

Exploration of a Novel Biomarker in Endometrial Carcinoma

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Research

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Abstract

Background

The adenomatous polyposis coli (APC) gene, located on chromosome 5q21, is the chromatin-remodeling related gene and a typical tumor suppressor. As reported, patients with high expression of programmed death-ligand 1 (PD-L1) or a high level of tumor mutational burden (TMB) may benefit from immunotherapy in endometrial cancer. The objective of this study was to demonstrate that APC as a new target for the diagnosis and treatment of endometrial cancer, by analyzing the correlation of APC with PD-L1 expression or TMB.

Methods

We performed an integrative analysis of a commercial panel including 520 cancer-related genes on 99 tumors from an endometrial cancer cohort in China and DNA-seq data from The Cancer Genome Atlas (TCGA) to identify new gene mutations as endometrial cancer immunotherapy markers. To determine the effect of gene mutations on endometrial cancer, we explored the correlation between gene mutations and tumor immune microenvironment, and explored the immune microenvironment in endometrial cancer, including TMB, PD-L1 expression and lymphocytic infiltration.

Results

We found that the significant mutant genes were related to the chromatin state and generated a discovery set including 12 mutated genes that significantly correlated with PD-L1 expression and TMB. We identified the APC gene, with 19% (18/99) mutation rate, as a new marker for immunotherapy. Further analysis revealed that tumors with the APC mutation had a high TMB, increased expression of PD-L1 and increased lymphocytic infiltration (all $P < 0.02$). By assessing the relationship between immunotherapy biomarkers and APC, we verified that APC have inactive mutation in endometrial cancer, which may affect the immune response, including PD-L1 expression, microsatellite instability and lymphocytic infiltrate. Using TCGA data, we found that patients with the APC mutation had longer overall survival.

Conclusion

Our study demonstrates that APC could play an important role in enhancing the response to endometrial cancer treatment, particularly immunotherapy.

Background

Endometrial cancer is the one of the most common malignant tumors of the female reproductive system. In recent years, the morbidity and mortality of endometrial cancer have increased worldwide. Early diagnosis, surgery, and chemotherapy reduce endometrial cancer mortality. However, there is a subset of low-grade, early stage, well-differentiated endometrioid tumors in which unexpected recurrences and poor outcomes do occur. For women diagnosed with a clinically aggressive histologic subtype of the disease,

such as serous, clinical outcomes worsen considerably with recurrent or advanced disease (1, 2). Traditional endometrial cancer typing (Bokhman) has limited predictive value for prognosis, because it does not consider the genetic variation and heterogeneity of the tumor. The Cancer Genome Atlas (TCGA) published a comprehensive genomic study of serous and endometrioid histotypes and reported four genomic subtypes: polymerase-epsilon (POLE), microsatellite instability (MSI), copy-number low and copy-number high (3). Intracellular mutation accumulation caused by POLE mutations or MSI, leads to high tumor mutational burden (TMB), increased expression of new antigens and abundant tumor infiltrating lymphocytes, which result in sensitivity to immune checkpoint inhibitors (4).

Immunotherapy, the most promising research direction in cancer treatment, regulates the tumor immune microenvironment to kill tumor cells by activating or regulating the body's immune system. Multiple clinical trials have proven that the type of POLE hypermutation and MSI are potential beneficiaries of programmed cell death protein (PD-1) and programmed death-ligand 1 (PD-L1) immunotherapy in endometrial cancer (3, 5, 6). The expression of PD-L1 was typically detected by immunohistochemistry to predict the effect of immunotherapy for endometrial cancer. However, some clinical studies have indicated that patients with positive PD-1 expression do not respond to PD-1/PD-L1 immunotherapy (6, 7). Coincidentally, the latest research by Dou et al. found that the efficiency of antigen expression varies greatly in MSI-type endometrial cancer, and the down-regulation of antigen presentation ability would alter the effect of immunotherapy (7, 8). It is urgent to identify new targets for diagnosis and treatment to improve the effect of immunotherapy in endometrial cancer.

Epigenetics is the study of heritable phenotype changes, most often involving changes that affect gene activity and expression, but do not involve DNA sequence alterations. Due to epigenetic changes, tumor cell immunogenicity and immune recognition mechanisms are destroyed (9, 11). In addition, epigenetic silencing affects antigen processing and presentation (12). Chromatin remodeling is an important part of epigenetics. Impaired chromatin remodeling leads to the accumulation of epigenetic abnormalities. Several studies have found, in malignant tumors, that the gene related to chromatin remodeling has high mutation frequency and plays an important role in tumor immune escape (13, 14). The adenomatous polyposis coli (APC) gene, located on chromosome 5q21, is the chromatin-remodeling related gene and a typical tumor suppressor. APC protein is involved in the modification of transcription activation and cell cycle regulation. APC has an oligomerization domain, a 15- or 20-residue repeat domain important for binding to β -catenin, SAMP repeats for axin binding, a basic domain for microtubule binding and C-terminal domains that bind to EB1 and DLG proteins (15). The basal region and the C-terminal region, combined with microtubules, interact with EB1 to promote chromosome aggregation (16). APC inactivation leads to the loss of spindle function in mitosis and the instability of the genome and chromosome (17). Aberrant structure or expression of APC has been reported to be associated with various cancers. For example, high APC expression is an unfavorable prognostic factor for T4 gastric cancer and may be used as a novel biomarker for pathogenesis research, diagnosis, and treatment of gastric cancer (18). Loss of APC function leading to Wnt/ β -catenin signaling hyper-activation is considered one of the driving forces of colorectal cancer tumorigenesis (19). APC mutation occurs in 20–45% of endometrial cancer (20), and the methylation of APC is associated with endometrial cancer

occurrence (21). Recent studies have shown that APC mutation can induce endometrial hyperplasia and endometrial cancer by preventing estrogen signal transduction in endometrial epithelium (22). Therefore, we speculated that APC may have an important role in the pathogenesis and clinical progression of endometrial cancer.

