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ORIGINAL RESEARCH

Exploration of the Correlation Between *GRHL1* Expression and Tumor Microenvironment in Endometrial Cancer and Immunotherapy

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Introduction: *GRHL1* belongs to the family of Grainyhead-like (GRHL). Previous studies have shown that dysregulation of growth and survival pathways is associated with the GRHL family of gene cancers. Immunotherapy with checkpoint inhibitors has changed the treatment paradigm for many tumors, including endometrial cancer (EC). However, the effect of *GRHL1* on immunotherapy in EC and its relationship with immune cell infiltration are poorly understood.

Methods: Differential expression of *GRHL1* between EC and normal EC tissues was analyzed by searching the TCGA database, and the results were verified utilizing immunohistochemistry analyses. Next, the relationship between *GRHL1*, CD8+ T cells and tumor microenvironment (TME) was also investigated, and the effect of *GRHL1* expression on immunotherapy in EC was evaluated.

Results: According to the findings, EC tissues had elevated expression levels of *GRHL1* relative to normal tissues. Patients with EC who expressed *GRHL1* at high levels experienced worse overall survival (OS) and Progression-free survival (PFS) than those whose expression was lower. In addition, *GRHL1* expression was negatively correlated with CD8+ T cells, and patients with high *GRHL1* expression were less effective in receiving immunotherapy.

Conclusion: The expression of *GRHL1* was high in EC patients, and high expression of *GRHL1* inhibits the proliferation of CD8+ T cells in the tumor microenvironment of EC and affect the efficacy of immunotherapy.

Keywords: *GRHL1*, endometrial cancer, tumor microenvironment, CD8+ T cells, immunotherapy

Introduction

Endometrial cancer (EC) is the sixth most common neoplasm in women worldwide.[1] In the US, EC is the most prevalent type of gynecological cancer.[2] In the US, the mortality rate for people of the white race was 4.3–4.5 per 100,000 individuals, while this value for people of the black race was 8.2–8.9 per 100,000 population. In China, the mortality rate for people of the yellow race was 2.5 per 100,000 individuals.[3] Even though high-grade EC often has a positive prognosis, it tends to recur. Because recurrent EC has such a dismal prognosis, preventing it is crucial. Currently, traditional surgery and minimally invasive surgery are the two most crucial treatment options for EC.[4,5] The use of radiotherapy, immunotherapy, targeted therapy, and chemotherapy in the treatment of EC has increased in recent years.[6–8]

The GRHL family of genes has been implicated in the development of many cancers,[9–13] and is involved in the regulation of embryogenesis, and growth and survival pathways in cancer,[14] where it leads to dysfunction through dedifferentiation or loss of functional integrity.[15] As a member of the GRHL family, *GRHL1* is mainly associated with neuroblastoma and esophageal cancer.[16,17]

In this study, the possible involvement of *GRHL1* in EC and its expression in TME were validated. Using the TCGA database and in vitro tests, we discovered that *GRHL1* expression was considerably upregulated in EC tissues. We also

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explored the level of *GRHL1* expression and its relationship with prognosis. In addition, the correlation of *GRHL1* with the tumor microenvironment and CD8+ T cells was analyzed, and the effect of *GRHL1* expression on EC immunotherapy was evaluated.

Materials and Methods

Dataset Source and Pre-Processing

The TCGA database was searched to retrieve clinical information on patients with uterine corpus endometrial carcinoma (UCEC), covering information on common gene expression, total mortality, and prognosis.[18] 33 TCGA pan-cancer tumor mutation burden (TMB) and microsatellite instability (MSI) data downloaded from the UCLA Xena Data Portal (https://xenabrowser.net/). RNA sequencing (FPKM values) was downloaded from the TCGA Genome Data Commons (GDC, https://portal.gdc.cancer.gov/) and then scrutinized utilizing the R package TCGAbiolink.[18] The FPKM values were converted to transcript per kilobase million values. To account for the non-biological technical bias-related batch effect, the "ComBat" method from the sva package was implemented. R (v 4.1.2) and the R Bioconductor package were applied to analyze all of the data.

Clinical Sample Collection

Patients undergoing EC surgery at the First Affiliated Hospital of Bengbu Medical College, Department of Oncology Gynecology, from January 2021 to December 2021, were recruited for this research, and samples were obtained from those patients. Immunohistochemistry (IHC) labeling was performed on 66 EC tissue specimens and 10 surrounding normal tissues. Before or after surgery, no patient had chemotherapy, radiation, or biological treatment, and there was no history of EC in any of the patients. Tissue samples collected after surgery were preserved in a refrigerator at −80 degrees Celsius until protein extraction.

Experimental Materials

CUSABIO (CSB-PA868368LA01HU) provided rabbit anti-human antibody *GRHL1* (50μL, WUHAN, CH). Primary antibodies against actin and alpha rabbit monoclonal antibodies against CD8+ T cells were purchased from Cell Signaling Technology Inc. (Danvers, MA, United States). Jackson ImmunoResearch Inc. provided an anti-rabbit antibody coupled to horseradish peroxidase (HRP, West Grove, PA, US). Sigma-Aldrich provided bovine serum albumin (BSA, St. Louis, MO, United States). Skim milk and Tween-20 were supplied by Sangon Biotech Co., Ltd (Shanghai, China).

Immunohistochemistry (IHC)

Paraformaldehyde (4%) (PFA) was employed to fix all tissue samples, followed by embedding them in paraffin, cutting them into sections, and attaching them on slides. Extraction of antigens was performed by soaking the slides in citrate buffer (pH 7.8, 0.1M) for 24 mins at about 82°C after they had been deparaffinized, rehydrated, and exposed to xylene density gradients. To inhibit peroxidase activity, slides were coated uniformly with an endogenous blocking solution for 15 min at room temperature (RT). Overnight, slides were treated with anti-*GRHL1* primary antibody, then rinsed gently in PBS. Following a 10-minute incubation at RT with a biotin-conjugated secondary antibody, the samples were treated with streptavidin peroxidase for 5 mins. Next, the slides were rinsed with hematoxylin dye to eliminate the remaining debris. After the slides had been dried and washed, an IHC examination was performed. Sections were scored by two pathologists with extensive experience in double-blind reading. To determine the extent of staining, a quantity score (0–4) denoted 0, 0%; 1, 1–10%; 2, 11–50%; 3, 51–80% and 4, 81–100% of positive cells, was used. The staining intensity was divided into three grades: weak, moderate, and strong staining. It should be noted that the corresponding intensity scores ranged from 1 to 3. The final IHC score was calculated by multiplying the quantity and intensity scores.

