

The Clinicopathological Characteristics, Prognosis and Immune Microenvironment Mapping in MSI-H/MMR-D Endometrial Carcinomas

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Abstract

Endometrial cancer had a relatively high prevalence of MMR deficiency. MMR-D/MSI-H endometrial cancer patients are suggested to be potential beneficiaries of PD-1/PD-L1 inhibitor therapy. Here, we explored the prognostic value of MSI subtype in endometrial cancer and its correlation with immune environment. Based on expression and clinical data of 78 POLE, 123 MSI and 299 Other EC samples from the TCGA-UCEC project, we found that the MSI tumors were identified more often in early stage, had a lower age, better patient survival, enriched CD8⁺ T cells, and regulatory T cells and less M2 macrophages and activated dendritic cells than the Other subgroup, and shared a relatively similar expression profile with POLE subgroup by differential analysis. In addition, we established the immune landscape of an MMR-D endometrial cancer tissue using unbiased single-cell RNA-seq analysis of 4518 cells. By immunohistochemistry analysis, we found that the MMR-D tumors showed a higher trend of CD20⁺ B cells infiltration. Our study might expand our understanding of the role of immune subsets in MSI endometrial carcinomas and provide guidance of immunotherapy for endometrial cancer.

Introduction

Endometrial carcinomas (ECs) have been broadly categorized into two subtypes according to clinicopathologic characteristics, hormone receptor expression and grade in the past 30 years [1]. Type I tumors account for 70–80% of ECs, and are endometrioid, hormone-receptor-positive, low-grade ECs with a good prognosis. Type II ECs are non-endometrioid, hormone-receptor-negative, high-grade tumors, which exhibit higher risk of metastasis and poor outcomes [1, 2]. However, the prognostic value of this dualistic classification remains limited, proper subtyping is critical for selecting appropriate treatment [1, 3]. In 2013, molecular classification by The Cancer Genome Atlas characterized endometrioid and serous endometrial cancer into four distinct molecular subgroups: POLE ultramutated, microsatellite instability hypermutated (MSI-H), copy number low (endometrioid) and copy number high (serous-like) [4]. Molecular classification is one of the most important developments in the study of endometrial carcinoma in recent years. The ESGO/ESTRO/ESP (the European Society of Gynaecological Oncology (ESGO), the European Society for Radiotherapy & Oncology (ESTRO) and the European Society of Pathology (ESP)) 2020 classification of endometrial carcinoma revised the pathological subtypes and integrated the molecular typing of endometrial cancer [5].

MSI-H endometrial cancer patients are potential beneficiaries of PD-1/PD-L1 inhibitor therapy [6]. MSI is usually as a result of defects in the mismatch repair (MMR) system, a group of enzymes that is responsible for monitoring and repairing the error incorporations in microsatellites [7]. The MMR genes consist of MLH1, MSH2, MSH6 and PMS2. Lynch syndrome results from germline mutations in four MMR genes [8]. Determination of the MSI phenotype in endometrial carcinoma patients by testing MMR status/microsatellite instability (MSI) has been shown to be relevant for four clinical applications: as a surrogate marker for histopathological typing; for Lynch syndrome screening; helping to predict the EC patient's prognosis and determine treatment decisions such as the potential utility of immune checkpoint inhibitor therapy. The International Society of Gynecological Pathology (ISGyP) has suggested universal testing for MMR status/MSI routinely in all endometrial carcinoma cases [9].

There are two assays used to determine MMR-deficient/MSI-H phenotype (simply MSI hereafter) in current clinical testing: MMR - immunohistochemistry (IHC), and PCR-based DNA microsatellite instability analysis (MSI-test) [9, 10]. MMR-IHC is now the first-line approach to identify patients with MMR deficiency. MMR-IHC is a widely available, cost-effective and reliable method to assess MMR status, which can provide direct information on the absence of expression of the altered gene/protein. Alternative MSI-test and subsequent reflex *MLH1* hypermethylation, panel testing for germline mutations in >20 cancer-causing genes (which include the MMR genes) would be undertaken when indicated.

Identification of the MSI phenotype in colorectal cancer has been well used to help determine treatment decisions for targeted immunotherapy. Some studies also have proven that mismatch-repair deficiency can predict PD-1 blockade response in solid tumors [6, 11–13]. Importantly, the UCCN (National Comprehensive Cancer Network) and ESGO/ESTRO/ESP guidelines in 2020 recommended anti-PD-1 targeted therapy for some advanced MSI EC patients [5]. But its clinical effect remains to be well investigated and improved. Thus, owing to the high prevalence of MMR deficiency in endometrial cancer and potential applications of immunotherapy in EC [1, 14], deeper understanding of MMR-D/MSI-H subtype of endometrial cancer is extremely important.