The objective of this study was to demonstrate that APC as a new target for the diagnosis and treatment of endometrial cancer. We used data from TCGA and tumor samples from an endometrial cancer cohort to characterize endometrial cancer with and without the APC mutation. And our findings support that APC may play an important role in the immunotherapy of endometrial cancer.

Methods

Research data source

We randomly selected 100 endometrial cancer tumors from 1000 in the endometrial cancer cohort biorepository of Shanghai First Maternity and Infant Hospital. Specimens were kept on dry ice to maintain specimen integrity and then cryo-pulverized. The cryo-pulverized specimen and paired blood sample were prepared for DNA isolation for molecular characterization. Given that one sample failed the quality test, a total of 99 endometrial cancer patients were enrolled in this study, with an average age of 58 years. The cohort study of China included 88 cases endometrioid adenocarcinoma, 6 cases uterine carcinosarcoma, 3 cases serous carcinoma and 2 cases other types of endometrial cancer. Among them, 74.2% of patients were grade I, followed by 19.1% grade II and 6.7% grade III. Most of the samples submitted for inspection are early-stage (71.6% for stage I and 10.1% for stage II), and 18.1% for late-stage. The data from TCGA, including high throughput RNA sequencing (RNA-Seq) and clinical follow-up information for uterine corpus endometrioid carcinoma, were downloaded from TCGA on May 31, 2020. Samples from TCGA with more than 30 days of follow-up were screened for clinical follow-up data to further match the RNA-seq expression profile.

DNA extraction and fragmentation

DNA isolation and subsequent sequencing procedures were performed in the laboratory of Burning Rock Biotech (Guangzhou, China) accredited and certified by the College of American Pathologists and Clinical Laboratory Improvement Amendments. Genomic DNA (gDNA) was extracted from tumor tissues and white blood cells by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Qubit fluorometer with the dsDNA high-sensitivity assay kit (Life Technologies, Carlsbad, CA, USA) was used to measure DNA quality following the manufacturer's instructions. DNA fragmentation was performed using a Covaris M220 Focused-ultrasonicator (Woburn, MA, USA), followed by end repair, phosphorylation, and adaptor ligation. Fragments between 200 to 400 bp were selected using AMPure beads (Agencourt AMPure XP Kit, Beckman Coulter, Brea, CA, USA). Subsequently, the hybridization with capture probe baits, hybrid selection with magnetic beads, and PCR amplification were performed. A high-sensitivity DNA assay was then performed to assess the quality and size of the DNA fragments (Agilent 2100 bioanalyzer instrument, Agilent, Santa Clara, CA, USA).

Next-generation sequencing (NGS) library preparation, and capture-based targeted DNA sequencing

The NGS library was constructed for the DNA isolated from tumor tissues and white blood cells according to an optimized protocol. A minimum of 50 ng of DNA was required for NGS library construction. Target capture was performed using commercially available panels of 520 cancer-related genes (OncoScreen Plus, Burning Rock, Guangzhou, China), spanning 1.64 Mb of the human genome (Burning Rock Biotech, Guangzhou, China). These panels comprehensively and accurately detect single nucleotide variants, insertion-deletions, copy number variations (CNV), and structural variations of genes that are clinically relevant to cancer. The average sequencing depths were 1,000X for tissue DNA. Indexed samples were sequenced on Nextseq500 sequencer (Illumina, Inc., San Diego, CA, USA) with paired-end reads. Sequencing data were analyzed using proprietary computational algorithms optimized for somatic variant calling as described previously (23, 24).

Sequencing data analysis

The sequencing data in FASTQ format were mapped to the human genome (hg19) using Burrows-Wheeler Aligner v.0.7.10 (25). Local alignment optimization and variant calling were performed using GATK v.3.2 (26) and VarScan v.2.4.3 (27), respectively. Variants were filtered using the VarScan ffilter pipeline; loci with sequencing depth less than 100 were eliminated. Matched white blood cells were used to filter out germline mutations. Variant calling in plasma and tissue samples required at least 8 supporting reads for single nucleotide variants and 2 and 5 supporting reads for insertions and deletions, respectively. Variants with population frequency over 0.1% in the ExAC, 1000 Genomes, dbSNP or ESP6500SI-V2 databases were grouped as single nucleotide polymorphisms and excluded from further analysis. The remaining variants were annotated with ANNOVAR (2016-02-01 release) (28) and SnpEff v.3.6 (29). DNA translocation analysis was performed using Factera v.1.4.3 (30). Gene-level CNV was assessed using an in-house-developed algorithm based on a statistic after normalizing read depth at each region by the total read number and region size and correcting the GC-bias using a LOESS algorithm. CNV was called if the coverage data of the gene region was quantitatively and statistically significantly different from its reference control. The limit of detection for CNVs was 1.5 for copy number deletions and 2.64 for copy number amplifications. TMB was calculated as the ratio of mutation count to the size of coding region of the panel (1.26 Mb), excluding CNV, fusions, large genomic rearrangements and mutations occurring on the kinase domain of EGFR and ALK. The MSI phenotype detection method used a read-count–distribution-based approach according to a previously published protocol and algorithm (31).

Immunohistochemistry

Paraffin-embedded tissue sections (4 μ m) of the endometrial cancer samples were processed for immunohistochemistry. First, specimens were deparaffinized and dehydrated, and then sections were stained with anti-APC antibody from Abcam (1:100 dilution); anti-PD-L1 antibody from Ventana (prediluted); anti-CD3 + antibody and anti-CD8 + antibody from Springs, (1:200 dilution); and anti-

MLH1 antibody, anti-MSH2, anti-MSH6, and anti-PMS2 from Maixin (1:100 dilution). After washing, the sections were incubated with biotin-conjugated secondary antibodies and subsequently with streptavidin–HRP. The sections were finally visualized by incubation with 3,3'-diaminobenzidine substrate. Images were obtained with the Mantra System (PerkinElmer, Waltham, Massachusetts, USA) with identical exposure times. The integrated optical density was used to quantify the protein levels of APC, PD-L1, CD3⁺, CD8⁺, MLH1, MSH2, MSH6 and PMS2 in tumor tissue, and this integrated optical density was calculated by staining intensity dividing the staining area (brown stained area).