Assessment of the Immune Characteristics of the EC Tumor Microenvironment

Immunological features of TME in EC encompass the immunomodulator expression, the cancer immune cycle activity, TIIC infiltration levels, and suppressive immune checkpoint expression. Information on 48 immunomodulators was collected, including chemokines and their receptors.[19] To examine the link between *GRHL1* expression and the

infiltration levels of immune cells, we employed the Tumour Immune Estimation Resource (TIMER)[20] and CIBERSORT algorithm to investigate how *GRHL1* expression relates to immune cell activity. When the p-value was <0.05, it was considered to be significant. To avoid errors, correlations between *GRHL1* and CD8+ T cells were calculated based on 7 different algorithms: XCELL, QUANTISEQ, CIBERSORT−ABS, CIBERSORT, MCP−COUNTER, TIMER and EPIC algorithms.[21–27]

Immune Response Analysis

The Cancer Genome Atlas (TCGA) and other sources of next-generation sequencing (NGS) data for 20 solid tumors are provided with an extensive immunogenomic analysis by the Cancer Immunome Database (TCIA) (https://www.tcia.at/home).[28] Using the ggpubr R program, the immunological phenomenon scores (IPS) of 560 EC patients from this database were used to assess immunotherapy.

Statistical Analysis

R software (version 4.1.2) and Perl (version 5.32.1.1) were utilized for data analysis. P <0.05 indicated the significance level.

Results

Expression of *GRHL1* in Endometrial Cancer and Adjacent Normal Tissues and Its Prognostic Assessment in Endometrial Cancer

In the TCGA cohort, the *GRHL1* mRNA expression level was found to be elevated in EC tissues compared to nearby normal tissues ($p < 0.001$, Figure 1A). The comparison of the mRNA expression of *GRHL1* in 35 pairs of EC and adjacent normal tissues in the TCGA database showed that *GRHL1* expression was higher in EC tissues than in adjacent normal tissues ($p < 0.05$, Figure 1B). IHC staining tests confirmed this finding at the tissue level (Figure 1C). KM analysis of survival of patients exhibiting varying *GRHL1* expression levels based on TCGA-derived data was performed to evaluate the prognostic significance of *GRHL1* in EC. According to the results, the 10-year OS was substantially higher in patients whose *GRHL1* expression was lower as compared to those whose *GRHL1* expression was higher ($p =0.035$, Figure 1D). We also discovered that *GRHL1* overexpression was a poor predictor of EC PFS ($p = 0.042$, Figure 1E). Overall, remarkably higher expression levels of *GRHL1* were found in EC, in comparison to nearby normal tissues. *GRHL1* upregulation was associated with a shorter OS, and PFS for patients.

Clinicopathological and Predictive Value of GRHL1 Expression in EC Patients

Using box plots and heatmaps, we revealed that *GRHL1* expression levels were correlated with clinicopathological parameters of EC patients (Figure 2A–E). The results showed that the expression level of *GRHL1* did not correlate well with the clinicopathologic stage. Patients' clinical stage, grade, and age were shown to have a substantial association with their OS, and *GRHL1* was found to have statistically significant predictive value for EC patients, as shown in a univariate analysis ($p = 0.044$, Figure 2F). In addition, multivariate analysis demonstrated that clinical stage, grade, and age were substantially linked to EC patients' OS, but the prognostic value of *GRHL1* for EC patients was found to be insignificant ($p = 0.165$, Figure 2G).

GRHL1 Inhibits CD8+ T Cell Proliferation and is Associated with Immune Escape in EC

Herein, we assessed the correlation of chemokines and their receptors with *GRHL1* expression (Figure 3A and B). We found that CCL2, CCL4, CCL4, CCL5, CXCL9, CXCL10, CXCR3, and CCR5 were negatively correlated with the expression of *GRHL1*. Chemokines that bind to CXCR3 (such as CXCL9 and CXCL10) are essential and necessary for the trafficking of activated CD8+ T cells to tumor sites.[29] Subsequently, we used the TCGA-derived data to calculate the TME scores using the EC expressions and the "estimate" package included R. Furthermore, we plotted TME differential analysis. The ImmuneScore, StromalScore, and ESTIMATEScore of TME were downregulated in the high *GRHL1* expression group (Figure 3C).

