6
Pelvic cavity	5
Abdominal cavity	2
Bone	1
Others	0
Smoking	
Negative	13
Positive	1

blocking with 5% skim milk, the membranes were incubated overnight at 4°C with primary antibodies as listed in Table S3. After incubation with HRP-conjugated α-rabbit or α-mouse secondary antibodies for 1 hour, protein bands were detected using chemiluminescence substrates (Perkin Elmer) and visualized using a ChemiDoc imaging system (Bio-Rad, Hercules, CA, USA). See Table S3 for details.

Sphere formation assay

By inoculating 1000 cells per well into 12-well plates, the cells were incubated at 37°C in a 5% CO_2 environment for one week. To transfer primary pellets to secondary ones, we utilized 0.25% trypsin (Gibco) and reseeded 1000 cells into ultra-low adherent plates of size 6-well for an additional week. The quantification of tumor spheres per well was performed using an inverted microscope (×100 or ×40, Leica, USA).

Invasion and migration assays

The invasiveness was evaluated using Matrigel gels and Trans-Well plates. Cells were seeded in Matrigel at a density of 2 × 10^4 cells with 200 μL of serum-free RPMI-1640. In the lower chamber of a 24-well plate transwell system, cells were seeded on an 8 μm polycarbonate filter with medium containing 10% FBS. After incubation for 48 hours, the cells on the submembrane surface were fixed with methanol and stained with crystal violet. Microscopic images were captured to visualize the stained membranes. Cell counts were performed in five randomly selected areas, and the mean value was calculated from three replicate wells.

The cells were seeded in 6-well plates and allowed to reach a cell density of 90-100%. Subsequently, a "scratch" was created on the monolayer of cells using a 20 μL pipette tip. After removing debris by washing with PBS, the cells were cultured in fresh medium. At both 0 and 48 hours, five fields were selected for measurement under the microscope. Each scratch distance was measured three times to obtain an average value. The cell migration rate was calculated as (0-hour scratch distance - 48-hour scratch distance)/0-hour scratch distance × 100%.

Patient samples

Fourteen postoperative specimens of uterine sarcoma patients, confirmed by pathology at Wuxi People's Hospital from 2010 to 2020, were collected. The study received approval from the Ethics Committee of Wuxi People's Hospital. See **Table 1** for details.

Multiplex immunohistochemistry (mIHC) assays

The slides were initially heated, followed by removal of residual paraffin using xylene and rehydration in graded alcohol. Antigen retrieval was performed using PBS buffer and microwave treatment. Subsequently, blocking solution was applied for blocking. Then, the first primary antibody (position 1) was applied and incubated. As a secondary antibody, opal polymer HRP Ms + Rb (Aifang, hubei, China) was used. After washing the slides, tylamine signal amplification (TSA) dye (Aifang, hubei, China) was applied at position 1. To strip off primary and secondary antibodies, the slides were microwaved again before being washed and blocked with blocking solution once more. The second primary antibody was then applied at position 2 following the same procedure as before. DAPI staining occurred after washing off unbound DAPI and finally the slides were

covered with LongtTM Gold anti-quenching reagent (Invitrogen, Carlsbad, CA, USA). Five fields of view at 200× magnification on monochromatic slides were captured for imaging purposes while generating a spectral library for unmixing through StrataQuest image analysis software. See Table S3 for details.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism V8.3.1 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 26.0 software. The correlations among the indicators measured by RT-qPCR were evaluated via the Spearman rank test. A linear regression analysis was employed to elucidate the mathematical dependencies among variables. Univariate Cox regression and multivariate Cox regression analyses were utilized to identify common genes associated with overall survival (OS), while the Kaplan-Meier method was applied to determine the cumulative survival rate and the Log-rank test was applied to assess differences between groups. Continuous variables were presented as mean ± standard deviation (mean ± SD). All data from cell experiments were sourced from three independent replicates. Statistical significance was defined as: **P<0.05, **P<0.01, ***P<0.001* (two-tailed test).

Pattern diagram

We also thank the drawing tools provided by Figdraw (https://www.figdraw.com/#/paint_msgs).

Results

High expression of GTSF1 was associated with recurrence of UCS

UCS is an aggressive cancer, and postoperative systemic chemotherapy is effective for early stage UCS [12], but the higher recurrence and lower survival rates suggest that we need to find new targets [13, 14]. Through the analysis of differential gene expression between recurrent and non-recurrent UCS patients in The Cancer Genome Atlas (TCGA) public database, we identified that GTSF1 is significantly upregulated in recurrent cases (Log2FC≥|1.5|, P≤0.05) (**Figure 1A**). We then validated our findings in The Genotype-Tissue Expression (GTEx) and TCGA databases, where we found that GTSF1 expression was significantly upregulated in cancer tissues compared to normal tissues, and was significantly correlated with Disease-free survival (DFS), suggesting that GTSF1 may be involved in the development and progression of UCS (**Figure 1B**, **1C**). UCS is a high-grade, low-differentiated tumor, so we suspect that its high recurrence rate may be related to its tumor stemness. Using Sangerbox 3.0 (http://sangerbox.com/home.html) to calculate the mRNA expression and methylation signature of UCS in TCGA database, we found a correlation between GTSF1 gene expression and four tumor stemness indexes, suggesting that GTSF1 may promote its recurrence by inducing tumor stemness (**Figure 1D**). The samples from TCGA were categorized into two groups ($GTSF1^{Low}$ and $GTSF1^{High}$) based on the expression levels of the GTSF1 gene, followed by conducting Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) based pathway enrichment analysis. Most of the differentially expressed genes were enriched in cytokine-cytokine receptor interaction pathways, immune response, cell differentiation and other pathways (**Figure 1E**, **1F**). These results suggest that GTSF1 may maintain tumor stemness by regulating cytokines and immune microenvironment.