Statistics

Categorical data were described by frequency and percentage. Quantitative variables were expressed as means \pm SEM. Fisher's exact or Chi-square test was performed to compare categorical variables. The Student t-test was used to compare continuous variables between two groups. Pearson's correlation coefficient was used to assess correlations. All the data were analyzed via R statistics package (R version 3.5.3; R: The R-Project for Statistical Computing, Vienna, Austria). All statistical tests were two-sided, and P-values < 0.05 were considered significant. R package survival KM (Kaplan-Meier) was used to estimate the survival rate, and Cox proportion hazards regression was used to calculate a hazard ratio.

Results

Comprehensive analysis of gene mutation in endometrial cancer

The clinical and pathological characteristics of the 99 tumors from the cohort biorepository are summarized in Table S1. We used tissues and peripheral blood to detect gene mutations. The sequencing results showed that the high-frequency mutant genes of somatic cells were PTEN, PIK3CA and ARID1A, with 82%, 62% and 53% mutation rates, respectively. DNA damage repair (DDR) gene mutations were detected in 94% of samples (Fig. 1A). We also found that 17% of tumors had high levels of MSI, and the tumors with MSI had higher TMB (mean TMB: 53.25 mut/mb in MSI, 37.37 mut/mb in Microsatellitestable). (Fig. 1B). In addition, we found that TMB was significantly higher in tumors with the POLE mutant and those with MSI than those without (both $p < 0.01$; Fig. 1C). In our study, the total mutation rate of mismatch repair genes, including MSH2, MSH6, MLH1 and PMS2, was 26%. (Figure D). Through analyzing the genes with more than 10% mutations, we found that most of the mutated genes related to chromatin status, including covalent modification of chromatin, chromosome organization and chromatin organization (Fig. 1E).

Immune microenvironment correlated with chromatin status related genes

To determine the effect of chromatin-related gene mutations on endometrial cancer, we explored the correlation between gene mutations and tumor immune microenvironment. From the mutated chromatin-

related genes, we identified 12 mutant genes that were significantly positively correlated with PD-L1 expression ($R > 0.25$; $p < 0.05$) or TMB ($R > 0.8$; $p < 0.05$). Mutations of these 12 genes (STAG2, TAF1, ARID1A, KMT2C, KMT2D, APC, KMT2A, JAK2, BRCA2, PRKDC, ATRX, BCORL1) were detected in 66% of the endometrial cancer samples (Fig. 2A-B). The 66 samples that contained these mutated genes comprised the discovery set, and the remaining 33 samples were used as the control set. Next, we compared the discovery set with the control set in terms of TMB, PD-L1 expression and lymphocytic infiltration. The mean TMB of the discovery set (58.57 mut/mb, $\log_2\text{TMB} = 4.45$; $\text{SD} = 1.96$) was significantly higher than that of the control set (4.92mut/mb, $\log_2\text{TMB} = 2.16$; $\text{SD} = 0.64$; $p < 0.001$; Fig. 2C). We then quantified the expression of PD-L1 in tumor cells and immune cells. PD-L1 expression was higher in the discovery set than in the control set in both immune cells and tumor cells. PD-L1 expression increased significantly (both $p = 0.001$), especially in the immune cells (PDL1.TC: 0.074 in control and 0.130 in discovery; PDL1.IC: 0.225 in control and 0.378 in discovery; Fig. 2D). When comparing the discovery set and the control set, we found no significant difference in lymphocytic infiltration for CD3^+ T cell ($p = 0.11$) or CD8^+ T cell ($p = 0.07$; Fig. 2E).

Chromatin remodeling-related gene APC affects the immune microenvironment

To evaluate the effects of the 12 specific gene mutations, we explored the immune microenvironment, including TMB, PD-L1 expression and lymphocytic infiltration, in endometrial cancer (Fig. 3A). Four genes (KMT2C, APC, KMT2A, JAK2) were selected for significance and relevance. The mutation rates were 18% for KMT2C, 18% for APC, 17% for KMT2A, and 11% for JAK2 (Fig. 3B). Unlike the other three genes, the mutations of the chromatin remodeling-related gene APC were associated with significant increases in TMB, PD-L1 expression and CD3^+ T cell infiltration (all $p < 0.02$; Fig. 3C-E). To further verify the impact of APC mutations on the immune microenvironment, we analyzed the gene mutations of endometrial cancer from TCGA, which yielded similar results (Fig. 4A-C).

Chromatin remodeling-related gene APC expression in endometrial cancer

To further investigate expression of the APC endometrial cancer-specific immunotherapy markers, we assessed the protein levels of APC, MLH1, MSH2, MSH6, PMS2, PD-L1, CD3^+ and CD8^+ by immunohistochemistry using tissue from 99 endometrial cancer tumors. We quantified staining with the integrated optical density value that combined the staining intensity and the percentage of positive cells. In endometrial cancer, most APC mutations are inactive mutations which lead to reduced protein levels (22). In our study, the low expression of APC was accompanied by high levels of PD-L1 expression and increased infiltration of CD3^+ and CD8^+ T cells. To evaluate MSI, we quantified the expression levels of MLH1, MSH2, MSH6 and PMS2 by immunohistochemistry. Results indicated that samples with APC inactivated mutations were MSI type, which was consistent with gene sequencing (Fig. 5A-B). The results

of the survival analysis among the 526 TCGA samples suggested that the APC mutation was associated with longer survival ($p = 3.5e-06$; Fig. 5C).