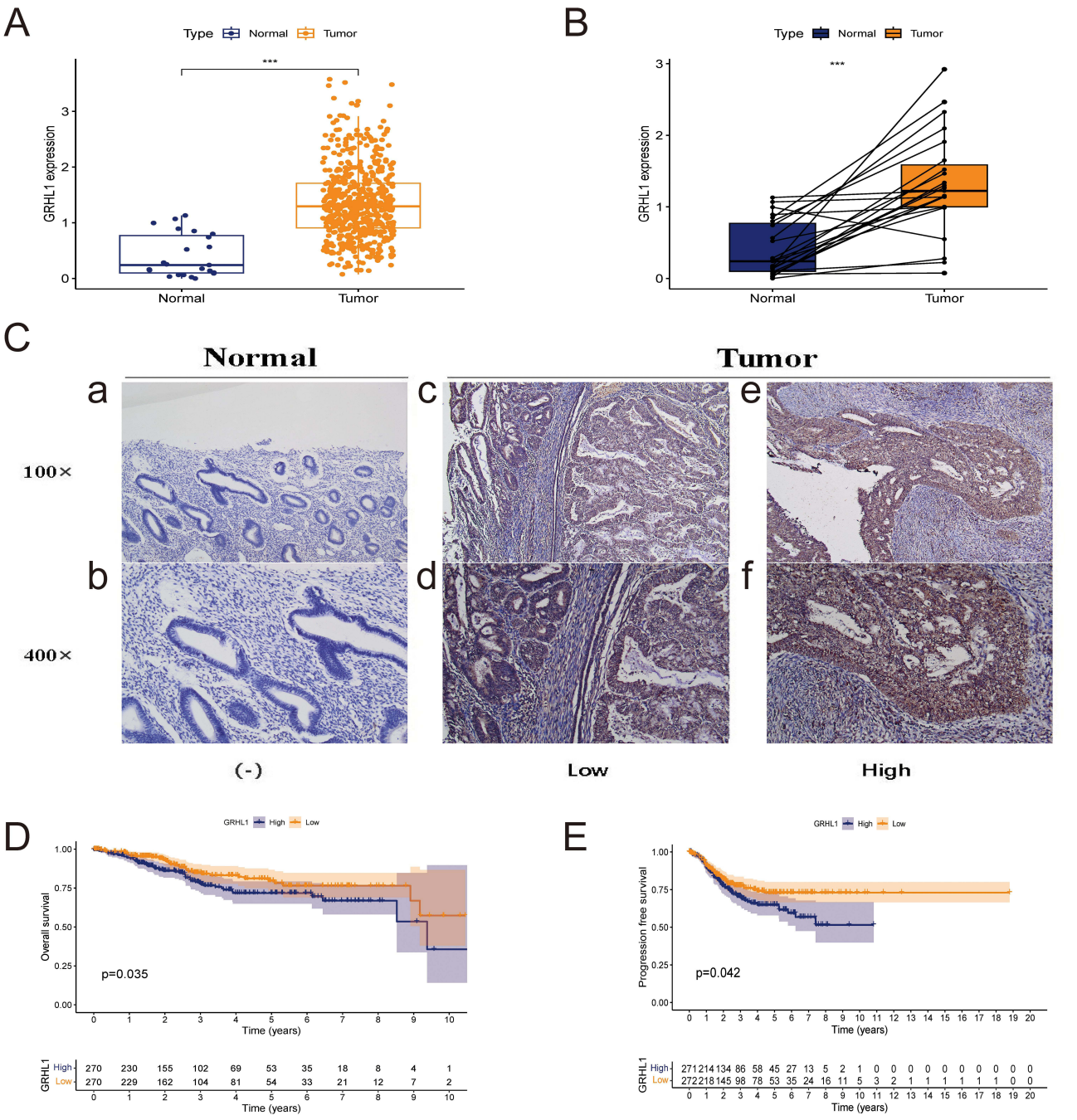

Figure 1 Expression of *GRHL1* in endometrial cancer and adjacent normal tissues and its prognostic assessment in endometrial cancer. (**A**) *GRHL1* was overexpressed in the TCGA cohort. (**B**) In the TCGA cohort, *GRHL1* was over-expressed in the tumor relative to the noncancerous tissues. (**C**) IHC staining analysis showed that *GRHL1* expression was low in paracancerous tissues (a and b) and up-regulated in EC tissues (c–f). (**D**) Kaplan-Meier analysis of OS of individuals with EC with *GRHL1* gene expression in the TCGA database. (**E**) Kaplan-Meier analysis of PFS of individuals with EC with *GRHL1* gene expression in the TCGA database. (***p < 0.001).

The CIBERSORT algorithm was used to verify the correlation between *GRHL1* and immune cells (Figure 4). The abundance of 22 different types of immune cells was measured and compared between the two groups (Figure 4A). *GRHL1* was negatively linked to CD8+ T cells, T cells regulatory (Tregs), NK cells activated, and Mast cells resting, and positively correlated with Neutrophils, T cells CD4 memory resting, Dendritic cells activated, B cells naive, and Mast cells activated (Figure 4B–K). In previous studies, TME has been categorized into two subtypes as follows: an inflammatory TME dominated by T-cell infiltration and a non-inflammatory TME dominated by T-cell suppression.