In the present study, we aimed to explore the relationships between the MSI status and EC clinical features, prognosis, mutation profile, immune infiltrates based on The Cancer Genome Atlas (TCGA) data, and to explore the cell landscape of an MMR-D/MSI-H

Tissue sections were collected and fixed in 10% formalin. 5 µM slides were used for immunohistochemistry analysis. The “UltraVision Quanto Detection System HRP DAB” IHC kit (TL-125-QDH, Thermo Fisher Scientific) was used for the tyramide signal amplification according to the manufacturer’s protocol. Primary antibodies used in this assay are as follows: anti-CD3 (ab16669, Abcam), anti-CD8 (ab17147, Abcam), anti-CD20 (M0755, Dako), anti-CD68 (ab955, Abcam), anti-CD163 (ab156769, Abcam), anti-MLH1 (MAB-0789, MXB Biotechnologies), anti-MSH2 (MAB-0836, MXB Biotechnologies), anti-PMS2 (GT215902, Gene Tech) and anti-MSH6 (MAB-0831, MXB Biotechnologies). Images were taken and quantitative image analysis was performed using ImagePro software. For comparison for CD20 between two groups, statistical evaluation was done by two-tailed Student’s t-test, error bars show standard error of the mean (SEM).

Results

Association between EC molecular subtypes and survival and clinical features

To explore the relationship between the distinct molecular subtypes of endometrial cancer and the clinical features, we analyzed clinical data of 500 EC patients from TCGA database, including clinical stage, histologic grade, age, history of radiation therapy, diabetes, hypertension, menopause state and BMI (Table 1). The 500 patients were classified into three molecular subgroups: the POLE, MSI and Other subgroup. We discovered that the POLE subgroup has a lower age, BMI, lower fraction of diabetes and hypertension. The tumors in MSI subgroup were identified more often in the early stage, and had a lower age than the Other subgroup (Figure 1A-1B). These results were consistent with the prognosis analysis between patient survival and molecular subtype, age and clinical stage (Figure 1A-1C). But there is no difference in the proportion of histologic grade across the subtypes (Figure 1A). The POLE and MSI subtypes, lower age, grade and clinical stage were all correlated with better patient survival (Figure 1C).

Landscape of somatic mutation files in POLE, MSI, and Other ECs

Tumors with POLE mutation or MSI have been suggested to be hyper or highly mutated. The somatic mutation profiles of 500 EC patients in the TCGA database were obtained from MuTect2[™] analysis. The top 50 mutated genes were shown in the waterfall plot; around 498 (99.6%) samples in total possessed somatic mutations, and most of the genes had higher mutation frequency in the POLE group (Figure 2A and supplementary table 1). The MSI group had few mutations in TP53, FBXW7, CTNBN1 and PPP2R1A, which was consistent with previously described (Figure 2B) [4]. 6 genes showed more frequent mutations in MSI group, including 4 genes (KRAS, ARID1A, JAK1 and RNF43) [4, 16] that have been previously reported in endometrial cancer and 2 novel genes (KMT2D and SETD1B) (Figure 2B). Except for KRAS with predominantly missense mutation in all groups, other 5 genes showed more frequent frameshift deletions in MSI group than POLE and other groups (Figure 2C). JAK1 and RNF43 with polymerase slippage-associated deletions have been reported previously [16]. KMT2D and SETD1B are chromatin remodeling-related genes, and appear to help predict the degree of myometrial invasion [17].

Enriched immune infiltrates in POLE and MSI ECs than Other ECs

We analyzed the global gene expression profile of 500 EC patients, and identified the differentially expressed genes (DEGs) between the three molecular groups. POLE group and MSI group share a similar expression profile (Figure S1). 1138 genes were upregulated in both POLE and MSI groups, and 792 genes were upregulated in Other group (Supplementary table 1). The gene set enrichment analyses (GSEA) using Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms showed that genes up-regulated in POLE and MSI samples were mainly enriched for immune-related functions, such as T cell activation, T cell differentiation, cytokine-cytokine receptor interaction and T cell receptor signaling pathway (Figure 3).

POLE mutation or MSI subtypes in cancers indicate hypermutations, and they are suggested to harbor more tumor-specific neoantigens and higher lymphocytes infiltrates [18]. Thus, we calculated the immune scores of all 500 samples using the ESTIMATE algorithm, and found that immune scores of both POLE and MSI EC groups were significantly higher than the Other group (Figure 4A). To investigate the association between molecular subtypes and specific immune infiltrates in endometrial cancer,

we then estimated the percent of 22 immune cell types of all 500 samples by CIBERSORT algorithm and compared immune cell fractions in 3 molecular subtype groups (Figure 4B). Furthermore, the Kaplan-Meier curve with log-rank test was used to analyze the correlation between each immune infiltrate level and the overall survival (OS) time of EC patients. The fractions of 10 immune cell types varied across molecular subtypes (Figure 4C). Importantly, both POLE and MSI groups were found to have more CD8⁺ T cells, follicular helper T cells, resting NK cells, M1 macrophages, while less activated NK cells, M2 macrophages, activated dendritic cells and mast cells (Figure 4C). POLE groups had more plasma cells (Figure 4C). MSI group had more regulatory T cells (Figure 4C). Among these immune cell types, high infiltrates level of CD8⁺ T cells, and regulatory T cells were found to be significantly positively related with OS, while high infiltrates level of M2 macrophages and activated dendritic cells were negatively related with OS (Figure 4D). These results were consistent with better clinical outcomes in POLE and MSI EC groups than Other group (Figure 1C).

