MUC16 mutations improve patients’ prognosis by enhancing the infiltration and antitumor immunity of cytotoxic T lymphocytes in the endometrial cancer microenvironment

Jing Hu and Jing Sun

Department of Gynecology, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, China

ABSTRACT
The incidence and mortality rates of endometrial cancer are increasing during recent years. CA125 (gene symbol MUC16) is a well-known diagnostic and prognostic serum marker of endometrial cancer. High serum CA125 level is associated with poor prognosis. MUC16 is one of the most frequently mutated genes in endometrial cancer. However, the potential relationship and underlying mechanism between MUC16 mutations and endometrial cancer patients’ prognosis and disease progression remain unclear. In present study, we analyzed the whole exome sequencing data, RNA sequencing data and patients’ clinical information in TCGA database and demonstrated that MUC16 mutational status was an independent prognostic factor for endometrial cancer patients. Patients with somatic MUC16 mutations had a prolonged overall survival time. MUC16 mutations promoted patients’ antitumor immune responses. Cytotoxic immune cells mediated pathways were enriched in endometrial cancer samples with MUC16 mutations. Elevation of two pathways, NO2-dependent IL 12 pathway in NK cells and T cytotoxic cell surface molecules, significantly correlated with a higher rate of MUC16 mutations and a significantly favorable patients’ prognosis. An increased level of cytotoxic T lymphocytes, not NK cells, infiltration was observed in the tumor microenvironment of patients with MUC16 mutations. High expression of molecular markers of T cells and CD8+ T cells associated with a higher rate of MUC16 mutations and a better patients’ prognosis. These findings may provide deeper insight into potential endometrial cancer immunotherapy approaches.

Introduction
Endometrial cancer is the most common gynecological malignancy in developed countries and the fifth most common cancer in women worldwide, with an estimated 320,000 new cases and 76,000 deaths each year worldwide.1 The incidence and mortality of endometrial cancer are increasing during recent years, likely due to rising obesity rates and an aging population.2

Mucins are high molecular weight glycoproteins that are divided into two subfamilies, secreted and transmembrane mucins.3 Mucins normally express on various types of epithelial cells and play numerous physiological roles ranging from protection against pathogenic infections to regulation of cellular signaling and transcription.4 Many studies also demonstrated that mucins were overexpressed and aberrantly glycosylated in diverse epithelial cancers to promote cancer cell growth and invasion.5–8 MUC16 is the largest transmembrane mucin and its secreted counterpart is CA125, the well-known diagnostic and adverse prognostic serum marker of gynecological malignancy.9,10 However, its roles in carcinogenesis and progression of endometrial carcinoma are not fully clear.

The core protein of MUC16 contains a large extracellular tandem repeat domain at its N-terminus, and a transmembrane domain with a short cytoplasmic domain at its C-terminus.4 Emerging evidence suggests that MUC16 might be involved in regulating immune responses in various cancer types. Gubbels JA et al reported that MUC16 protected ovarian cancer cells from NK cells targeting by inhibiting synapse formation between these tow types of cells.11 Later, Belisle JA et al identified that MUC16 can also bind to NK cells, B cells and monocytes via Siglec-9, which is an inhibitory receptor that attenuates T cell and NK cell function.12 A study indicated that circulating Treg proportion was related to the serum CA125 level and that MUC16 C terminal promoted Foxp3 expression and tumor-associated Treg enrichment in tumor tissues through tumor-secreted IL-6 activation of the Jak2/Stat3 pathway in pancreatic cancer.13 These findings suggest that MUC16 might play important roles in inhibiting anti-cancer immune responses. There also was study showed that the pro-inflammatory cytokines TNFα and IFNγ stimulate MUC16 expression in breast, endometrial and ovarian cancers through NFκB in vitro and elevated MUC16 expression is associated with elevated cytokine levels in breast and ovarian cancer tissues.14 Pro-inflammatory stimuli (oxidative stress and treatment with the cytokines IFNγ, IL-1α, and TNFα) altered MUC16 glycosylation
of pancreatic cancer cells.\(^5\) These indicate that MUC16 might be involved in promoting pro-inflammatory signaling in cancer.