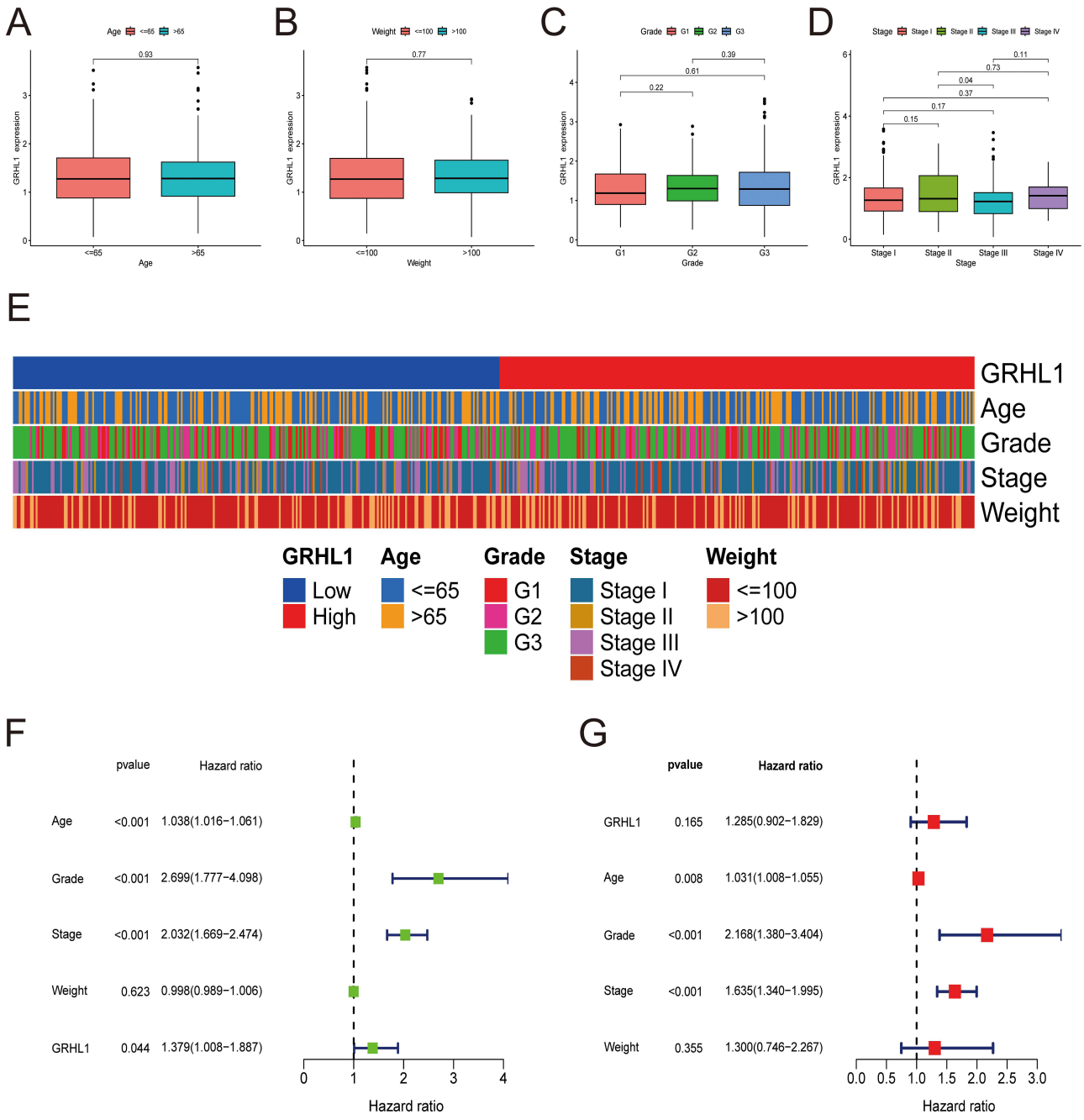

Figure 2 Association of clinicopathological characteristics with *GRHL1* expression levels. (**A**–**D**) The association of *GRHL1* expression level with the clinicopathological features of individuals with EC for age (**A**), weight (**B**), Grade (**C**), and stage (**D**). (**E**) Heatmap illustrating the link between the clinicopathological characteristics of EC patients and the *GRHL1* expression. (**F** and **G**) Forest plots show the results of univariate (**F**) and multivariate (**G**) Cox regression analyses for the OS of patients with EC.

Tumors with T-cell inflammation contain abundant CD8+ T cells. Tumors without T-cell inflammation lack these cells but contain blood vessels, fibroblasts, and macrophages, which support tumor growth.[30,31]

Next, we showed *GRHL1* correlation with CD8+ T cells based on ssGSEA, MCPcounter, TIMER, ESTIMATE, CIBERSORT, XCELL, and EPIC algorithms (Figure 5A–H). Among the six algorithms, *GRHL1* correlated negatively with CD8+ T cells (Figure 5B–G). TME cell infiltration is characterized by three types: immunoinflammatory phenotype (immunoinflammatory), immune exclusion phenotype (immune), and immune desert. The immune exclusion and the immune desert phenotypes are also known as non-immune inflammatory phenotypes.[32] Taken together, we define the

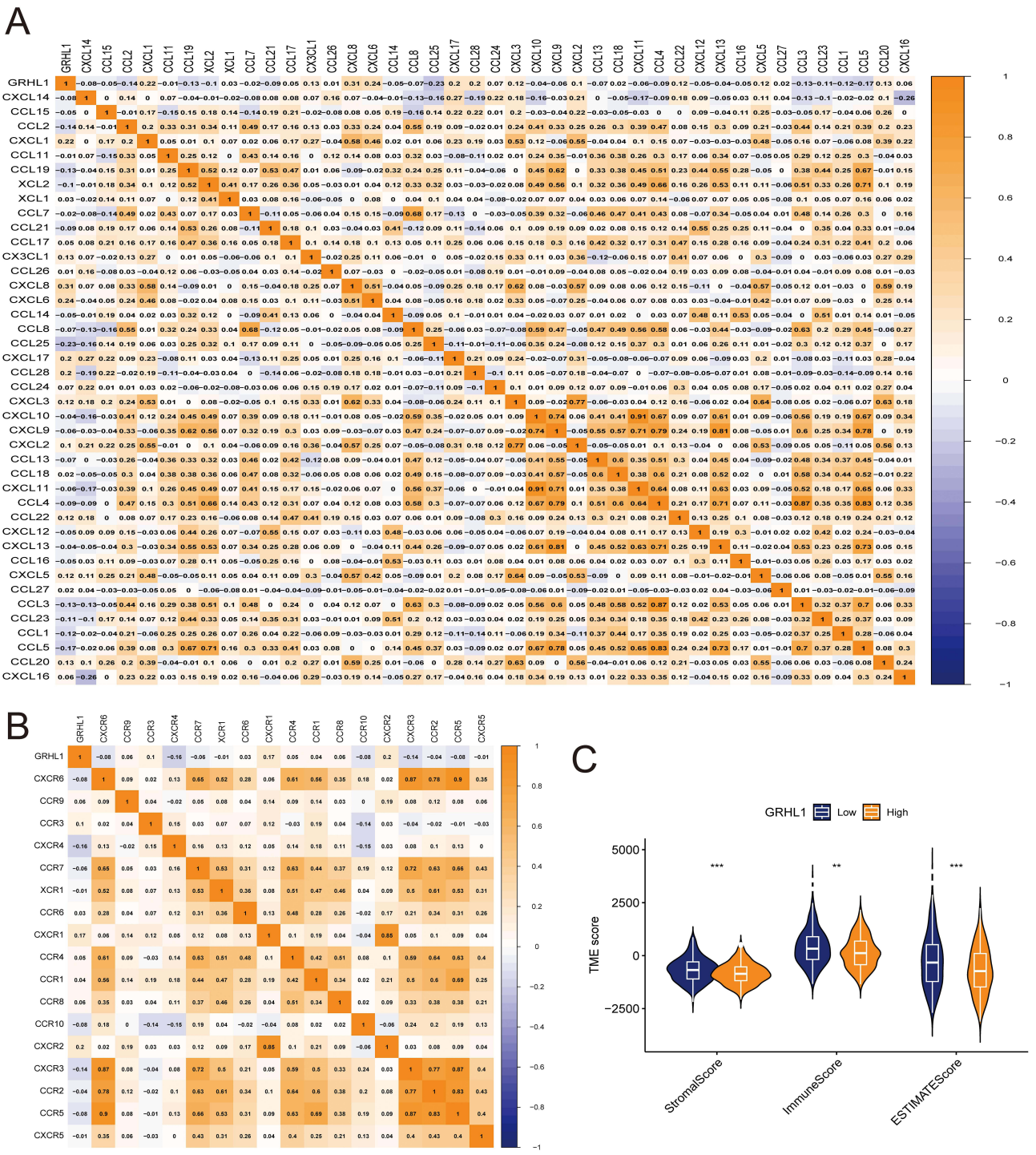

Figure 3 Correlation of *GRHL1* with chemokines and tumor microenvironment in EC. (**A** and **B**) Correlation of *GRHL1* with the immunomodulator chemokine (**A**) and its receptor (**B**) in EC. (**C**) The TME's ESTIMATEScore, ImmuneScore, and StromalScore varied between the groups with high and low *GRHL1* expression. (**p < 0.01; ***p < 0.001).

immune microenvironment shaped by *GRHL1* as an immunodeficient phenotype (immune exclusion phenotype), that is, a non-immune inflammatory phenotype.