Cell mapping of immune ecosystem in an MMR-D EC sample

Our previous study [15] also indicated high lymphocytes infiltration in an MMR-D cancer tissue whose MSH6 expression is negative (Figure S2A). The existence of different tumor-infiltrating immune cell types in this sample was also assessed by immunohistochemistry (IHC) staining with CD3, CD8, CD20, CD68 and CD163 antibody (Figure 5A). We further analyzed the cellular composition and immune microenvironment of this sample deeply based on its single-cell RNA-seq data using Seurat R package (Figure 5B). After quality filtering, 4518 cells were used for downstream analysis. Graph-based clustering was used to classify cells into groups. We identified 4 major cell types through marker genes: epithelial cells (KRT18), endothelial cells (VWF), fibroblasts (COL6A2), immune cells (PTPRC) in this sample (Figure S2B). The immune cells were further analyzed by identifying subsets in T cells, B cells and myeloid cells (Figure 5C). T cells included naive CD4⁺ T cells (cluster 5; *IL7R⁺*), regulatory T cells (cluster 6; *FOXP3⁺*), exhausted CD8⁺ T cells (cluster 0; *PDCD1⁺*) and natural killer T cells (NKT cells) (cluster 3; *GNLY⁺*) (Figure 5C-D and Figure S3). Cluster 11 had proliferative cells (*MKI67⁺*) of both lymphocyte and myeloid lineages (Figure 5E). Two distinct populations in macrophages (*CD163⁺*) were observed: macrophage population 1 (cluster 1) with enriched expression of C1QA, C1QB, C1QC; and macrophage population 2 (cluster 7) with enriched expression of FTL, CXCL8, SPP1 (Figure 5C, 5F and Figure S3). One cluster of monocytes was characterized by enriched expression of LYZ, S100A8 and S100A9 (Figure 5C and Figure S3) [19]. Two subtypes in B cells were further analyzed. Follicular B cells were enriched for the expression of CD19 and MS4A1 [20], while plasma B cells were characterized by the expression of MZB1 and CD38 (Figure 5C, 5G and Figure S2) [19]. These results formed the basis description of the immune subsets in endometrial carcinomas with MSI.

Brooke E et al. reported that the MSI tumors exhibited higher infiltration of CD3⁺ and CD8⁺ T cells compared to MSS tumors [21]. However, little is known about the relationship between MSI status and tumor infiltrating B cells. Here, we assessed the infiltration of B cells by IHC staining of MMR-D and MMR-I tumor sections with CD20 antibody (Figure 5H). The MMR-D tumors exhibited a higher trend in the number of B cells than MMR-I tumors, but with no significance (Figure 5I). Patient overall survival analysis from TCGA-UCEC data indicated that higher expression of CD20 (MS4A1) was associated with good prognosis (Figure 5J). The function of B cells in endometrial carcinomas remains to be further explored.

Discussion

Distinct factors affect the prognosis of endometrial cancer such as tumor type, grade and stage status [22, 23]. MMR-D subtype has been reported to be related with a good prognosis in endometrial cancer patients [14]. Moreover, promising clinical trials of immunotherapy (anti-PD-1/anti-PD-L1) indicate good response in advanced MMR-D/MSI-H EC patients [24, 25]. But the underlying mechanism between the MMR-D status and endometrial cancer patient prognosis is not well investigated and understood. In this study, we related the molecular subtype to clinical data and prognosis of EC patients based on 78 POLE, 123 MSI and 299 Other EC samples in the TCGA-UCEC project. The tumors in MSI subgroup were identified more often in early stage, had a lower age, better patient survival and enriched immune infiltrates than the Other subgroup. Besides, using the unbiased single-cell RNA-seq analysis for an MMR-D endometrial cancer tissue, an immune atlas was established.