Discussion

We analyzed gene expression profiles in endometrial cancer samples from our cohort by using a commercial panel of 520 genes that are closely associated with cancer pathogenesis. We comprehensively analyzed the mutation profiles of endometrial cancer and confirmed the somatic and germline mutations of previously study described at the genomic level (3). In recent years, PD-1/PD-L1 treatment that blocks immune checkpoints has developed rapidly and attracted attention broadly. TMB, MSI and PD-L1 expression all have been reported as markers for PD-1/PD-L1 immunotherapy (4, 6, 32). However, as a result of paradoxical clinical trials (7), the issue has been the identification of markers that accurately predict the efficacy of immunotherapy in endometrial cancer. Our results revealed that the samples characterized by MSI or POLE mutation were accompanied by an increase of TMB (Fig. 1B-C). Beyond that, clustering analysis of gene mutations profiles showed that 94% of the samples had mutations of the DDR gene, and we clearly demonstrated that genes with more than 10% mutations are related to chromatin status (Fig. 1D-E). These findings suggested that mutations of chromatin state-related genes may have an impact on the immune microenvironment of endometrial cancer.

Feature selection is a data preprocessing technique that has been widely used in many bioinformatics applications (33). Here, we modeled marker discovery as an approach to select the best feature subset for the immunotherapy of endometrial tumors. It is not easy to choose a reliable feature subset due to the multi-dimensionality of QIAamp DNA FFPE tissue kit data. Therefore, we designed a cross feature selection schema, based on the correlation of chromatin status related genes with PD-L1 expression or TMB, to select a reliable subset (Fig. 2A-2B). Filters may lead to locally optimum sets but not the best discriminative subset, which may make it impossible to find diagnostic markers with high sensitivity and specificity. We further analyzed the TMB level, PD-L1 expression and lymphocytic infiltration of the selected subset to verify its reliability, which was identified containing 12 chromatin state-related genes (Fig. 2C-2E). The results indicated that the cross method performed extremely well for identifying a discovery set of genes associated with immune microenvironment in endometrial cancer, except for lymphocytic infiltration.

As cancer treatments, immunotherapy approaches have been highly successful but can be affected by immune microenvironment. Our results indicate that the gene APC may serve as a new marker to assessing the impact on the immune microenvironment (Fig. 3A-3B). By analyzing the relationship between individual mutant genes in the discovery set and the immune microenvironment, we found that the APC mutations was significantly associated with the immune microenvironment, including TMB, PD-L1 expression and lymphocytic infiltration (Fig. 3C-3E). Our results showed that APC mutations might suggest elevation of the TMB, PD-L1 expression and lymphocytic infiltration, which may help identify patients who may benefit from immunotherapy. Moreover, we confirmed our findings regarding the effect of the APC mutation on the immune-microenvironment using TCGA (Fig. 4A-4C). Further biological

validation and clinical trials are needed to evaluate the clinical significance of chromatic status-related gene APC, along with studies to understand the mechanism by which APC regulates the immune microenvironment.

In our study, the significantly decreased expression of APC was further confirmed in human endometrial cancer tissues by immunohistochemistry (Fig. 5A-5B). The APC mutation may contribute to increased expression of new antigens and abundant tumor infiltrating lymphocytes, which may result in the sensitivity of immune checkpoint inhibitors. This may explain why patients with APC mutation have a more favorable prognosis (Fig. 5C).

Conclusion

In summary, immune therapy, including checkpoint inhibition and tumor vaccination, plays a crucial role in cancer treatment. The sensitivity of existing target molecules is insufficient, such that a significant proportion of patients fail to respond to immunotherapy. Our results suggest that APC may affect the immune microenvironment of endometrial cancer; thus, patients with APC mutations may be more sensitive to immunotherapy. Consequently, this information could improve selection of endometrial cancer patients who will have a better response to immune checkpoint therapy and thus a better prognosis. Although our results are observational, they provide the basis for multiple hypotheses of clinical relevance that can and should be further explored by the scientific community.

Abbreviations

APC
Adenomatous polyposis coli
DDR
DNA damage repair
EC
Endometrial cancer
MSI
Microsatellite instability
PD-1
Programmed cell death protein
PD-L1
Programmed death-ligand 1
POLE
Polymerase-epsilon
TMB
Tumor mutational burden
TCGA
The Cancer Genome Atlas

Declarations

Ethics approval and consent to participate

This study was approved by the Clinical Research Ethics Committee of College of Medicine, Tongji University. Written informed consent was received from participants prior to inclusion in the study.

Consent for publication

The authors confirm that we have obtained written consent from the patients to publish this manuscript.

Availability of data and material

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that no conflict of interest exists.

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Authors' contributions

YRL carried out the molecular genetic studies and immunoassays, participated in the sequence alignment, performed the statistical analysis, and drafted the manuscript. KW participated in the design of the study and the sequence alignment. XPW conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figures