Analyses of Immune Cell Infiltration in High- and Low-GRHL1-Expression Groups

Since *GRHL1* was negatively correlated with CD8+ T cells, we investigated its potential role in the tumor immune microenvironment (TIME) of EC. IHC staining was performed to compare the tissue samples with low (+) and high (+++) *GRHL1*

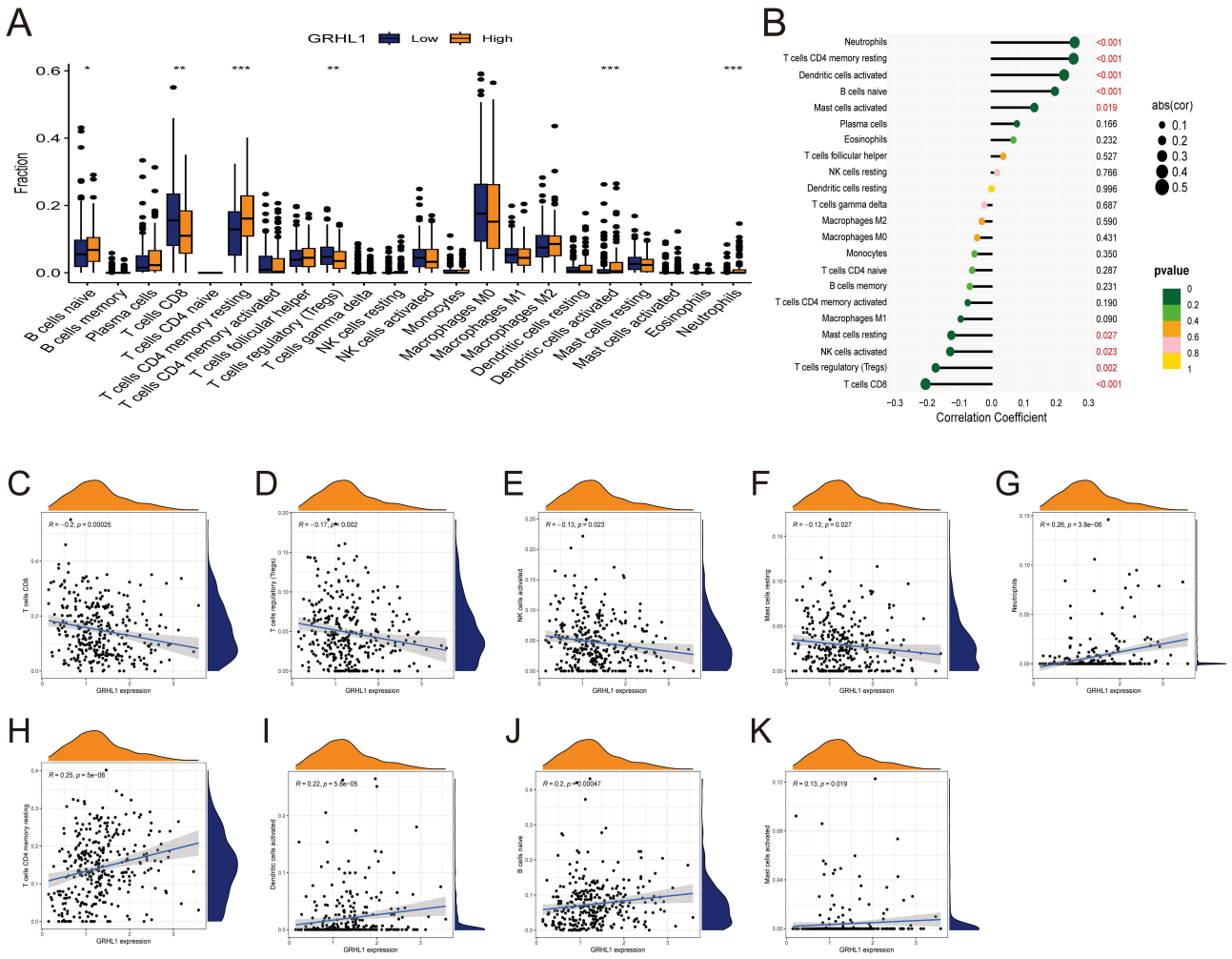

Figure 4 Link between *GRHL1* and immune cell infiltration. (**A**) Box plots showing the differences between 22 immune cell types and *GRHL1* expression analysis in EC. (**B**) Correlation between *GRHL1* expression and 22 immune cell types in EC. (**C**–**F**) *GRHL1* expression was negatively linked to CD8+ T cells (**C**), T cells regulatory (Tregs) (**D**), NK cells activated (**E**), Mast cells resting (**F**). (**G**–**K**) *GRHL1* expression was positively correlated with Neutrophils (**G**), T cells CD4 memory resting (**H**), Dendritic cells activated (**I**), B cells naïve (**J**), and Mast cells activated (**K**). (*p < 0.05; **p <0.01; ***p < 0.001).

expression levels (Figure 1C). To determine whether there was a link between high *GRHL1* expression and CD8+ T cells in TIME, we used IHC labeling to measure the infiltration of CD8+ T cells into EC and the surrounding tissues. CD8+ T cells in EC tissues with high (+++) *GRHL1* levels were particularly clustered in the peripheral tumor stroma, with just a small proportion of immune cells infiltrating the stroma to reach the tumor's parenchyma (Figure 6A). Conversely, a greater number of immune cells infiltrated the stroma of the tumor parenchyma, and fewer CD8+ T cells were clustered throughout the tumor parenchyma in EC tissues with low (+) *GRHL1* levels (Figure 6B). In summary, high expression of *GRHL1* inhibited the proliferation of CD8+ T cells in the TIME of EC, as shown by the analysis.

Correlation Analysis of GRHL1 Expression with TMB and MSI and Evaluation of Immunotherapy

After exploring immune phenomenon scores (IPS) through TCIA,[28] it was observed that patients with high *GRHL1* expression were less effective in receiving immunotherapy (Figure 7A–D, $p < 0.05$). To exclude the influence of confounding factors on immunotherapy efficacy, we also assessed the correlation between TMB/MSI and *GRHL1* expression. (Figure 7E and F, $p > 0.05$).