Tumors with defects in MMR proteins indicated higher mutation frequency, and were suggested to harbor more tumor-specific neoantigens and higher lymphocytes infiltrates [18]. Here, our enrichment analysis of up-regulated genes in POLE and MSI subtype also suggested the enrichment of immune-related functions. Diversity of tumor infiltrating immune cells in tumor influences tumor

development, progression, and treatment response to targeted agents. CD8⁺ T cells are essential for successful tumor killing. Regulatory T cells suppress anti-tumor immune response, and are usually associated with poor clinical outcomes [26]. A previous study reported that tumor-associated macrophages (TAMs) revealed a pro-tumor role in endometrial cancer [27]. Dendritic cells could be modulated by tumor cells, and thus drive immune tolerance [28]. In this study, both CD8⁺ T cells and regulatory T cells showed high infiltration in MSI subtype and were related with good OS. M2 macrophages and activated dendritic cells were found to be negatively correlated with MSI subtype and patient prognosis. The high infiltration of regulatory T cells might result from high neoantigens. Further investigations are necessary to clarify the exact functions of regulatory T cells and activated dendritic cells in EC.

B cells are the other major type of tumor-infiltrating lymphocytes (TILs) besides T cells, and are recognized to play an anti-tumor role by secreting immunoglobulins, promoting T cell response, and killing cancer cells directly [29]. In this study, we tested MMR status of 98 EC patients, and identified 15 MMR-D cases. By comparing the infiltration of B cells between MMR-D and MMR-I EC tumors, we found that the MMR-D tumors showed a higher trend in the number of B cells. The statistical analysis showed no significance thus necessitating more samples to confirm the result.

Last, we provided a baseline description of the immune transcriptomes by single-cell RNA-seq analysis for an MMR-D EC tumor, which formed the basis for further examination into the role of immune subsets in endometrial carcinomas. Future studies are needed to determine the specific roles these complex immune cell types play in the regulation of EC development and progression.

Abbreviations

EC: Endometrial cancer; ECC: endometrioid carcinoma; MMR: mismatch repair; PTEN: phosphate and tension homology deleted on chromosome ten; POLE: DNA polymerase e. BMI: Body Mass Index.

Declarations

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DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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Tables

Table 1 is not available with this version.