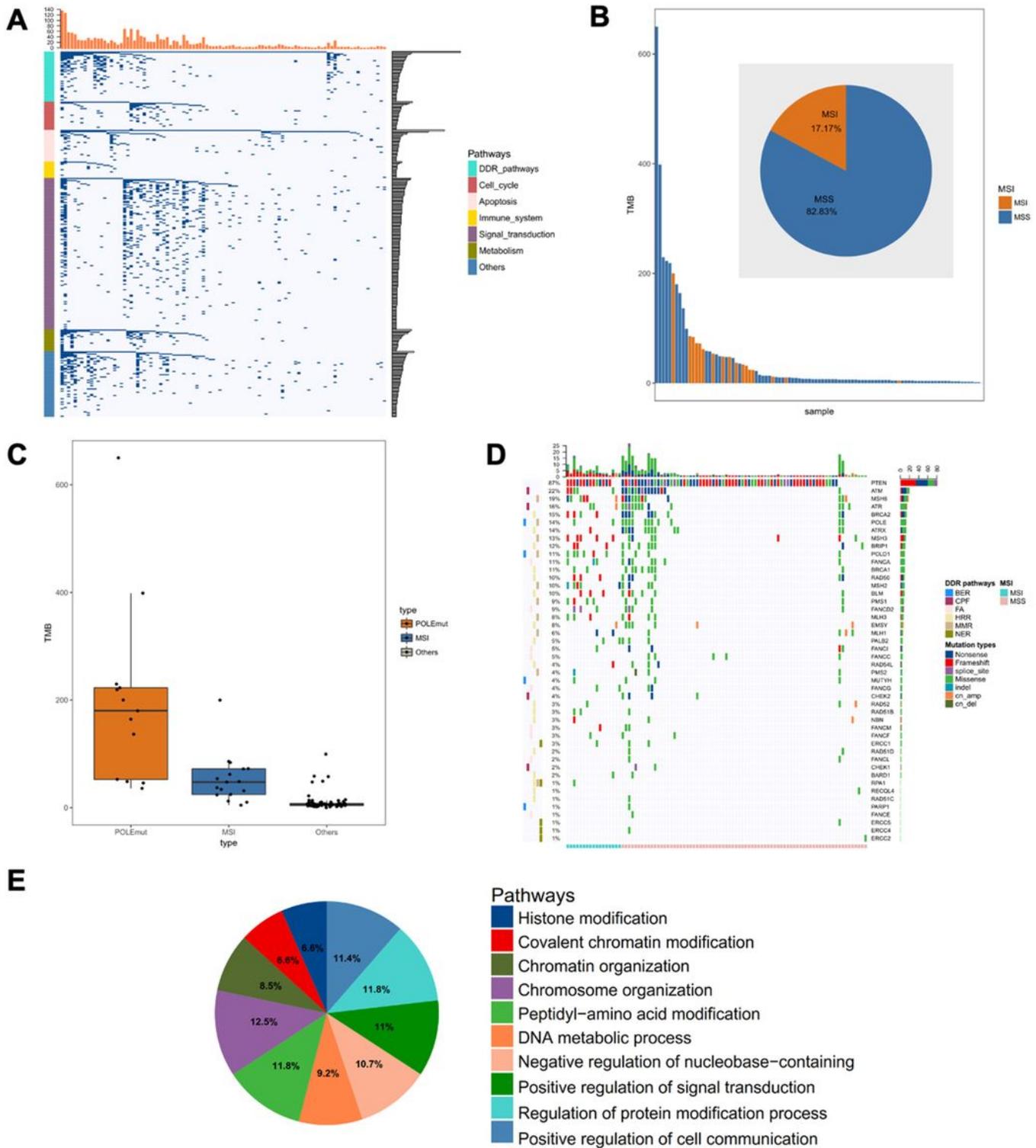


Figure 1

Unsupervised clustering of gene mutations in endometrial cancer. Samples are presented in columns, and the 520 cancer-related genes are presented in rows. B. Analysis of microsatellite instability in 99 samples C. Analysis of tumor mutational burden level in POLE (DNA polymerase epsilon, catalytic subunit) mutant group, MSI (microsatellite instability) group and other groups D. Analysis of mutated genes related to DDR pathway E. Pathway analysis of significantly mutated genes (mutation >10%).