Cancer is characterized by a sequential accumulation of genetic alterations including somatic mutations.\(^6\) Cancer progression is driven by a handful of mutations in cancer-related genes (oncogenes and tumor suppressors), termed ‘driver mutations’ which facilitate uncontrolled proliferation and other hallmarks of cancer.\(^7,8\) Driver mutations, however, arise alongside thousands of other mutations, called “passenger mutations” that have previously been assumed to be neutral and largely ignored in cancer research.\(^9\) Yet growing evidence suggests that passenger mutations can be deleterious to cancer cells and play an important role in both cancer progression and clinical outcomes.\(^20–22\)

MUC16 is one of the most frequently mutated genes in malignant tumors of the female reproductive system through preliminarily analyzing The Cancer Genome Atlas Cancer Genome (TCGA) exome sequencing data via its online exploration tool. However, the studies on MUC16 mutational effects on endometrial cancer tumorigenesis and progression have not been performed to our knowledge. It is also not known whether MUC16 mutations have influences on endometrial cancer patients’ prognosis. In this study, we addressed these questions through a combinatorial analysis of whole exome sequencing data, RNA sequencing data and patients’ clinical information of the Uterine Corpus Endometrial Carcinoma (UCEC) project in TCGA database to investigate MUC16 mutational effects on endometrial cancer.

Results

**MUC16 frequently mutates in endometrial cancer**

530 endometrial cancer patients in TCGA-UCEC project were included in this study. 530 patients contained the whole exome sequencing data, 528 patients contained clinical and survival information and 524 patients contained RNA sequencing data.

Exome sequencing of 530 endometrial cancer samples identified 498,711 somatic mutations based on consensus calls from mutect2 mutation-detection algorithm (Supplementary Table 1). The types of these mutations included short insertions (INS), short deletions (DEL) and single nucleotide polymorphisms (SNPs), which mainly were nonsynonymous and splice site SNPs (Supplementary Table 1). Synonymous, 5’UTR, 3’UTR and intronic SNPs were not included in our analysis. The major type of variants was SNPs (Number = 464454), compared with insertions and deletions (Figure 1A). The majority of mutations were missense (Number = 415573) and nonsense mutations (Number = 40375) (Figure 1B). The top ten mutated genes in endometrial cancer included PTEN (57%), PIK3CA (48%), TTN (44%), ARID1A (43%), TP53 (36%), MUC16 (30%), PIK3R1 (30%), KMT2D (27%), CTCF (24%), and CSMC3 (24%) (Figure 1C). MUC16 was the sixth most frequently mutated gene in endometrial cancer with a mutational frequency of 159 in 530 tumor samples (30%) and it mutated more than once in one third of samples with mutant MUC16 approximately (Figure 1C). We identified 744 mutations in MUC16 gene, including 621 single nucleotide variants (SNVs) that were mainly substitutions between cytosine and thymine (C/T), cytosine and adenine (C/A), adenine and guanine (A/G) and thymine and guanine (T/G) (Figure 1D). 736 mutations occurred in exon regions and more than 86% mutations occurred in exon1 (21.60%), exon3 (54.35%) and exon5 (10.87%) (Figure 1E).

Most of MUC16 mutations were missense mutations and these mutations were equally distributed from N-terminal to C-terminal of MUC16 protein (Figure 1C, F). The total tumor mutational burden of these patients ranged from 1 mutation to 19526 mutations per sample and the mean of variants per sample was 940.964 (Figure 1G). Patients with MUC16 mutations had a higher tumor mutational burden compared with patients without MUC16 mutations (the means of variants per sample 2709.044 vs. 183.2156, \(P < 2.2e-16\)) (Figure 1G).

**MUC16 mutations are associated with favorable prognosis of endometrial cancer patients**

The detailed clinical characteristics included age at diagnosis, histological type, histologic grade, clinical stage, race and history of neoadjuvant treatment (Table 1). The relationship between MUC16 mutational status and clinical features was evaluated through chi-square test. The results showed that MUC16 mutational status was significantly associated with patients’ age at diagnosis (\(P = 0.0002825\)) and histological type (\(P = 0.002432\)); No significant difference was found between MUC16 mutational status and histologic grade or clinical stage (\(P > 0.05\)) (Supplementary Table 2).