GTSF1 knockdown suppresses the stemness of UCS cells in vitro

In order to further investigate the cellular function of GTSF1, we utilized recombinant lentiviruses with GTSF1 shRNA sequences to knockdown the expression of GTSF1 gene in UCS cell lines ($MES\text{-}SA^{SH\text{-}GTSF1}$ and $SK\text{-}UT\text{-}1^{SH\text{-}GTSF1}$). We compared the expression of GTSF1 in two types of UCS cells infected with the control lentivirus and SH-GTSF1 lentivirus and found that the mRNA and protein expression of GTSF1 was significantly reduced in SH-GTSF1 group (**Figure 2A**, **2B**). Subsequently, we used the sphere-forming assay to examine the tumor stemness, and found that GTSF1 knockdown significantly reduced the tumor sphere-forming ability, suggesting that the tumor stemness was inhibited (**Figure 2C**, **2D**).

GTSF1 Knockdown suppresses invasion and migration of UCS cells in vitro

In addition, we evaluated the roles of GTSF1 in cell migration and invasion via the trans-well

GTSF1-CCL1 axis drives UCS stemness via M1 macrophages

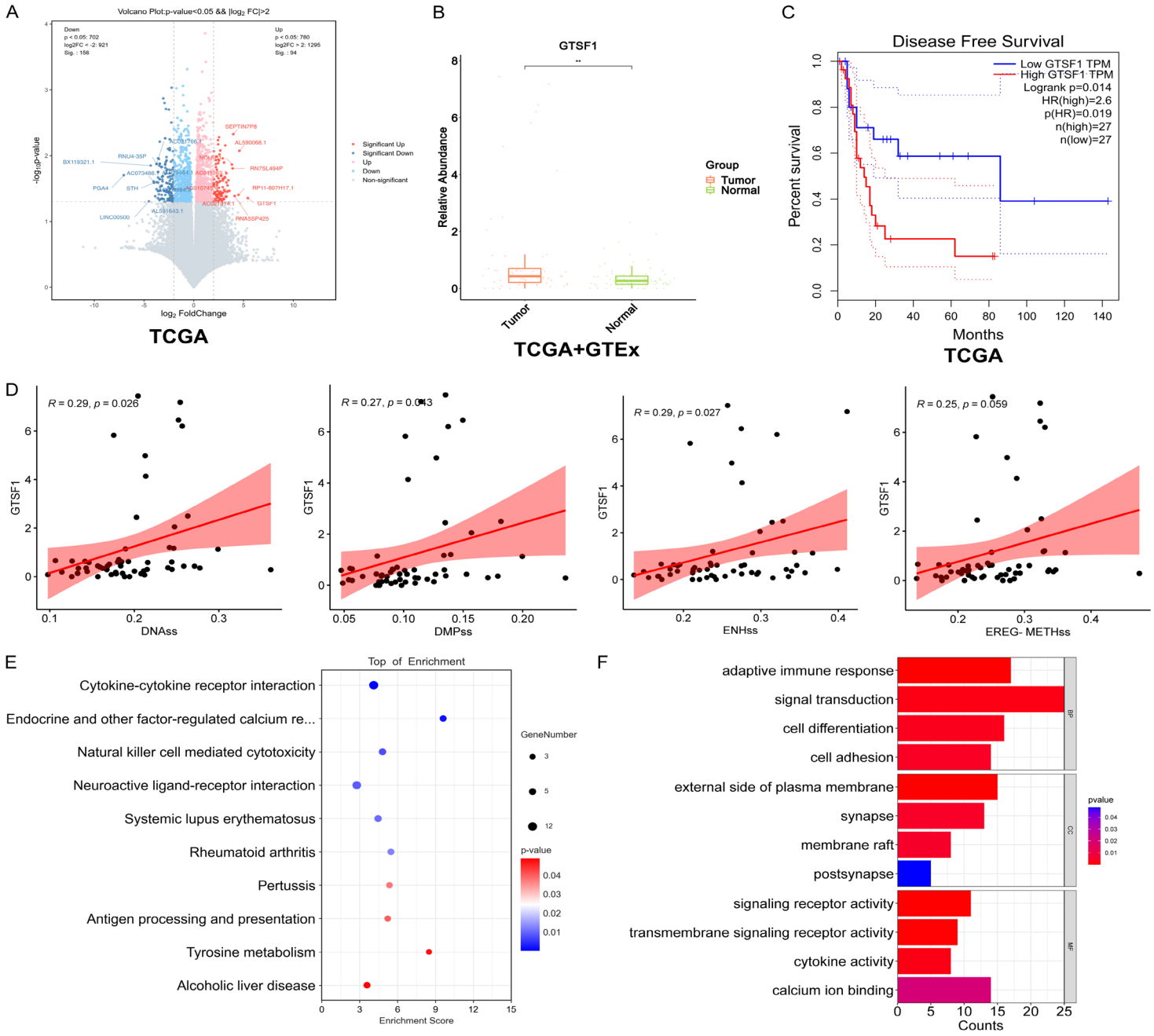


Figure 1. High expression of GTSF1 is related to the stemness and recurrence of UCS. A. The Sangerbox platform was utilized for the analysis of clinical and sequencing data from the TCGA dataset, specifically focusing on UCS. UCS cases were stratified into recurrence and non-recurrence groups, followed by identification of differentially expressed genes (Log2FC≥|1.5|, P≤0.05). B. The expression of GTSF1 was found to be significantly elevated in tumor tissues compared to normal tissues, based on a comparative analysis of the TCGA and GTEx databases. C. Through analysis of the GEPIA database, a significant correlation was observed between high GTSF1 expression and tumor progression in UCS. D. The Sangerbox database was utilized to investigate the correlation between GTSF1 and stemness, revealing that elevated expression of GTSF1 promotes tumor stemness. E, F. According to the mRNA expression of GTSF1 in UCS from the TCGA database, the samples were stratified into high and low expression groups based on the median value. Subsequently, DAVID was employed for KEGG and GO pathway enrichment analysis. **P<0.05, **P<0.01, ***P<0.001*.