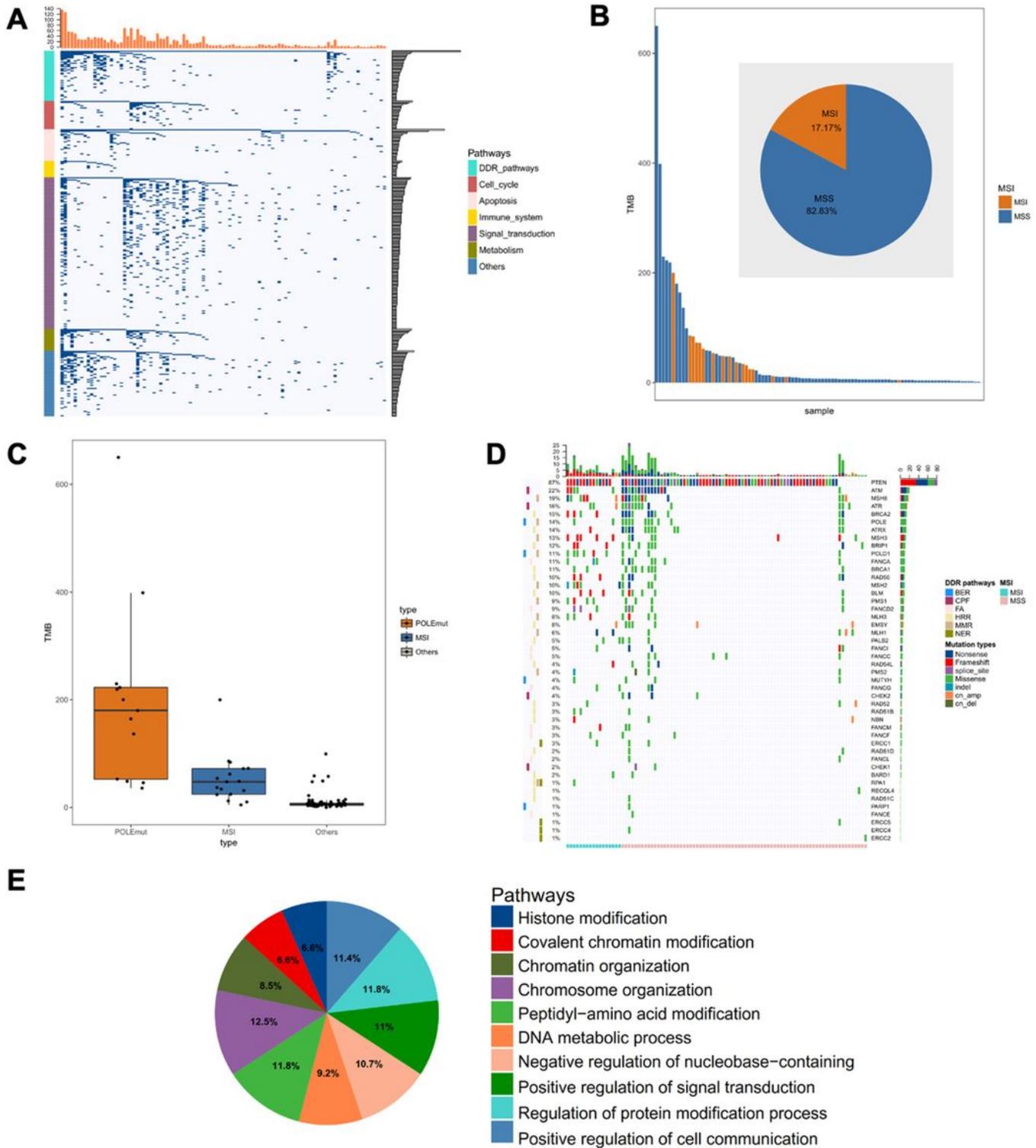


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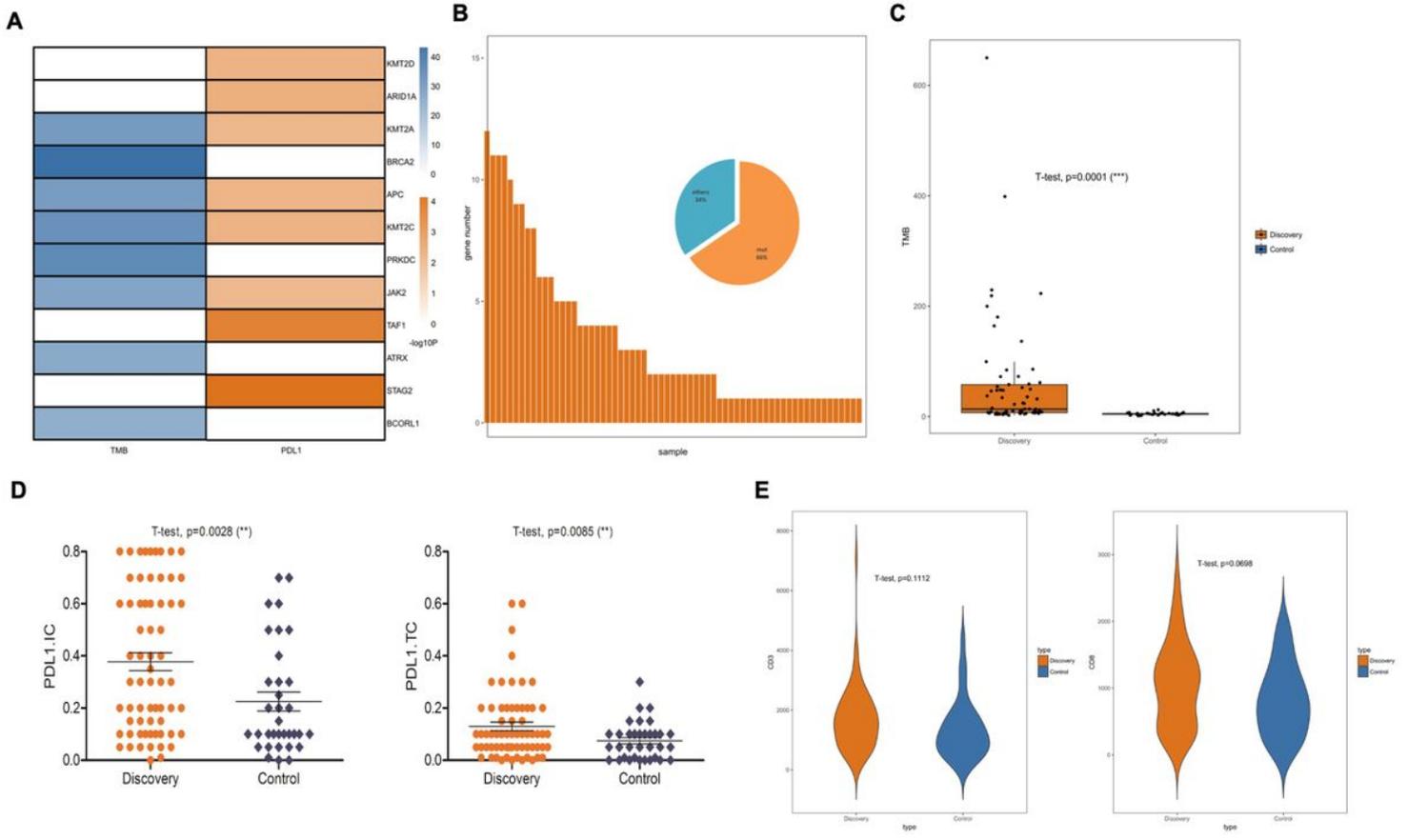


Figure 2

A. Specific mutated genes screened by significant correlation with PD-L1(programmed death-ligand 1) expression or TMB(tumor mutational burden), Pearson's correlation coefficient was used to assess correlations B. Mutation of 12 specific mutated genes identified as discovery set C. Relationship between discovery sets and TMB D. Relationship between discovery sets and PD-L1 Expression. E. Relationship between discovery sets and CD3+ and CD8+ T cell infiltration