Among all clinical features and top ten mutated genes, there were seven genes (PTEN, PIK3CA, TNN, ARID1A, TP53, MUC16 and CTCF) and four clinical features (age, histological type, histologic grade and clinical stage) might correlate with patients’ overall survival. In univariate analysis, all the features’ \(P\) values were less than 0.05, indicating that these features may relate with patients’ overall survival in endometrial cancer (Table 2). Therefore, we included all these features in the following multivariate analysis and used a much more stringent cut-off criterion \(P < 0.01\). In multivariate analysis, there were only histologic grade (HR = 7.5872, \(P = 0.00543\)), clinical stage (HR = 3.1203, \(P = 6.44e-07\)) and MUC16 mutational status (HR = 2.4534, \(P = 0.00872\)) that were showed to be independent prognostic factors in endometrial cancer patients (Table 2). We then plotted patients’ survival curve based on the stratifications of three independent prognostic factors separately using Kaplan-Meier curve and Log-rank test. The results showed that low histologic grade (G1), early clinical stage (I+ II) and MUC16 mutations were positively related to patients’ overall survival (Figure 2). Despite its smallest \(P\) value among three independent prognostic factors, clinical stage, which had an intersect in its survival curve, was a poor stratified factor of patients’ prognosis (Figure 2A). Compared with clinical stage, histologic grade and MUC16 mutational status were good prognostic
stratified factors, which had not intersects in their survival curves (Figure 2B, C).

**MUC16 mutations are involved in antitumor immune responses**

Patients with MUC16 mutations had a prolonged overall survival time, indicating that mutant MUC16 might be critically involved in the tumorigenesis and progression of endometrial cancer. To further study the roles of MUC16 mutations in endometrial cancer, we analyzed the global gene expression profiling between 158 tumor samples with mutant MUC16 and 366 tumor samples with wild type MUC16 using RNA sequencing data downloaded from TCGA database. Gene Set Enrichment Analysis (GSEA) using GO terms as the gene sets revealed enrichment in categories like immune response, lymphocyte activation, adaptive immune response, cellular response to IFN-γ and others, in endometrial cancer samples with MUC16 mutations (Figure 3). Increasing evidence suggests that MUC16 might play important roles in inhibiting antitumor immune responses11–13 and in promoting pro-inflammatory signaling in many cancers14,15. It is possible that MUC16 mutations might abrogate its inhibitory immune effects. On the other hand, we observed that patients with MUC16 mutations had a much more higher tumor mutational burden (Figure 1G). It is also possible that the hypermutated status might enhance antitumor immune responses. To minimize the interference of hypermutated status on antitumor immunity, we excluded hypermutated phenotypes (more than 1000 mutations per
sample in Supplementary Figure 3A or more than 500 mutations per sample in Supplementary Figure 3B) and found that total mutational loads decreased significantly, but remained slightly higher in patients with MUC16 mutations. However, the exclusion of patients with hypermutated phenotypes did not alter the effects of MUC16 mutations on patients’ prognosis (Supplementary Figure 3C) and antitumor immune responses (Supplementary Figure 3D). A study reported that it was neoantigen quality, not quantity, that can predict patients’ survival and MUC16 neoantigens were enriched in long-term survivor of pancreatic cancer. These observations indicated that MUC16 mutations might play critical roles in patients’ prognosis and in regulating antitumor immunity.

### Table 1. Clinical characteristics of patients with endometrial cancer. NA, not available.

| Variables                        | Cases, N (%) |
|----------------------------------|--------------|
| Age at diagnosis                 |              |
| <60                              | 174 (32.95%) |
| ≥60                              | 352 (66.67%) |
| NA                               | 2 (0.38%)    |
| Histological type                |              |
| Endometrioid endometrial adenocarcinoma | 395 (74.81%) |
| Serous endometrial adenocarcinoma | 111 (21.02%) |
| Mixed serous and endometrioid endometrial adenocarcinoma | 22 (4.17%) |
| Histologic grade                 |              |
| G1                                | 97 (18.37%)  |
| G2 + G3                          | 431 (81.63%) |
| Clinical stage                    |              |
| I + II                           | 381 (72.16%) |
| III + IV                         | 147 (27.84%) |
| Race                             |              |
| American Indian or Alaska Native | 3 (0.57%)    |
| Asian                            | 20 (3.79%)   |
| Black or African American        | 106 (20.08%) |
| Native Hawaiian or Other Pacific Islander | 8 (1.52%) |
| White                            | 360 (68.18%) |
| NA                               | 31 (5.87%)   |
| Neoadjuvant Treatment History    |              |
| Yes                              | 2 (0.38%)    |
| No                               | 526 (99.62%) |