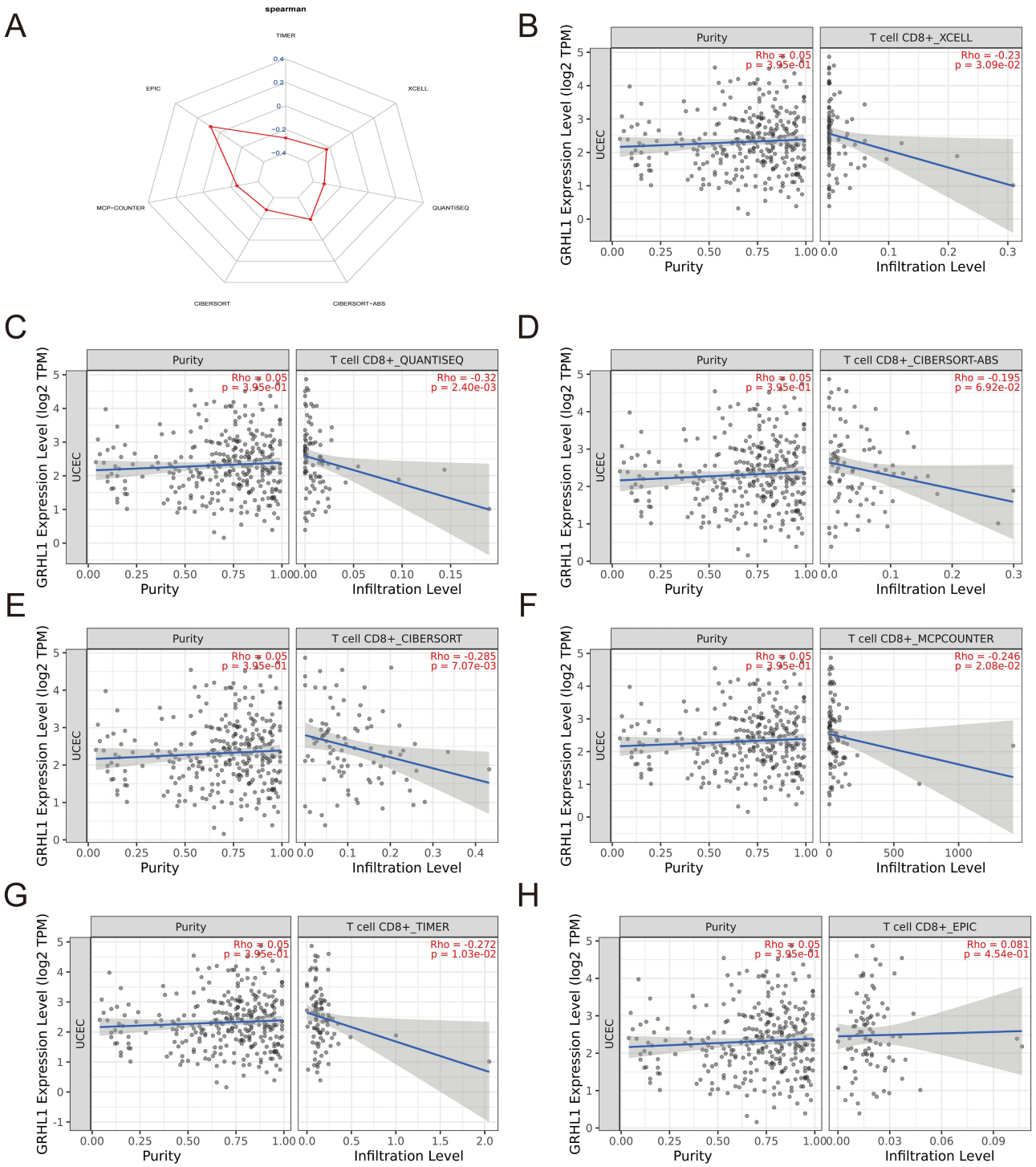

Figure 5 Investigation of the relationship between *GRHL1* expression and CD8+ T cells. (**A**) Radar plot of correlation analysis between *GRHL1* expression and CD8+ T cells. (**B**–**H**) Seven immunological algorithms were utilized to investigate the link between *GRHL1* expression and CD8+ T cells: XCELL (**B**), QUANTISEQ (**C**), CIBERSORT−ABS (**D**), CIBERSORT (**E**), MCP−COUNTER (**F**), TIMER (**G**) and EPIC algorithms (**H**).

Discussion

Endometrial cancer (EC) is among the three most prevalent gynecological malignancies.[33] It has now become the most common gynecologic cancer in developed countries.[33] Although the majority of patients are diagnosed at an early stage and have a favorable prognosis, there are still some patients with advanced stage at the time of initial diagnosis or