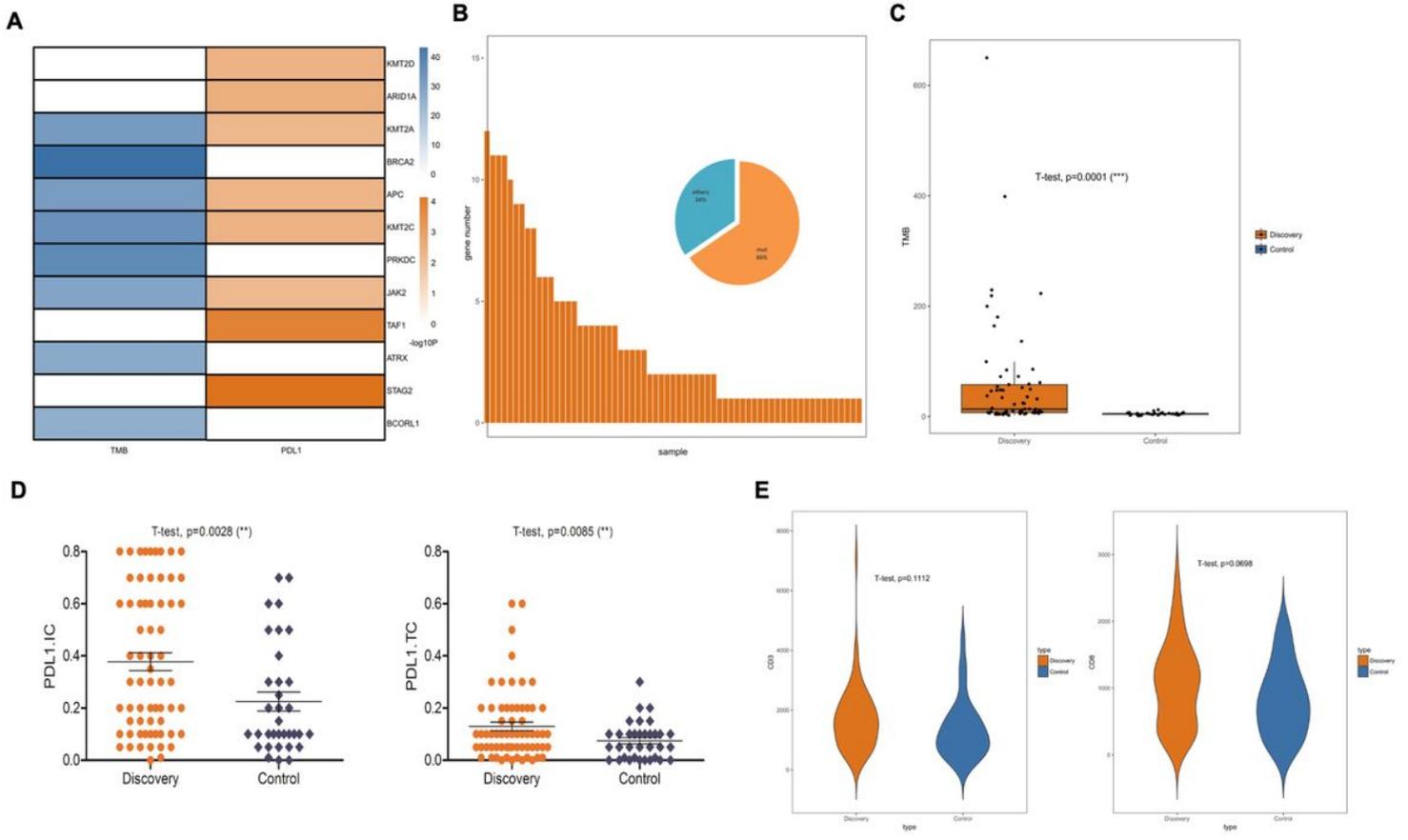


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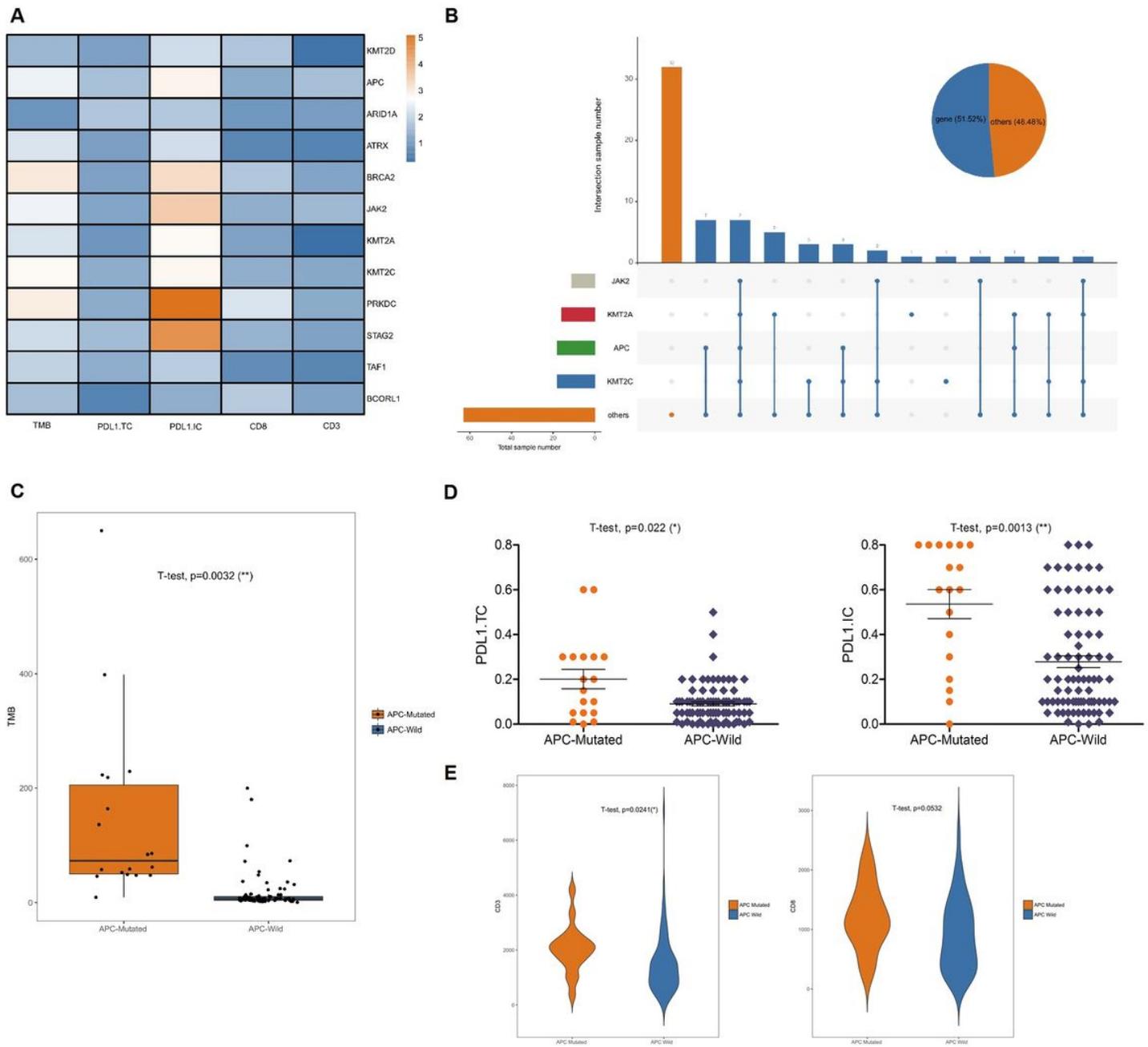