**MUC16 mutations are involved in cytotoxic immune cell mediated antitumor responses**

To guide our identification of immune pathways necessary for mutant MUC16 function, we performed GSEA using canonical pathways mainly from BIOCARTA, KEGG and REACTOME databases as the gene sets. The results demonstrated that cytotoxic immune cell mediated antitumor immune responses, such as genes involved in immunoregulatory interactions between a lymphoid and a non-lymphoid cell (Figure 4A), genes involved in phosphorylation of CD3 and TCR zeta chains (Figure 4B) and NO2-dependent IL 12 pathway in NK cells (Figure 4C), were enriched in endometrial cancer samples with MUC16 mutations. Next, we...
employed BIOCARTA pathway analysis of the differentially expressed genes (DEGs) via DAVID database. According to the cut-off criteria ($P < 0.05$ and log$_2$|FC| $\geq 0.5$), a total of 1666 DEGs were identified, including 420 up-regulated and 1246 down-regulated genes (Figure 4D and Supplementary Table 3). We identified that NO2-dependent IL12 pathway in NK cells was the most overrepresented pathway in tumor samples with MUC16 mutations (Figure 4E). The results also
showed that T cell mediated antitumor immune responses like T cytotoxic cell surface molecules, cytotoxic T lymphocyte (CTL) mediated immune response against target cells and others were significantly enriched in samples with MUC16 mutations (Figure 4E). These findings suggested that MUC16 mutations promote antitumor immune responses through cytotoxic immune cell mediated pathways in endometrial cancer.

To further investigate the involvement of mutant MUC16 in antitumor immune responses through cytotoxic immune cell mediated pathways, we analyzed the expression level of 17 genes in NO2-dependent IL 12 pathway in NK cells and 12
genes in T cytotoxic cell surface molecules between tumor samples with mutant and wild type MUC16. Next, we stratified tumors samples based on the expression pattern of genes in these two pathways and examined the mutation rate of MUC16. Two main groups were observed from the unsupervised hierarchical clustering of the expression matrix from 17 genes in NO2-dependent IL 12 pathway in NK cells of the 524 samples. Cluster I had a higher gene expression pattern and exhibited a higher rate of MUC16 mutations (35.38%) (Figure 5A). Cluster II had a lower expression pattern and a lower rate of MUC16 mutations (20.33%) (Figure 5A). Similarly, two main groups were also observed in T cytotoxic cell surface molecules. Cluster I had a higher gene expression pattern and exhibited a higher rate of MUC16 mutations (33.91%) (Figure 5C). Cluster II had a lower expression pattern and a lower rate of MUC16 mutations (22.73%) (Figure 5C).

To test the biological significance of elevation of NO2-dependent IL 12 pathway in NK cells and T cytotoxic cell surface molecules in tumors with MUC16 mutations, we examined whether up-regulation of these two pathways correlated with patients’ prognosis. It was striking that cluster I, which had higher expression level of the genes in these two pathways, was correlated with a more favorable prognosis, whereas cluster II, which had lower expression pattern, was associated with a significantly poorer survival probability.
Our GSEA and DEGs analyses also indicated that T cells in the tumor microenvironment of patients with MUC16 mutations, but these patients also had a significantly favorable survival.

**MUC16 mutations enhance the infiltration and antitumor immunity of cytotoxic T lymphocytes in the endometrial cancer microenvironment**

To guide our identification of the specific populations and subpopulations of cytotoxic immune cells infiltration in the endometrial cancer microenvironment of patients with MUC16 mutations, we analyzed the expression level of molecular markers of T cells and NK cells between patients with and without MUC16 mutations through the use of immune cell signatures built by a research group from expression data. Transcriptomic profiling analysis of 524 endometrial cancer samples revealed the upregulation of genes specific for T cell and CD8$^+$ T cells in the tumor microenvironment of patients with MUC16 mutations (Figure 6A, left panel). Cancer samples with MUC16 mutations did not display differences in the expression of molecular markers of NK cells (Figure 6A, right panel). The CD56$^{dim}$ NK cell subpopulation displays high cytotoxic capacity, secretes cytokines as interferon (IFN)-gamma after direct contact with target cells and is thought to mediate antitumor responses. The expression of genes specific for CD56$^{dim}$ NK cells was slightly higher in patients with MUC16 mutations, however, with no statistical significance (Figure 6A, right panel).