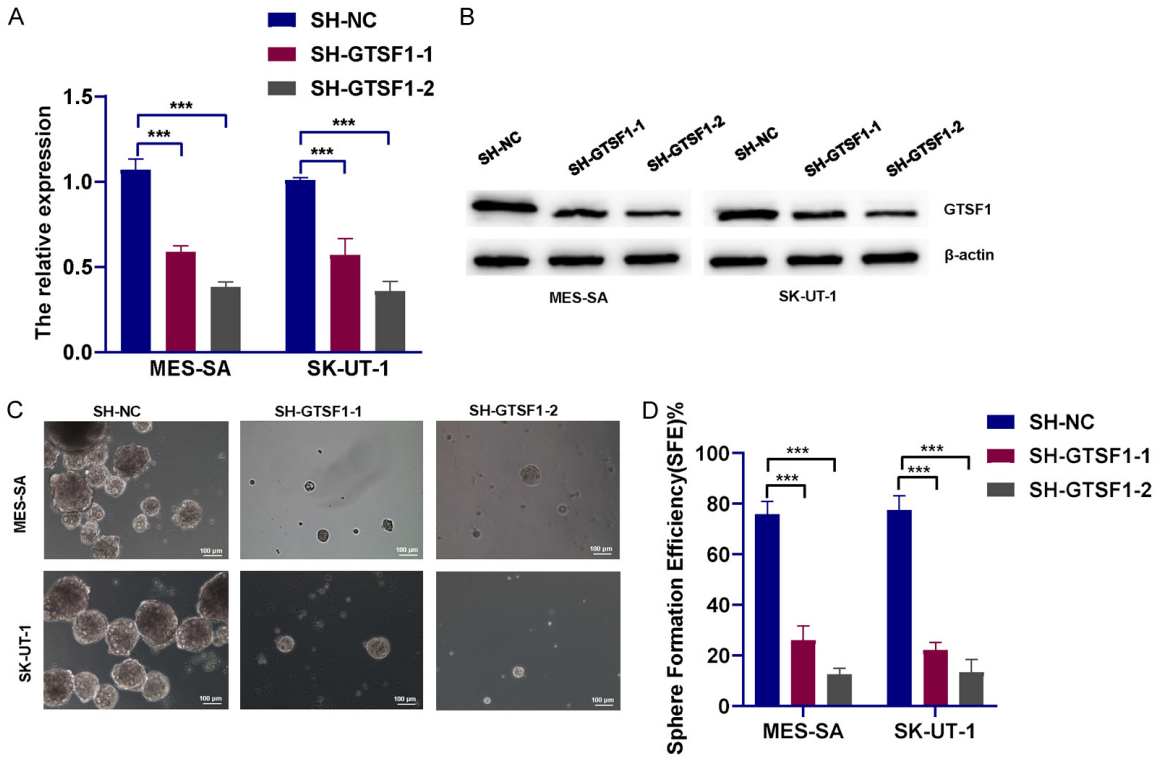


Figure 2. Knockdown of GTSF1 impedes UCS stemness in cellular-level. A, B. The knockdown efficiency of GTSF1 was assessed at the mRNA and protein levels in two UCS cell lines (MES-SA, SK-UT-1) following infection with SH-GTSF1 virus and its control virus. C. The virus-infected cells were subjected to a cell sphere formation assay on suspension culture plates in order to assess the efficiency of tumor sphere formation. D. Ratio of SFE. SFE = Number of cell balls greater than 75 μm in diameter per well/total number of original inoculated cells per well. Scale bar: 100 μm. **P<0.05, **P<0.01, ***P<0.001*.

invasion and wound healing assay. MES-SA$^{SH\text{-}GTSF1}$ and SK-UT-1$^{SH\text{-}GTSF1}$ showed a significant reduction in the number of cells migrating through the matrigel layer after 48 h, indicating that knockdown of GTSF1 significantly inhibited invasion ability (**Figure 3A**, **3B**). Similarly, the results showed that the wound closure rate (WCR) of MES-SA$^{SH\text{-}GTSF1}$ and SK-UT-1$^{SH\text{-}GTSF1}$ cells were significantly lower than that of the control cells (**Figure 3C**, **3D**). Collectively, knockdown of GTSF1 impedes invasion and migration capabilities of UCS cells in cellular level, indicating that GTSF1 could serve as a promising target for predicting recurrence and metastasis in UCS.

GTSF1 promotes stemness, invasion and migration of UCS cells by activating CCL1

We further analyzed the mechanism by heat mapping the differential genes in the cytokine-cytokine receptor interaction pathway, and found that GTSF1 gene expression was significantly positively correlated with CCL1 (**Figure 4A**, **4B**). The chemokine CCL1, also known as