Figures

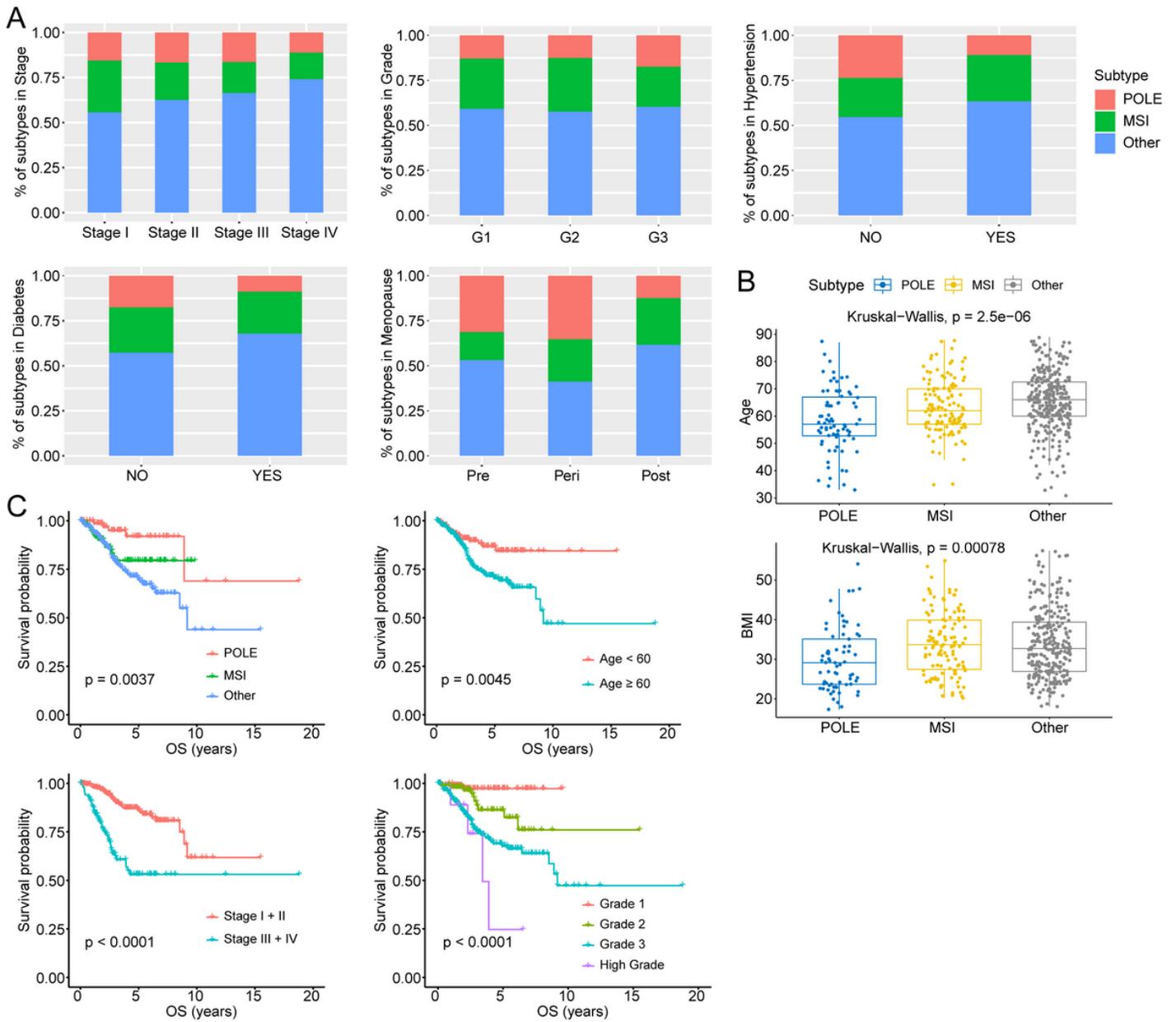


Figure 1

Association between molecular subtypes and clinical variables and survival outcome. (A) The fractions of molecular subtypes in clinical stage, histologic grade, diabetes, hypertension and menopause state. Chi-square test. (B) Distribution of age and BMI of EC molecular subtypes. Kruskal-Wallis test. (C) Overall survival curve and log-rank test for endometrial cancer patients based on molecular subtype, clinical stage, histologic grade and age classification.

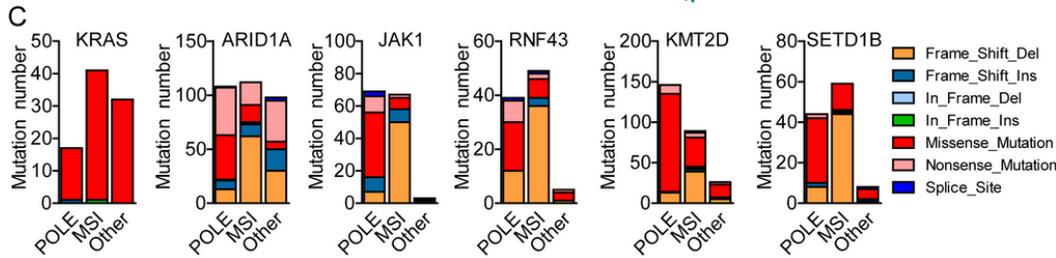
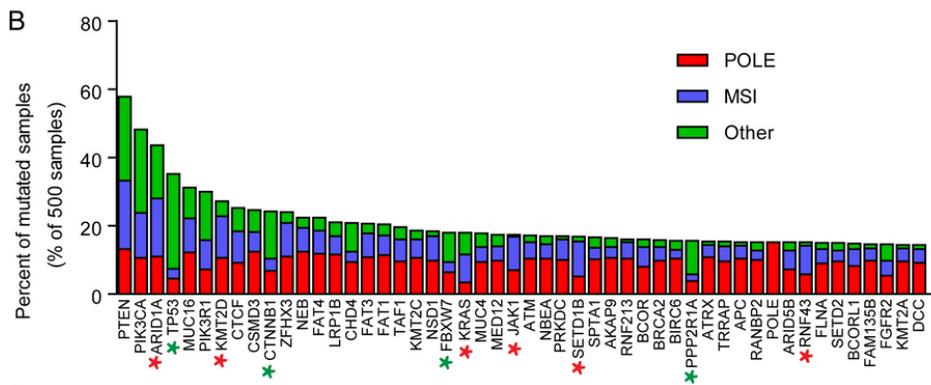
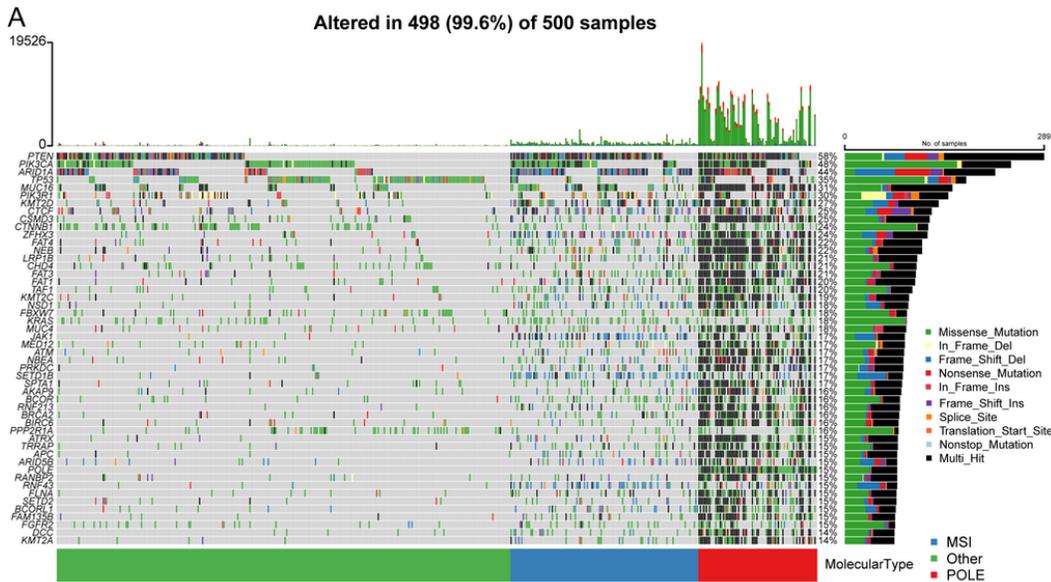