The unsupervised hierarchical clustering of the expression matrix from T cell and CD8$^+$ T cell signatures of 524 endometrial cancer samples showed that the higher gene expression cluster (Cluster I) exhibited a higher rate of MUC16 mutations (33.63%) and the lower gene expression cluster (Cluster II) exhibited a lower rate of MUC16 mutations (24.08%) (Figure 6B). Consistently, Cluster I was correlated with a more favorable prognosis, whereas cluster II was associated with a significantly poorer survival probability (Figure 6C). These findings demonstrated that patients with MUC16 mutations had an elevated level of cytotoxic T lymphocytes infiltration and enhanced T cell antitumor immunity in the tumor microenvironment and exhibited prolonged overall survival time.

**Discussion**

As one of the most well-studied tumor biomarkers over three decades, elevated serum CA125 level suggests patients’ poor prognosis in many cancers including endometrial cancer. A positive significant correlation was also found between increased levels of CA125 tissue expression and elevated serum CA125 levels in ovarian cancer patients. These findings demonstrate that CA125 expression levels are closely associated with patients’ prognosis. MUC16 mutated in various cancer types with a high mutational frequency. However, little is known about the relationship between MUC16 mutational status and patients’ prognosis. In our analysis, we found that although the gene expression levels of MUC16 between endometrial cancer samples with mutant and wild type MUC16 were no statistical significance (data not show), MUC16 mutational status was an independent prognostic factor for patients, indicating that patients with somatic MUC16 mutations had a prolonged overall survival time (Table 2 and Figure 2C).

Accumulating evidence supports that passenger mutations correlate with slower cancer progression, enhanced antitumor immunity and improved clinical outcomes. Consistently, we observed that in six genes (PTEN, PIK3CA, TNN, ARID1A, MUC16 and CTCF) were positively related to patients’ overall survival and only TP53 mutations negatively associated with patients’ prognosis ($P < 0.05$) (Figure 2C, Supplementary Figure 2). Simultaneously, we also found that many patients with MUC16 mutations also carried numerous passenger mutations resulting in higher mutational burden (Figure 1G), and these patients exhibited enhanced antitumor immune responses in the tumor microenvironment (Figure 3) and favorable prognosis (Figure 2C). However, the exclusion of these hypermutated cases did not alter the effects of MUC16 mutations on patients’ prognosis (Supplementary Figure 3C) and antitumor immune responses (Supplementary Figure 3D). Not only quantities, but also qualities of neoantigens generated by various mutations influence patients’ survival. This may be one possible interpretation for our observations in Supplementary Figure 3. These findings indicated the important regulatory roles of MUC16 mutations in patients’ survival and antitumor immunity.

Increasing evidence suggests that MUC16 actively interacts with immune system. Pro-inflammatory cytokines can stimulate MUC16 expression or alter its glycosylation in normal epithelial cells or epithelial cancer cells. MUC16 can repress antitumor immune responses through inhibiting NK cell or T cell function in many cancers. However, very little is known about the effects of MUC16 mutations on patients’ antitumor immunity. Several studies demonstrated that somatic mutations can generate cancer-specific neoantigens that are presented by HLA molecules and potentially recognized by the mature T cell repertoire as ideal targets for cancer immunotherapy and cancer vaccine. A study unveiled that MUC16 was the most frequently tumor specific antigen presented by HLA molecules on ovarian cancer cells and 85% of MUC16-derived HLA ligands are immunogenic and able to prime T cells, rendering MUC16 an unparalleled first-class antigen for ovarian cancer immunotherapy. Currently, a research identified that, compared with short-term survivors, the long-term survivors of pancreatic cancer had a higher frequency of MUC16 neoantigens and had T-cell responses against mutant MUC16, suggesting that MUC16 mutations generate neoantigens that are key antitumour targets of the immune system. Our GSEA and DEGs analyses also revealed that T cell mediated immune responses like T Cytotoxic Cell Surface Molecules and CTL mediated immune response against target cells are enriched in endometrial cancer samples with MUC16 mutations (Figure 4B, E). The analysis of molecular markers expression level of cytotoxic T lymphocytes indicated that increased T cells and CD8$^+$ T cells infiltration in the tumor microenvironment of patients with MUC16 mutations (Figure 6A). High expression of T cytotoxic cell surface molecules...
pathway, T-cell and CD8+ T-cell signatures, significantly correlated with higher rates of MUC16 mutations (Figure 5C, 6B) and favorable patients’ prognosis (Figure 5D, 6C). Collectively, our data demonstrated that patients with somatic MUC16 mutations had favorable prognosis than those with wild type MUC16 and exhibited enhanced infiltration and antitumor immunity of cytotoxic T lymphocytes. More in-depth studies are needed to investigate how to exploit mutant MUC16 in endometrial cancer immunotherapy to promote patients’ survival.