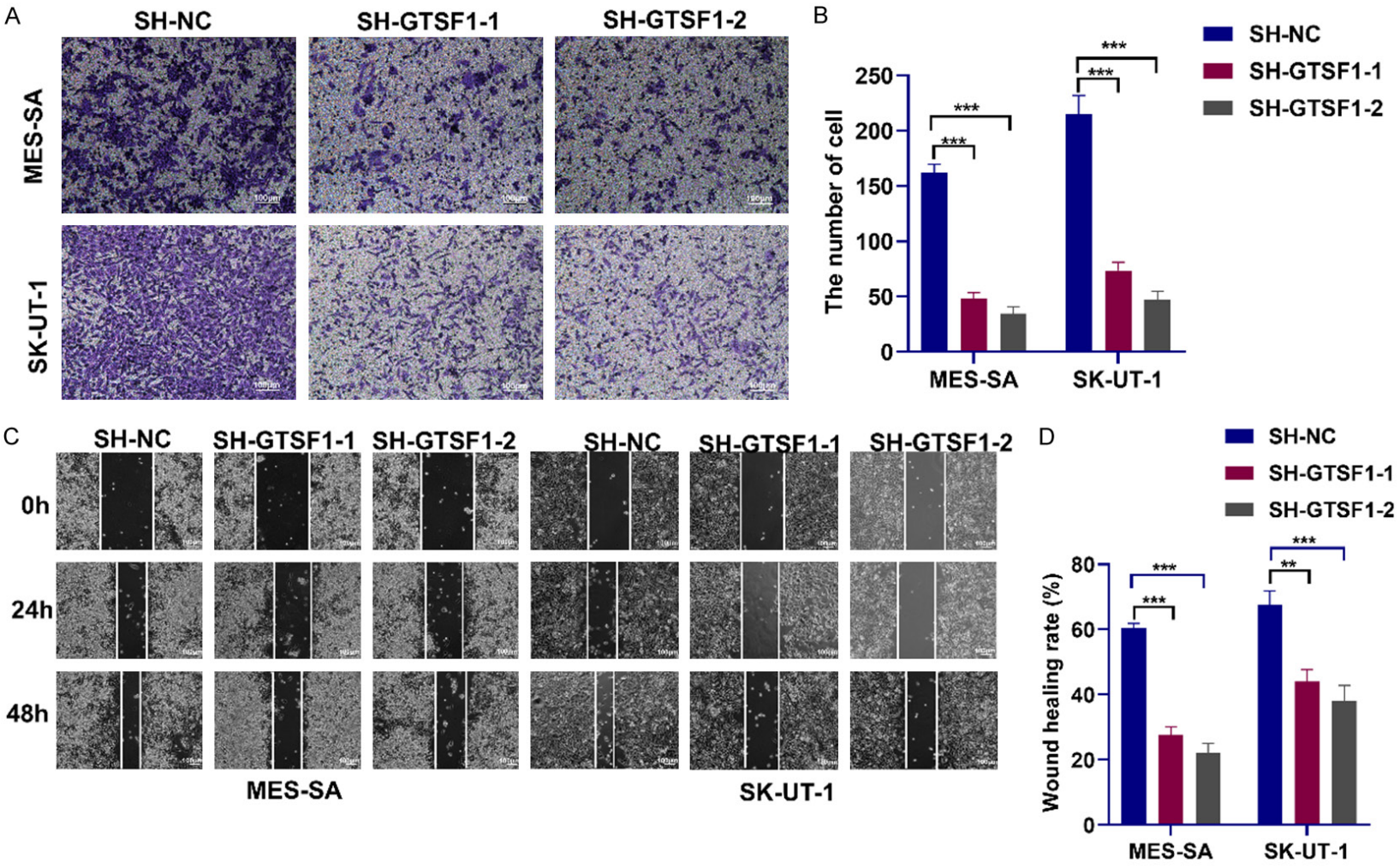
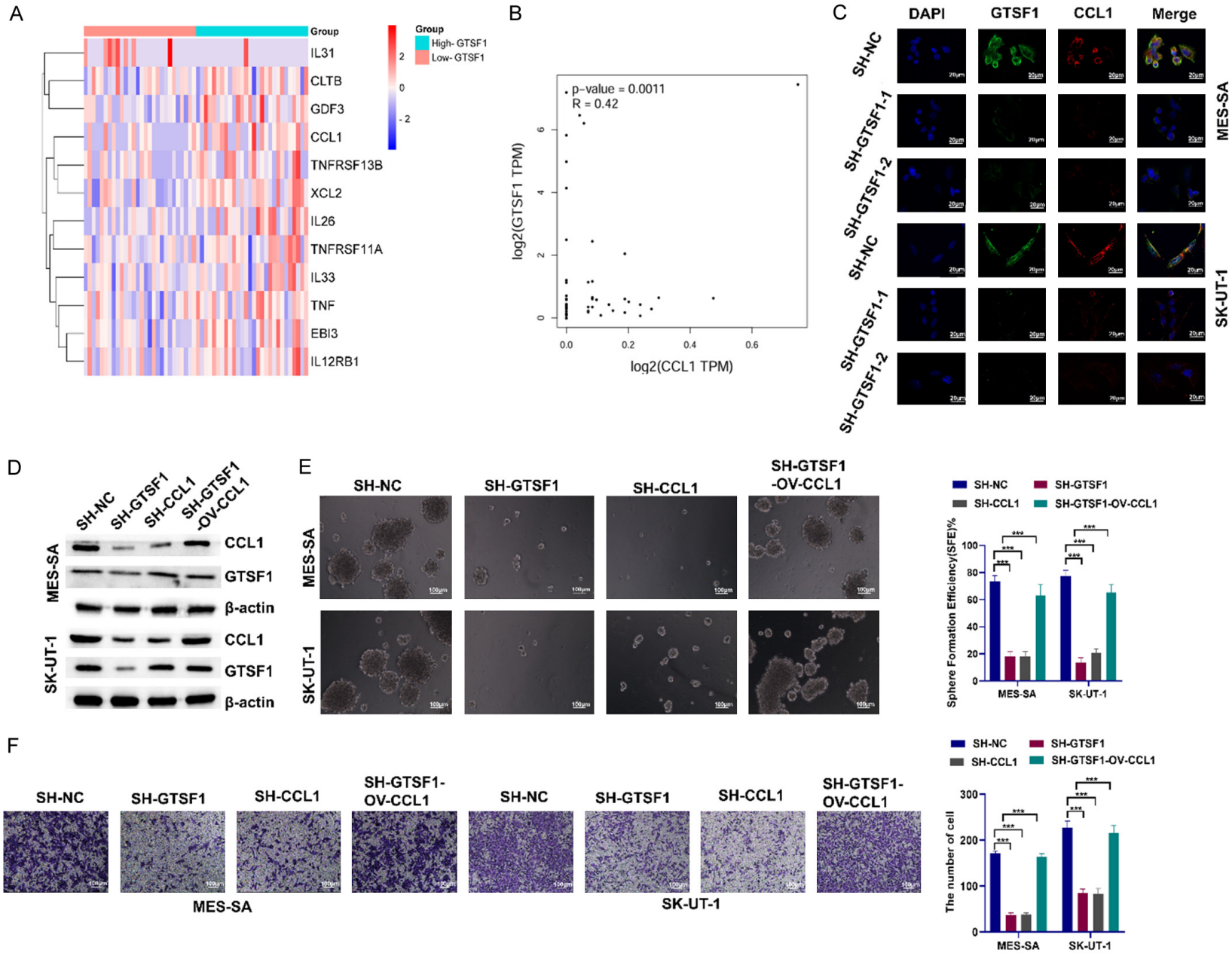


Figure 3. Knockdown of GTSF1 impedes the malignant progression of UCS in cellular-level. A. Representative images of the trans-well invasion assays. Scale bar: 100 μm. B. The number of cells in NC and GTSF1 knockdown groups. C. Representative images of the wound healing assays. Scale bar: 100 μm. D. The wound healing rate in NC and GTSF1 knockdown groups. **P<0.05, **P<0.01, ***P<0.001*.