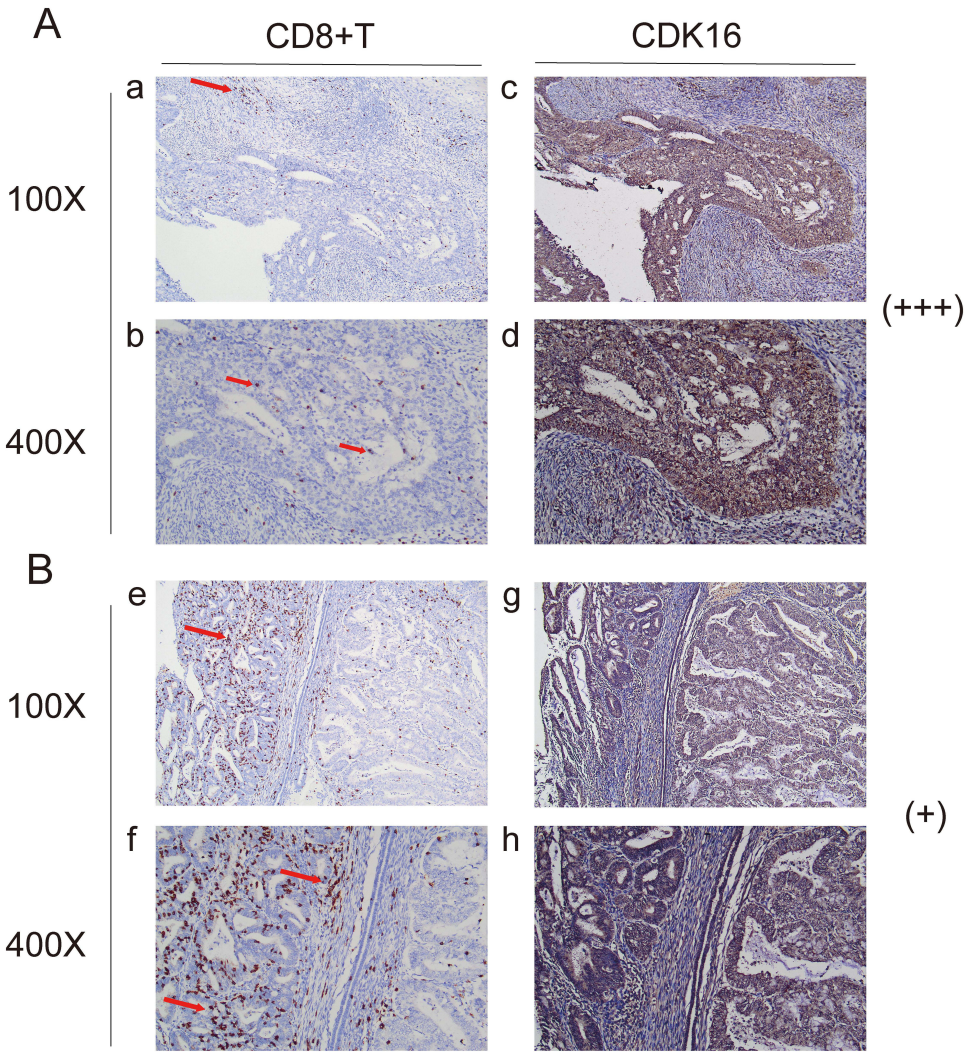

Figure 6 Immune cell infiltration in the groups with high and low *GRHL1* expression. (**A**) CD8+ T cell (a and b) infiltration in the group with high *GRHL1* expression (c and d). (**B**) CD8+ T cell (e and f) infiltration in the group with low *GRHL1* expression (g, h). The locations of immunological cells are indicated by the red arrows.

recurrence and metastasis after treatment, and the 5-year survival rate is only 20–26%.[34] The success of tumor immunotherapy has shown that immunotherapy plays a landmark role in cancer treatment.[35] In recent years, treatment guidelines for EC have been updated to include targeted treatments such as immune checkpoint inhibitors.[36,37]

Homologous sequencing has led to the identification of 3 GRHL genes in the human genome to date.[14] Recent years have seen a surge in the publication of multiple studies on GRHL and cancer, and these findings provide compelling evidence that GRHL is strongly associated with cancer.[15,38,39] *GRHL3* is closely associated with epithelial cancers, including skin, breast, and head and neck cancers.[10,11,40] *GRHL2* acts as an oncogene to suppress epithelial-to-mesenchymal transition (EMT).[41,42] In recent years *GRHL1* has also been found to be closely associated with skin cancer, esophageal cancer and neuroblastoma.[16,17,43]

We discovered that *GRHL1* expression was upregulated in EC tissues in comparison to non-EC tissues. *GRHL1* upregulation was associated with a shorter OS, and PFS for patients. Based on these results, *GRHL1* could be a potential target gene for EC. It has been shown that immune infiltration and immune escape of tumors correlate with both the prognosis of cancer and the patient's response to treatment.[44] The majority of tumor cells express antigens that may facilitate the identification of the tumor by the host CD8+ T cells. The TME allows for the categorization of immune escape into two distinct subtypes. One of the primary subsets has a phenotype that is indicative of T cell inflammation. These tumors can resist immune