Besides cytotoxic T cell, NK cell is also an important part of antitumor immune responses due to their potent cytolytic and cytokine-secreting abilities. We observed that NO2-dependent IL-12 pathway in NK cells was the most overrepresented pathway in tumor samples with MUC16 mutations through DEGs analysis (Figure 4E). High expression of this pathway was associated with higher rates of MUC16 mutations and improved patients’ overall survival (Figure 5A, B). However, patients with MUC16 mutations did not display differences in NK cells infiltration in the endometrial cancer microenvironment (Figure 6A). MUC16 was reported to inhibit immune synapse formation between NK cells and cancer cells. It can also bind to Siglec-9, an inhibitory receptor on NK cells. There are little researches focus on how mutated tumor-specific antigens recognized by NK cells and NK cells’ efficiency in related cancer immunotherapy. Further validations were needed to identify whether MUC16 mutations correlate with antitumor function of NK cells. And the applications of mutant MUC16 proteins in NK cells associated cancer immunotherapy need further investigations.

Interleukin-12 (IL-12) promotes cell mediated immunity by activating the cytotoxic activity of both T cell and NK cell and inducing Th1 cell differentiation which is believed to represent an important link between innate and adaptive immunity. In our analysis, we observed that two IL-12
mediated pathways in T cells and NK cells were upregulated in tumor samples with MUC16 mutations (Figure 4E). Studies reported that chimeric antigen receptors expressing T cells (CAR T cells), which secrete IL-12 and target retained MUC16 ectodomain, exhibited enhanced antitumor efficacy in recurrent ovarian cancer.\textsuperscript{44,45} The combination use of MUC16 and IL-12 provided novel and promising approaches for cancer immunotherapy. And, more researches are needed to explore appropriate protocols, the safety and efficacy of these methods in clinical application.

Methods

**TCGA data downloading**

The Mutation Annotation Format (MAF) data generated by mutect2 algorithm from whole exome sequencing data, RNA sequencing raw counts (HTSeq – Counts) data, RNA sequencing FPKM data (HTSeq – FPKM), clinical and survival information were downloaded from TCGA database (https://cancergenome.nih.gov/) through TCGAbiolinks R/Bioconductor package.\textsuperscript{46} In brief, 530 endometrial cancer patients were included in the following analyses, all of which contained the whole exome sequencing data, 528 patients contained clinical and survival information and 524 patients contained RNA sequencing data.

**Analyses and visualization of somatic mutations**

We used Maftools R/Bioconductor package\textsuperscript{47} to extract detailed mutational information from MAF file. The summary data of mutect2 mutational analysis were list in Supplementary Table 1. And, the plotmafSummary function was used to plot the summary of the MAF file, which displays number of variant type and variant classification. The oncoplot function\textsuperscript{48,49} and the lollipopPlot function were used to plot OncoPlot of the top ten mutated genes and lollipopPlot of MUC16 gene respectively.

**Patients’ prognostic analyses**

Survival curves were depicted using the Kaplan-Meier method and compared with log-rank test. Cox proportional hazards regression analysis was used for univariate and multivariate analyses to explore the association of clinical features, gene mutational status and patients’ prognosis. All the prognostic analyses were conducted by survival R package.