A
Group
High- GTSF1
Low- GTSF1
IL31
CLTB
GDF3
CCL1
TNFRSF13B
XCL2
IL26
TNFRSF11A
IL33
TNF
EBI3
IL12RB1
B
p-value = 0.0011
R = 0.42
log2(GTSF1 TPM)
log2(CCL1 TPM)
C
DAPI
GTSF1
CCL1
Merge
SH-NC
SH-GTSF1-1
SH-GTSF1-2
MES-SA
SK-UT-1
D
SH-NC
SH-GTSF1
SH-CCL1
SH-GTSF1 -OV-CCL1
CCL1
GTSF1
β-actin
E
Sphere Formation Efficiency(SFE)%
F
The number of cell

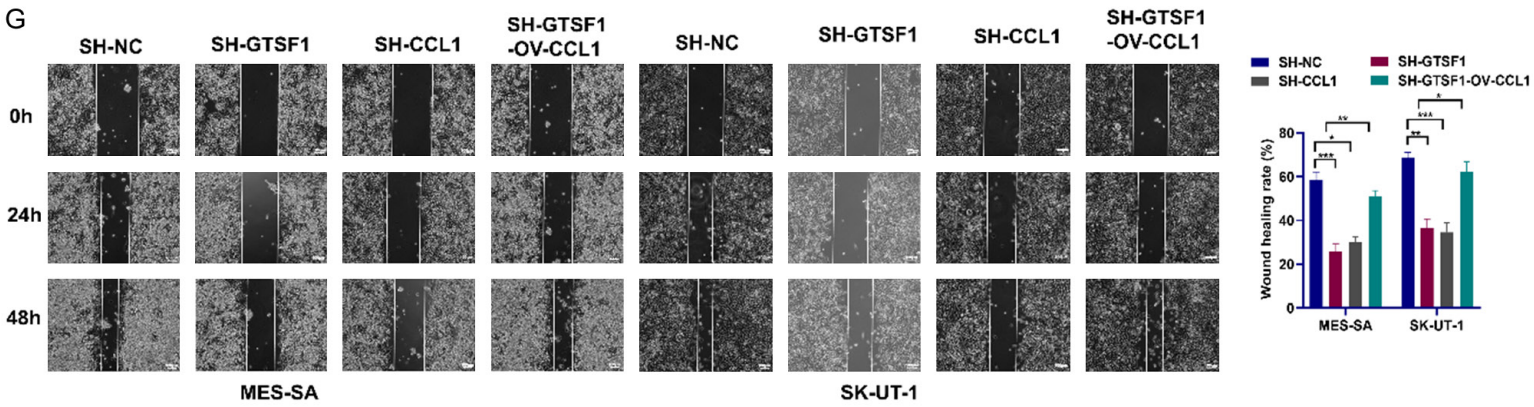


Figure 4. GTSF1 regulates the stemness and invasive potential of tumors via CCL1. A, B. The heat map analysis of differential genes in the cytokine-cytokine receptor interaction pathway within the KEGG database revealed a significant up-regulation of CCL1 in the high expression group of GTSF1. Subsequently, a noteworthy positive correlation between GTSF1 and CCL1 was observed at the mRNA level. C. Immunofluorescence analysis revealed a significant reduction in the fluorescence intensity of CCL1 following GTSF1 knockdown. Scale bar: 20 μm. D. Knockdown and overexpression of CCL1 cell lines were generated, followed by the detection of CCL1 and GTSF1 protein expressions. It was observed that GTSF1 exerted unidirectional regulation on CCL1 expression. E. The assay for evaluating stemness by forming spheres. F. Representative images of the trans-well invasion assays. Scale bar: 100 μm. G. Representative images of the wound healing assays. Scale bar: 100 μm. **P<0.05, **P<0.01, ***P<0.001*.

I-309, is the major ligand of C-C chemokine receptor 8 (CCR8), and CCL1 can be secreted into the tumor microenvironment to bind to CCR8, mainly leading to the recruitment of tumor-associated macrophages (TAMs) and regulatory T cells (Tregs) [15, 16]. TAMs and Tregs recruitment can promote tumor stemness and malignant progression [17-20]. We next used cellular immunofluorescence (IF) to detect the correlation between GTSF1 and CCL1 protein expression. We found that the fluorescence intensity of CCL1 was significantly reduced in GTSF1 knockdown cell lines, and subsequent western Blot (WB) experiments also confirmed that GTSF1 could regulate CCL1 protein expression, but CCL1 could not regulate GTSF1 expression (**Figure 4C**, **4D**). Subsequently, we found that knockdown of CCL1 resulted in decreased tumor sphere formation rate in both cell lines, and overexpression of CCL1 in SH-GTSF1 cell lines greatly restored tumor sphere formation rate, further suggesting that GTSF1 could play a role in promoting tumor stemness by regulating CCL1 (**Figure 4E**). Trans-well invasion and wound healing assay assays also demonstrated that overexpression of CCL1 in the SH-GTSF1 group restored tumor invasion and migration abilities and induced tumor malignant progression (**Figure 4F**, **4G**). The findings suggest that GTSF1 may play a crucial role in the development and progression of UCS by enhancing the expression of CCL1, thereby promoting tumor stemness, invasion, and migration capacity.