Figure 2

Landscape of somatic mutation profiles in 500 endometrial cancer samples from TCGA database. (A) The waterfall plot shows top 50 mutated genes in each sample. The upper barplot shows the number of mutations in each patient, while the right barplot indicates the number of mutated samples in each gene. (B) Proportion of tumors in three molecular subtypes of each gene. Green stars showed 4 genes with few mutations in MSI group, red stars showed 6 genes with frequent mutations in MSI group. (C) Frequently mutated genes in the MSI subgroup. Shown are the mutation numbers of different variant types in six genes.

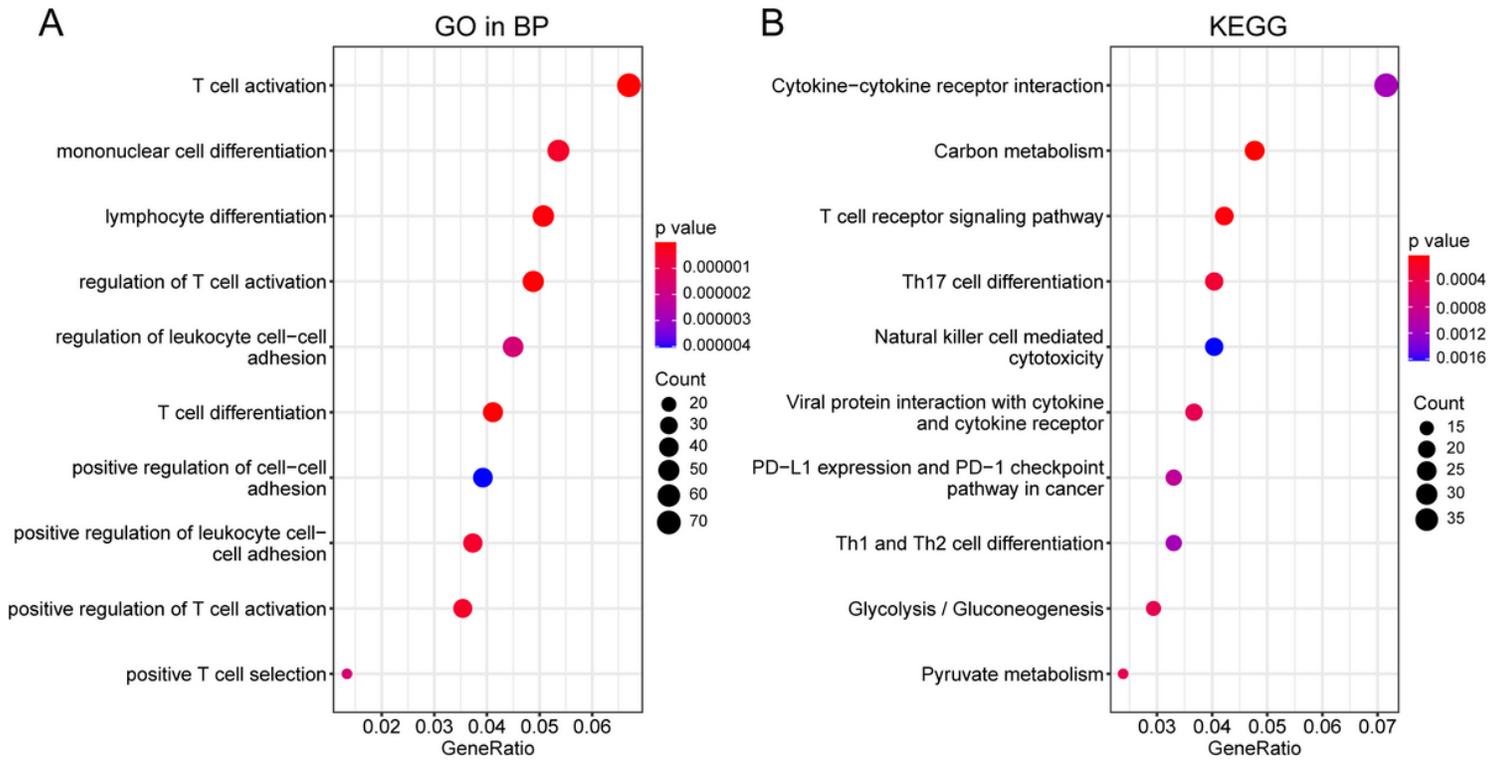


Figure 3

Enrichment analysis of differentially expressed genes (DEGs) between POLE, MSI and Other ECs. (A-B) The top 10 of biological processes GO terms (A) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway (B) enriched by 1138 genes those were upregulated in both POLE and MSI groups.