**Differentially expressed genes analysis and biocarta pathway analysis**

The differentially expressed genes (DEGs) between mutant and wild type MUC16 endometrial cancer samples were analyzed by edgeR R/Bioconductor package.\textsuperscript{49} Genes with \( \log_2|FC| \geq 0.5 \) and \( P < 0.05 \) were considered to be significantly DEGs. The DEGs data was list in Supplementary Table 3. BioCarta pathway analysis of DEGs was performed using DAVID database (https://david.ncifcrf.gov/).\textsuperscript{50}

**Gene set enrichment analysis**

Gene Set Enrichment Analysis (GSEA) was performed through the use of GSEAPreranked tool in GSEA software (http://www.broadinstitute.org/gsea/).\textsuperscript{51} The value of log\(_2\) (FC) calculated by edgeR package was used as ranking metric.

We used C5 collection that contains genes sets annotated by GO terms in the Molecular Signatures Database (MSigDB) as the gene sets in the analysis. The C5 collection, including 5917 gene sets, is divided into three sub-collections based on GO ontologies: Biological Process, Cellular Component, and Molecular Function.

We also used the canonical pathways sub-collection of C2 collection in the MSigDB as the gene sets in the analysis. This sub-collection contains 1329 gene sets, which are annotated pathways mainly from BIOMICARTA, KEGG and REACTOME databases.

**Hierarchical clustering**

The gene expression data for T cell, CD8\(^+\) T cell signatures and the following two pathways, NO2-dependent IL12 Pathway in NK cells and T Cytotoxic Cell Surface Molecules, were extracted from RNA sequencing FPKM data of 524 endometrial cancer samples in TCGA database. The data were subjected to log2 transformed. Unsupervised hierarchical clustering was used to discover groups based on the expression pattern of the genes in these two pathways and T cell, CD8\(^+\) T cell signatures. In total, 17 genes in NO2-dependent IL12 Pathway in NK cells, 12 genes in T Cytotoxic Cell Surface Molecules, 16 genes in T cell signature and 36 genes in CD8\(^+\) T cell signature were used in the unsupervised hierarchical clustering respectively. Expression values of these genes of 524 samples (where rows indicate the identity of the genes, columns indicate the identity of the patients) were clustered using hierarchical clustering with Euclidean distance and ward linkage by dendextend R package\textsuperscript{52} and heatmap.2 function in gplots R package. The MUC16 mutation rates of the resulting groups were calculated. The Kaplan-Meier survival curves were plotted for the resulting groups and compared with log-rank test.

**Cytotoxic t cell and NK cell populations and subpopulations analysis**

T cell, CD8\(^+\) T cell, NK cell and CD56\(^{dim}\) NK cell signatures were built by a research group, using genes specific for these immune cells.\textsuperscript{24} T cell signature genes include PRKCQ, CD3D, CD3G, CD28, LCK, TRAT1, BCL11B, CD2, ITM2A, SH2D1A, CD6, CD96, NCAIAD, GIMAP5, CD3E and SKAP1. CD8\(^+\) T cell signature genes include CD8B, CD8A, PF4, SF1, LIME1, PRR5, GGZM, SLC16A7, SRSP7, APBA2, HAUS3, LEPRTL1, GADD45A, ZFP36L2, KAT6A, ZEB1, ZNF609, MAPKAPK5-AS1, THUMP1, VAMP2, ZNF91, ZNF22, TMCC6, DNAJB1, FLTL3LG, CDKN2AIP, TSC22D3, TBCC, RBM3, ABT1, TMEM259, CAMLG, PPP1R2, AES, KLF9 and PRF1. NK cell signature genes include GAGE2, ZNF747, XCL1, XCL2, GNAS, SLC30A5, SGMS1, MCM3AP, TXA2R, CDC5L, FGF18, MRC2, PSMD4, PRX, ZNF205, APBB2, ZNF528, MAPRE3, BCL2, KANK2, ATL2, SPN, FZR1, PDLIM4, TRPV6, LDB3, ADARB1, PPP4R3A,
CD56α NK cell signature genes include SPON2, GZMB, TCTN2, IGFBP5, ALDH1B1, FUT5 and NCR1. mRNA z-scores were calculated via the function scale in R.

**Statistical analysis**

Univariate and multivariate cox proportional hazard regression analysis were carried out to compare clinical features, gene mutational status (mutated vs. not mutated) and patients’ prognosis. Survival curves were compared using log-rank test. Comparisons between two groups were performed using an unpaired two-tailed Student’s t-test (normally distributed parameters). Categorical variables were compared using a χ² test. All comparison groups had equivalent variances. P < 0.05 was considered to be statistically significant. All statistical analysis was performed using R programming language v. 3.4.3.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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