Positive correlations between GTSF1/CCL1 and M1 macrophages

The chemokine CCL1 can recruit tumor-associated macrophages (TAMs) and Tregs cells, thereby facilitating tumor angiogenesis and lymph node metastasis [16]. By analyzing the correlation between CCL1 and immune cells in TCGA UCS database, we found a positive correlation between CCL1 and M1 macrophages (**Figure 5A**, **5B**). It has been reported that M1-type TAMs can promote tumor stemness and epithelial-mesenchymal transition (EMT) by activating IL-6-Jak/Stat3 in oral squamous cell carcinoma (OSCC) cells [21]. In addition, M1 macrophages were found to promote the expression of tumor stemness related genes through pro-inflammatory factors in prostate cancer [20]. We performed tissue Multiplex immunohistochemistry (mIHC) staining on 14 samples of UCS pathological sections to analyze the correlation between GTSF1, CCL1 and CD86. We found a significant positive correlation between GTSF1 and CCL1 protein expression and M1 macrophages cell number (**Figure 5C**, **5D**).

CCL1 induces M1 macrophages recruitment and promotes tumor stemness and malignant progression

After incubation of virus-infected tumor cells with THP1 cells for 24 hours, we measured the molecular markers of M1 macrophages and found that GTSF1 knockdown inhibited the polarization of THP1 to M1 macrophages, and CCL1 overexpression could rescue the above phenomenon (**Figure 6A**). It further confirmed that GTSF1 could play a role in promoting cancer through CCL1-induced M1-like macrophage phenotype. IL-6 in the tumor microenvironment can promote tumor stemness and metastasis through a variety of ways, and is the core driver of tumor metastasis [22]. We subsequently measured the concentration of IL-6 in the supernatant of the co-incubated cells by ELISA and found that knockdown of GTSF1 inhibited the concentration of IL-6, but it was restored by overexpression of CCL1 (**Figure 6B**). To investigate whether IL-6 might influence UCS stemness and metastasis, we evaluated TCGA UCS database and found that IL-6 expression was positively correlated with stemness related genes (CD44 and PROM1) (**Figure 6C**). The above results suggest that GTSF1 recruits M1-type macrophages to secrete IL-6 through CCL1, which acts on tumor cells to induce tumor stemness and promote invasion ability (**Figure 7**).

Discussion

The UCS is an uncommon, high-grade endometrial cancer with limited treatment options and a propensity for recurrence and metastasis, resulting in a poor prognosis [23]. The GTSF1 is present in the genomes of various plant and animal species, playing a crucial role in the regulation of DNA methylation and retrotransposon activation within germ cells to finely modulate gene expression [8, 24]. At present, GTSF1 has only been reported as a prognostic biomarker in hepatocellular carcinoma [8]. We present novel findings demonstrating the regu-