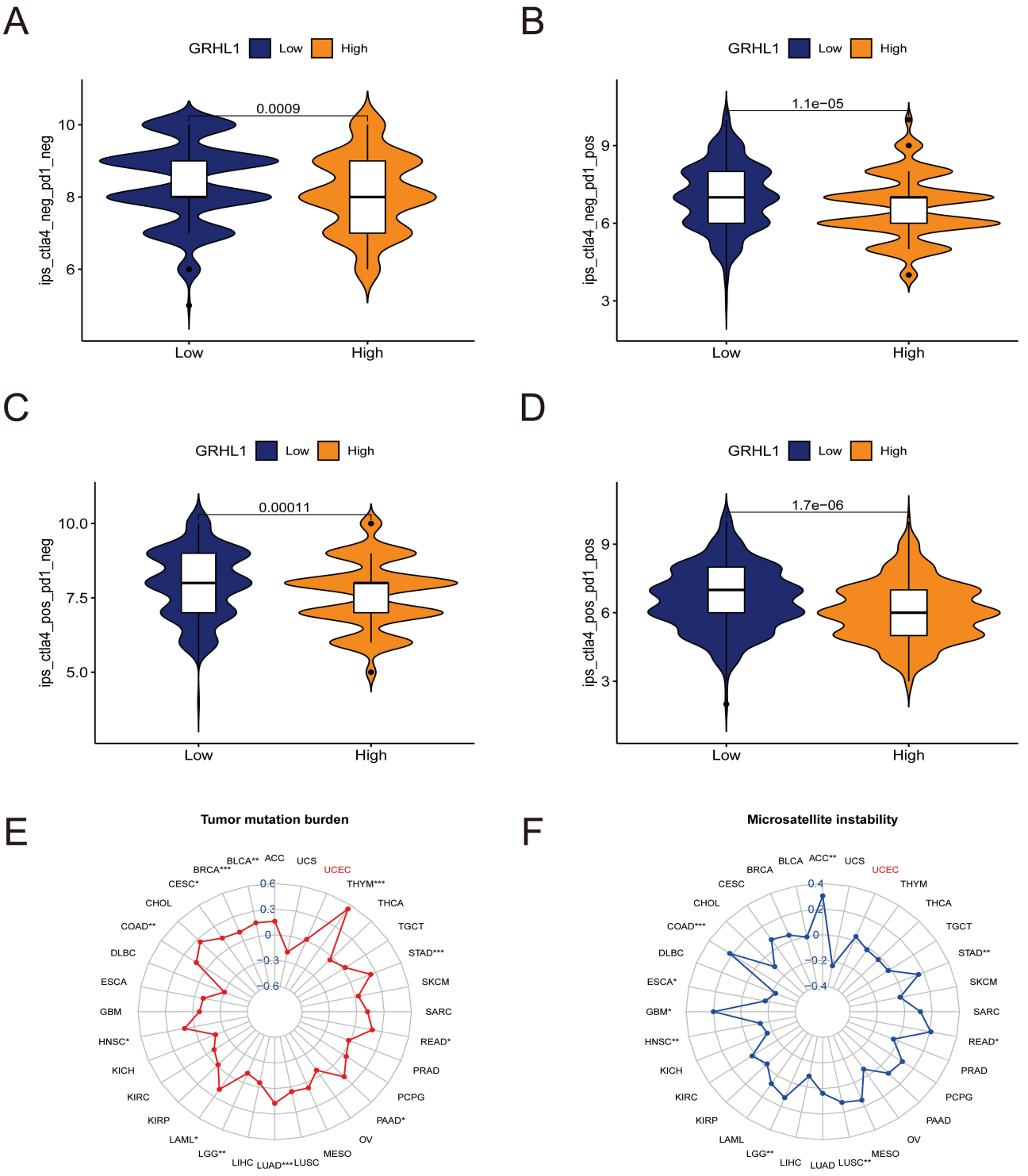

Figure 7 Correlation analysis of *GRHL1* expression with TMB and MSI and evaluation of immunotherapy. (**A**–**D**) Association of IPS with the *GRHL1* expression in individuals with EC based on the TCIA database; CTLA4- PD1- (**A**), CTLA4- PD1+ (**B**), CTLA4+ PD1- (**C**), CTLA4+PD1+ (**D**). (**E** and **F**) Correlation analysis of *GRHL1* expression with TMB (**E**) and MSI (**F**). (*p < 0.05; **p <0.01; ***p < 0.001).

attack through the dominant inhibitory effects of immune system-suppressive pathways. The other group does not exhibit this T cell inflammation profile and hence is resistant to immunological assault due to being excluded or ignored by the immune system.[45] Standardized cancer immunotherapy requires that a target molecule exhibit TME-specific upregulation and immunosuppression action.[46] To determine the potential role of *GRHL1* in EC and its expression in TME, we explored the

EC cohort from the transcriptome of specimens from the TCGA database dataset, and the results showed that EC expression was significantly overexpressed in tumor tissues. Additional research using IHC validated these findings. Our data also demonstrated a negative association between *GRHL1* and the immunological states of TME in EC. Using seven different algorithms, we found that in the high *GRHL1* group, T cell recruitment activity was remarkably downregulated, *GRHL1* was negatively linked to CD8+ T cell activation, and the amount of TIIC infiltration was substantially decreased. These results suggest that high expression of *GRHL1* in EC may inhibit the proliferation of CD8+ T cells, thereby affecting the efficacy of immunotherapy. Subsequent IHC experiments also verified this result. Therefore, targeting *GRHL1* in EC may improve the success of immunotherapy. Previous studies have shown that about 30% of endometrial cancers have mismatch repair defects, which increase the adverse effects of drug therapy, while the defects increase the likelihood of a positive response to immune checkpoint inhibitors.[47] And there is growing evidence that tumor mutational load is a promising predictor of immune checkpoint inhibitor therapy.[48] To rule out the influence of the confounding factors TMB and MSI on the efficacy of immunotherapy, we assessed the correlation between TMB/MSI and *GRHL1* expression. We found that there was no significant correlation between *GRHL1* expression level and TMB/MSI in endometrial cancer. This result also verified our conclusion from other aspects.

Some limitations remain in this study. The TCGA data served as the foundation for our investigation of *GRHL1*'s involvement in EC. Despite our success in using IHC assays to verify the link between *GRHL1* and immune cells infiltrating TME, the absence of equivalent confirmation in cellular and animal trials suggests where we should focus our future efforts. Second, the effect of *GRHL1* on patient immunotherapy was derived from an analysis of the TCGA database and lacks direct evidence. Third, although we analyzed a direct link between *GRHL1* expression and MSI, we did not stratify the mismatch repair status of the tumor on consecutive samples and TCGA data, and therefore could not obtain *GRHL1* results for the mismatch repair good and mismatch repair bad subgroups, which is a direction for our future work.

Conclusion

Our results show that *GRHL1* is highly expressed in EC tissues compared to normal samples and can be characterized as a specific biomarker to distinguish EC tissues from normal endometrial tissue. Unfavourable OS, and PFS were all highly correlated with *GRHL1* upregulation in EC patients. Immune cell infiltration analysis showed that high expression of *GRHL1* inhibited the proliferation of CD8+ T cells and affected the efficacy of immunotherapy. Our thorough analysis of *GRHL1*'s potential as an immune target for EC may offer a useful assessment system for clinical use.

Abbreviations

EC, endometrial cancer; GRHL1, Grainyhead like transcription factor 1; TCGA, The Cancer Genome Atlas; OS, overall survival; GSEA, Gene set enrichment analysis; IHC, Immunohistochemistry; RT, room temperature; DCs, resting dendritic cells; KM, Kaplan-Meier; FDR, false discovery rate; TME, tumor microenvironment; TIME, Tumor immune microenvironment; TMB, tumor mutation burden; ICI, Immune checkpoint inhibitor; ICB, immune checkpoint blockade; MSigDB, Molecular Signatures Database; FDRs, false discovery rates; TIMER, Tumour Immune Estimation Resource; TCIA, The Cancer Immunome Database; IPS, immune phenomenon scores.

Data Sharing Statement

The following online resources were screened based on this study's analysis of used clinical data: TCGA (https://www.cancer.gov/), TCIA (https://tcia.at/).

Ethics Approval and Informed Consent

The study involving human participants has been reviewed and approved by the First Affiliated Hospital of Bengbu Medical College's [2021] 143 ethics committee. The Helsinki Declaration, which outlines moral guidelines for medical research with human participants, was followed in conducting the study. Patients/participants signed a consent form to indicate their agreement to be included in the study with each patient providing their informed consent.

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Author Contributions

All authors contributed significantly to the research that was published, whether it was in the conceptualization, research design, implementation, data gathering, analysis, and interpretation, or each of these areas separately; participated in the report's drafting, revision, or detailed evaluation; approved the final version for publishing; decided upon the journal to which the manuscript has been submitted; and accept responsibility for all aspects of the project.

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Disclosure

The authors report no conflicts of interest in this work.

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