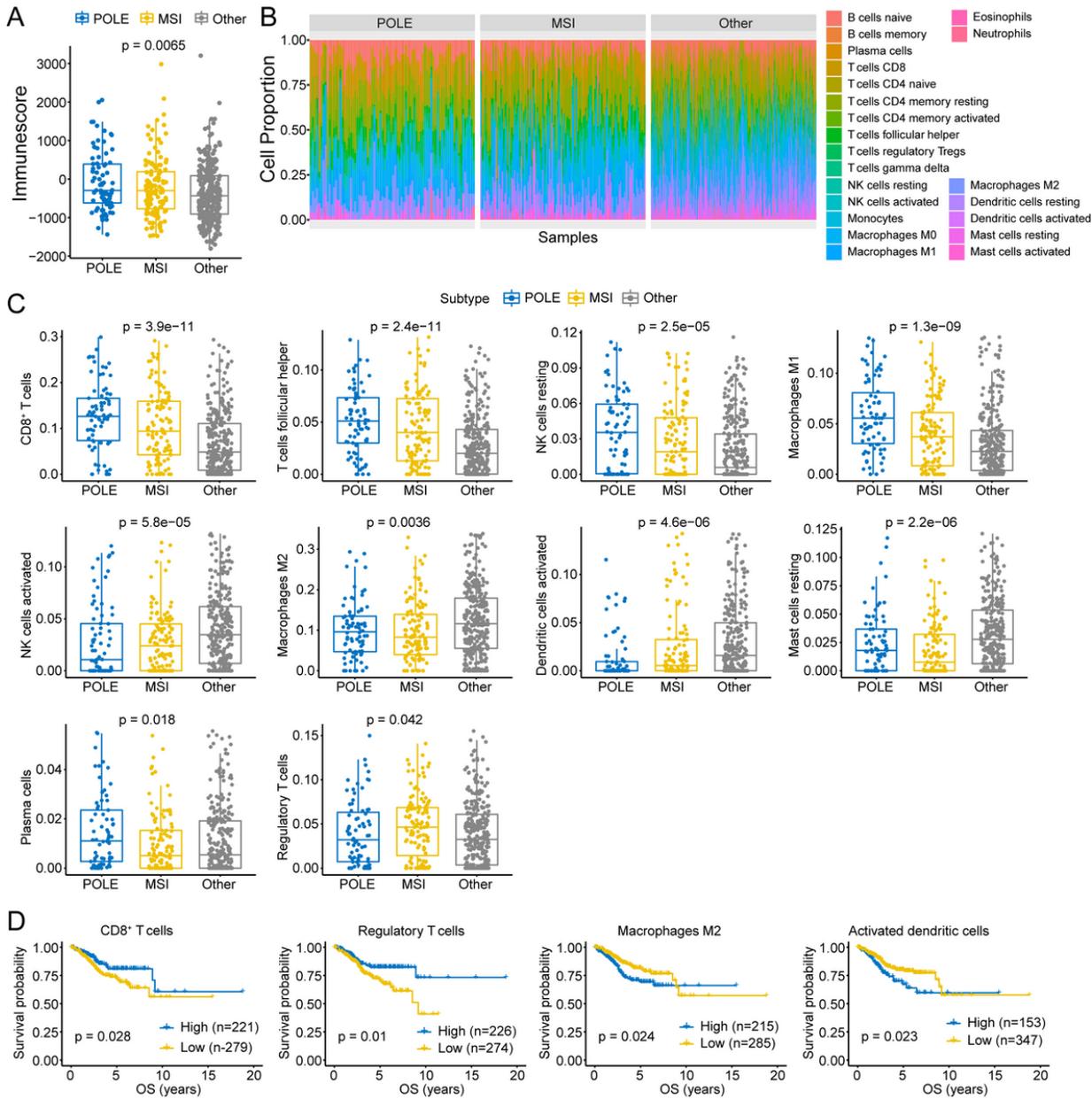


Figure 4

Immune infiltrates in EC samples from TCGA database. (A) Distribution of immune scores of EC molecular type, including POLE, MSI, and Other type. Kruskal-Wallis test. (B) The fractions of 22 immune cell types in 500 EC samples using CIBERSORT algorithm. (C) Relationship between different immune cell types and EC molecular type. Distribution of CD8+ T cells, follicular helper T cells, resting NK cells, M1 macrophages, activated NK cells, M2 macrophages, activated dendritic cells, mast cells, plasma cells and regulatory T cells across EC molecular type. Kruskal-Wallis test. (D) Kaplan-Meier curves with log-rank test for two immune cell types associated with good prognosis and two types associated with poor prognosis.