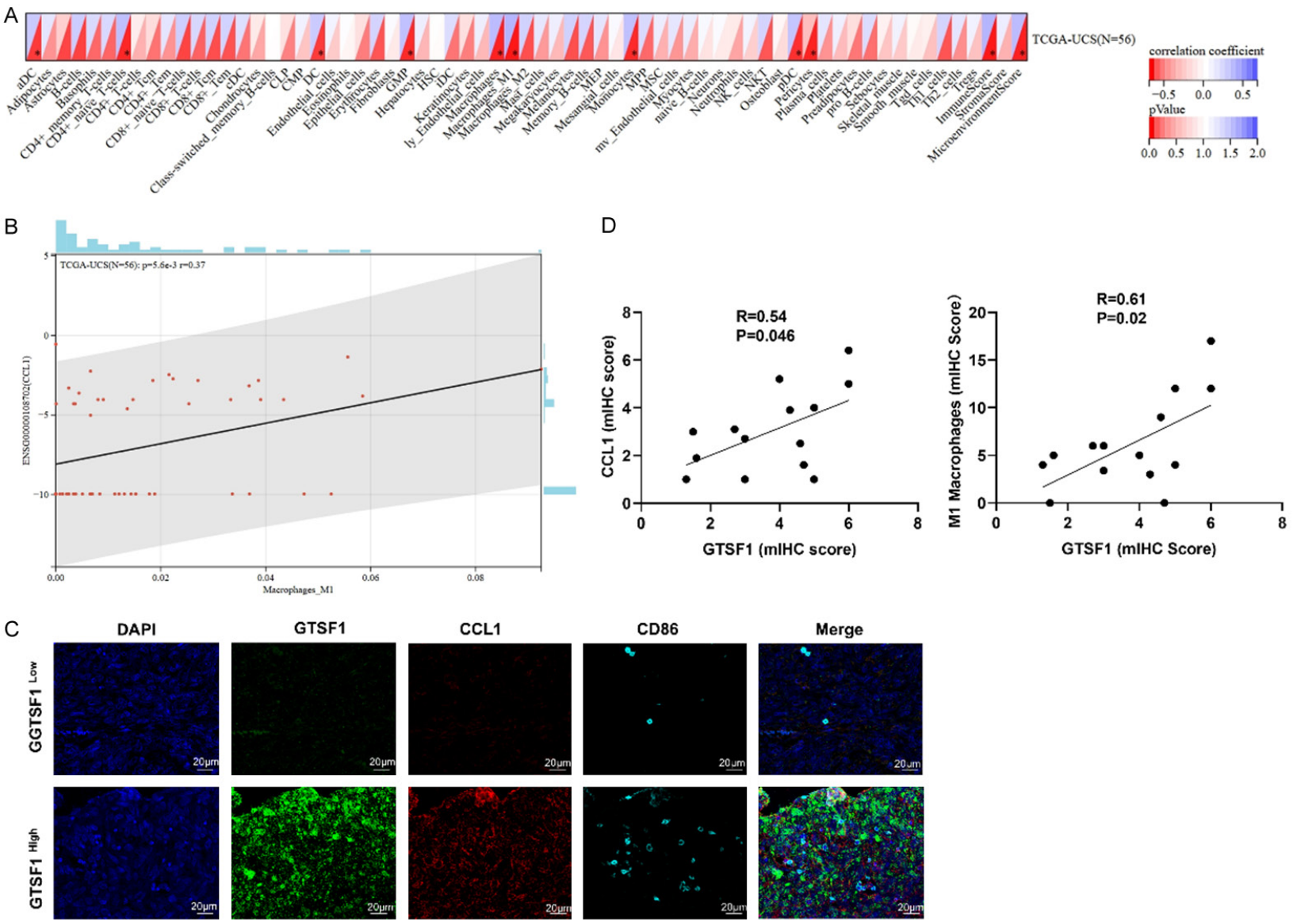


Figure 5. Correlation analysis for GTSF1/CCL1 and M1 macrophages. A. Sangerbox database was utilized to examine the association between CCL1 and infiltration of immune cells. B. There is a positive correlation between CCL1 and M1 macrophages in UCS. C. The expression of GTSF1 and CCL1, as well as the infiltration of M1-type macrophages in 14 samples of UCS, were detected using mIHC. Scale bar: 20 μm. D. The correlation between GTSF1 and CCL1 expression and M1 macrophages was quantified based on fluorescence intensity measurements and cell count analysis. **P<0.05, **P<0.01, ***P<0.001*.

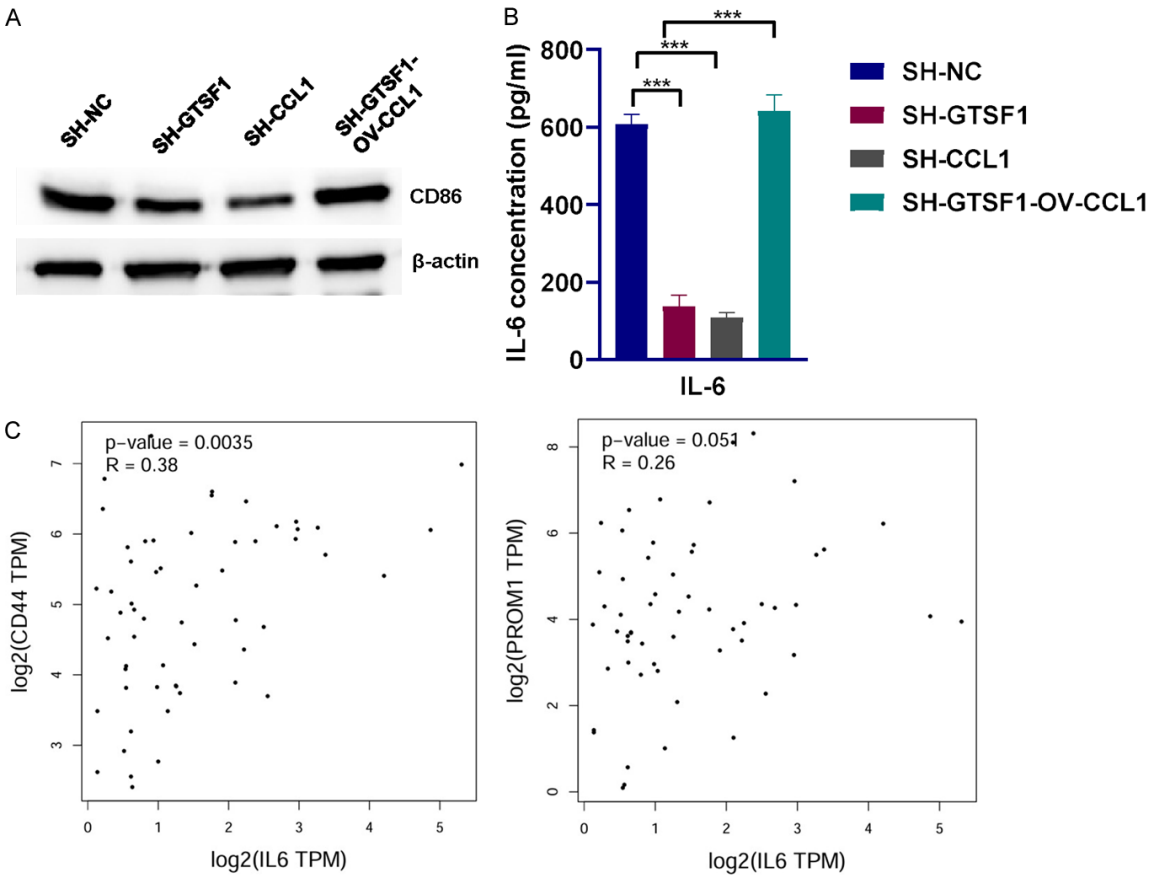


Figure 6. CCL1 induces the recruitment of M1-type macrophages. A. Virus-infected cells were co-cultured with Thp1 cells, and the Thp1 cells in the culture chamber were collected for Western blot protein detection of CD86, a molecular marker for M1 macrophages. B. ELISA was used to detect the concentration of IL-6 in the cell supernatant after co-culture. C. The correlation between IL-6 expression and stemness genes was investigated using publicly available databases. **P<0.05, **P<0.01, ***P<0.001*.

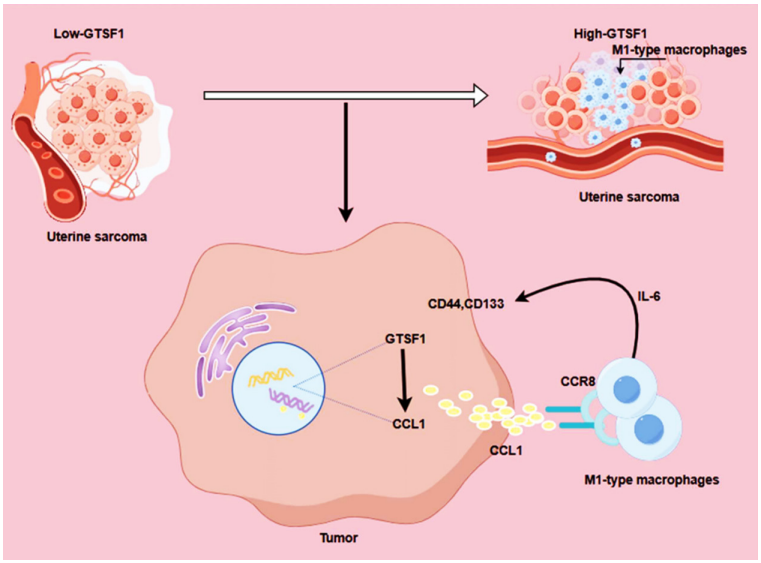


Figure 7. Schematic diagram of GTSF1 regulatory mechanism in uterine carcinosarcoma.

latory role of GTSF1 in modulating CCL1 expression, thereby facilitating recruitment of M1 macrophages for IL-6 release and promotion of tumor stemness and invasion.