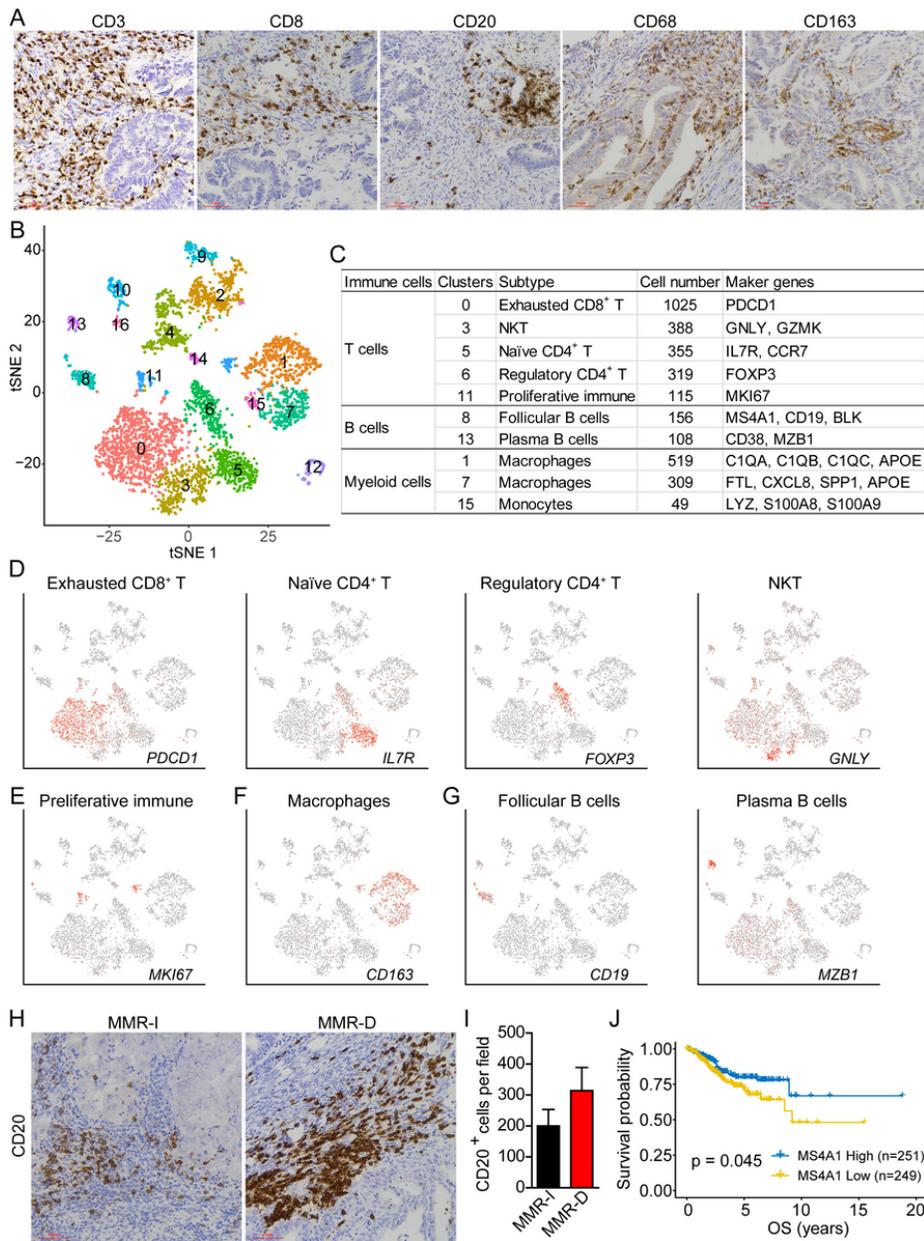


Figure 5

Atlas of multiple immune cell types in an MMR-D endometrial carcinoma sample and enrichment trend of B cells in MMR-D endometrial tumors. (A) IHC staining images of CD3, CD8, CD20, CD68 and CD163 in tumor slides isolated from the MMR-D endometrial tumors. Scale bars, 60 μ m. (B) t-SNE projection of all cells, color-coded by their associated cluster, and labeled with cluster number. (C) Cell cluster, number and marker genes of annotated immune cell types are summarized. (D-G) t-SNE map, colored by relative expression (lowest expression to highest expression, gray to red) of marker genes for immune cell subtypes: T cells (D), proliferative immune cells (E), macrophages (F), and B cells (G). (H) IHC staining images of CD20 in slides isolated from MMR-D and MMR-I endometrial carcinoma sections. Scale bars, 60 μ m. (I) Quantification of the numbers of B cells as presented in (B). Data are means \pm SEM (15 MMR-D sections and 15 MMR-I sections were analyzed). Student's t-test. (J) The overall survival curves with log-rank test for MS4A1 (CD20) expression based on TCGA-UCEC data.

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