The chemokine CCL1 interacts with the chemokine receptor CCR8 to regulate immune cell distribution and plays a role in the regulation of tumor angiogenesis and lymph node metastasis [16, 25]. The binding of c-Myc to the CCL1 promoter in glioma enhances the post-transcriptional interaction between CCL1 and CCR8, thereby facilitating the infiltration of immunosuppressive $CD4^+$

$CD25^+$ Tregs [15]. In breast cancer, SOX2 can bind to the promoter region of CCL1 and activate NF-κB-CCL1, inducing the aggregation of Tregs and promoting tumor stemness [19]. In cervical squamous cell carcinoma (CSCC), the activation of CCL1 is capable of facilitating the aggregation of TAMs and promoting tumor metastasis [26]. Activation of the CCL1-CCR8 axis recruited TAMs to promote liver metastasis of colorectal cancer [27]. In what manner does CCL1 contribute to its oncogenic function in UCS? Experimentally, we have demonstrated that GTSF1 promotes tumor stemness and invasive potential by regulating CCL1 expression. After conducting a comprehensive analysis of the correlation between CCL1 and immune cells in the TCGA UCS database, we have observed a significant positive association between the expression of CCL1 and M1 macrophages. Subsequent validation through mIHC experiments and cell co-culture studies have unequivocally confirmed the pivotal role played by CCL1 in recruiting and facilitating aggregation of M1 macrophages. While our *in vitro* and clinical correlation studies establish a mechanistic framework for the GTSF1-CCL1-macrophage axis in UCS progression, the absence of *in vivo* validation stands as a crucial constraint. Despite observing a notable positive association between GTSF1 and CCL1 protein expression and M1 macrophage cell count, forthcoming animal models will be indispensable to validate the causal link between this axis and metastatic activity in living organisms.

The tumor microenvironment (TME) contains a diverse population of TAMs, which can be classified into distinct subsets with varying functions, including M1 anti-tumor and M2 pro-tumor phenotypes [28, 29]. TAMs predominantly exhibit an M2-like phenotype within the TME, exerting regulatory control over several malignant processes such as angiogenesis, immunosuppression, and tumor metastasis [30-32]. However, the role of M1-type TAM in promoting tumor progression has also been substantiated in recent years. Bednarczyk et al. discovered that the secretion of M1-type macrophages induces epithelial-mesenchymal transition (EMT) in breast cancer cells T47-D and MCF-7, promoting cell migration and invasion. Targeting M1 macrophages or their secreted factors could potentially inhibit EMT and restrict the invasive capacity of breast cancer [33]. Both oral cancer and glioma have demonstrated that M1 macrophages are capable of secreting cytokines to activate tumor-related pathways and promote the expression of genes related to tumor stemness [20, 21]. The promoting or inhibiting effect of M1-type TAM on tumors remains a subject of ongoing controversy, highlighting the need for further research. Therefore, how does M1-type TAM regulate tumor stemness and invasion in UCS? The M1 macrophages have the capability to secrete various cytokines, including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), which play crucial roles in regulating tumor progression [34]. Among these cytokines, IL-6 has been extensively documented to be closely associated with tumor stemness and metastasis [35-39]. We also analyzed the cell supernatant post co-culture and observed that knockdown of GTSF1 resulted in suppression of M1 macrophage production and IL-6 secretion, whereas overexpression of CCL1 in the GTSF1 knockdown group restored these effects. The expression of tumor stemness markers (CD44 and CD133) in UCS was also found to be positively correlated with IL-6, indicating a potential role for IL-6 in promoting the stemness phenotype of UCS. In this study, we have demonstrated that GTSF1 facilitates the upregulation of CCL1 expression in UCS, thereby inducing the generation and recruitment of M1 macrophages. This process subsequently exacerbates IL-6 secretion, further augmenting cancer stem cell properties and invasive potential.

The present study for the first time suggested that GTSF1 could be a valuable molecular marker for predicting tumor recurrence and metastasis in the context of UCS. In addition, this study detailed the role of GTSF1 in promoting M1 macrophage generation and aggregation through CCL1 activation, thereby maintaining the tumor stemness phenotype and invasive potential. The blockade of CCL1 or IL-6 may present a promising therapeutic approach for relapsed or refractory UCS.

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Disclosure of conflict of interest

None.

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Table S1. Virus infection sequence

Lentivirus	Sequence
SH-GTSF1-1	5'-CACAAGCATCCTGTCTCATGTGT-3'
SH-GTSF1-2	5'-GGCAGGGTATCATCTTTCTATTC-3'
SH-CCL1	5'-GCTCGCGAGCTATAGAAGAAT-3'
OV-CCL1	5'-GCUAUCAGUCCACUGUGGUGGU-3'

Table S2. The sequences of primers for detection of gene expression

Gene Name	Forward Primer	Reverse Primer
GTSF1	5'-CACAAGCATCCTGTCTCATGTG-3'	5'-CTACACTTCTGGTCTGGGATTAC-3'
β-actin	5'-TGGCACCCAGCACAATGAA-3'	5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'

Table S3. The list of antibodies in the research

Position	Antibody	Company	Dilution	Incubation
Immunohistochemistry				
1	GTSF1	Proteintech	1:200	4°C overnight
2	CCL1	Proteintech	1:200	4°C overnight
3	CD86	Proteintech	1:200	4°C overnight
Western blot				
1	GTSF1	Proteintech	1:1000	4°C overnight
2	CCL1	Proteintech	1:1000	4°C overnight
3	CD86	Proteintech	1:1000	4°C overnight