Figure 3

A. Significant change of TMB, PD-L1 expression and lymphocytic infiltration inducing by mutation of discovery set. B. The mutation of KMT2C, APC, KMT2A and JAK2. C. Relationship between APC and TMB. D. Relationship between APC and PD-L1 Expression. E. Relationship between APC and CD3+ and CD8+ T cell infiltration

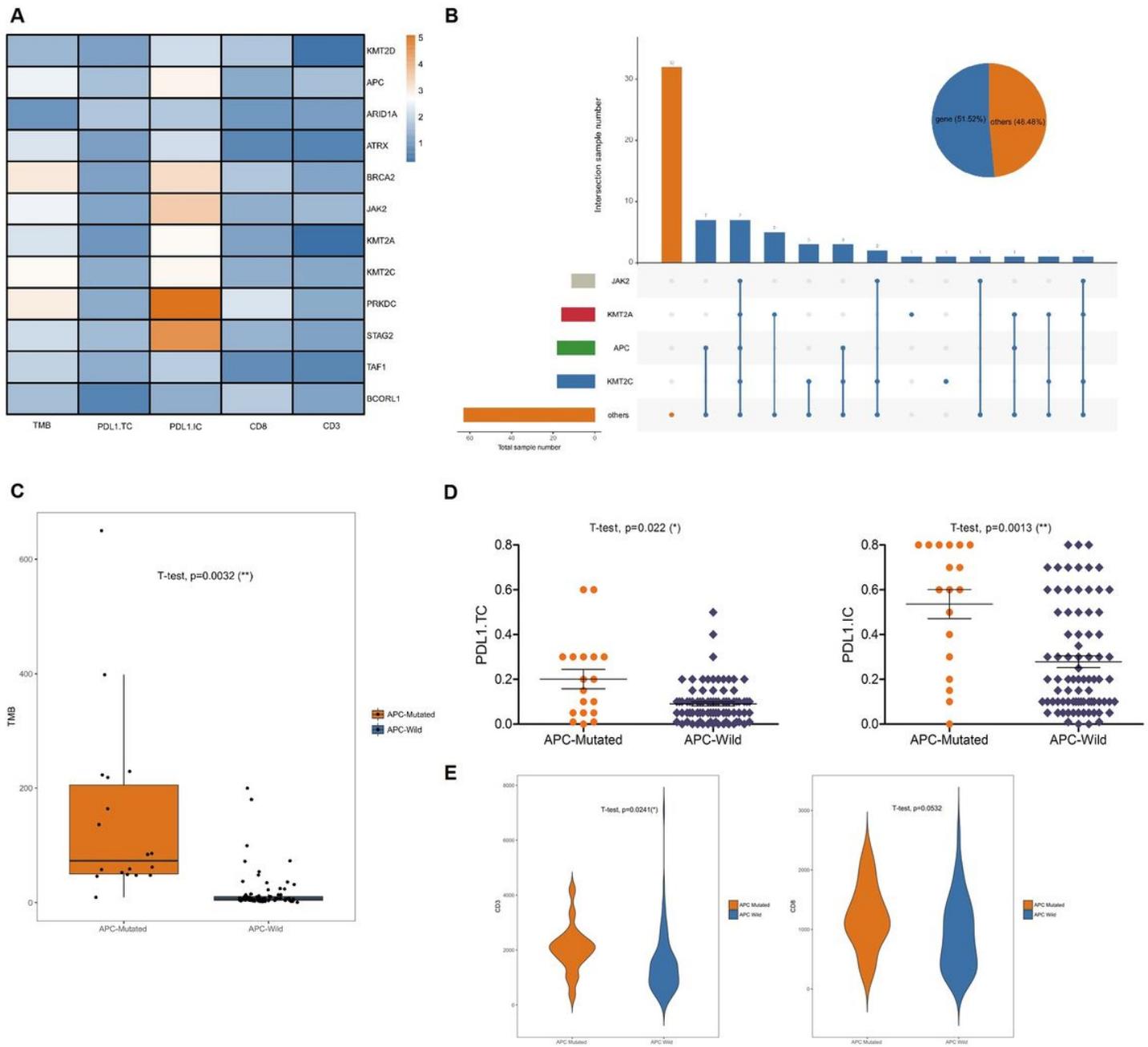


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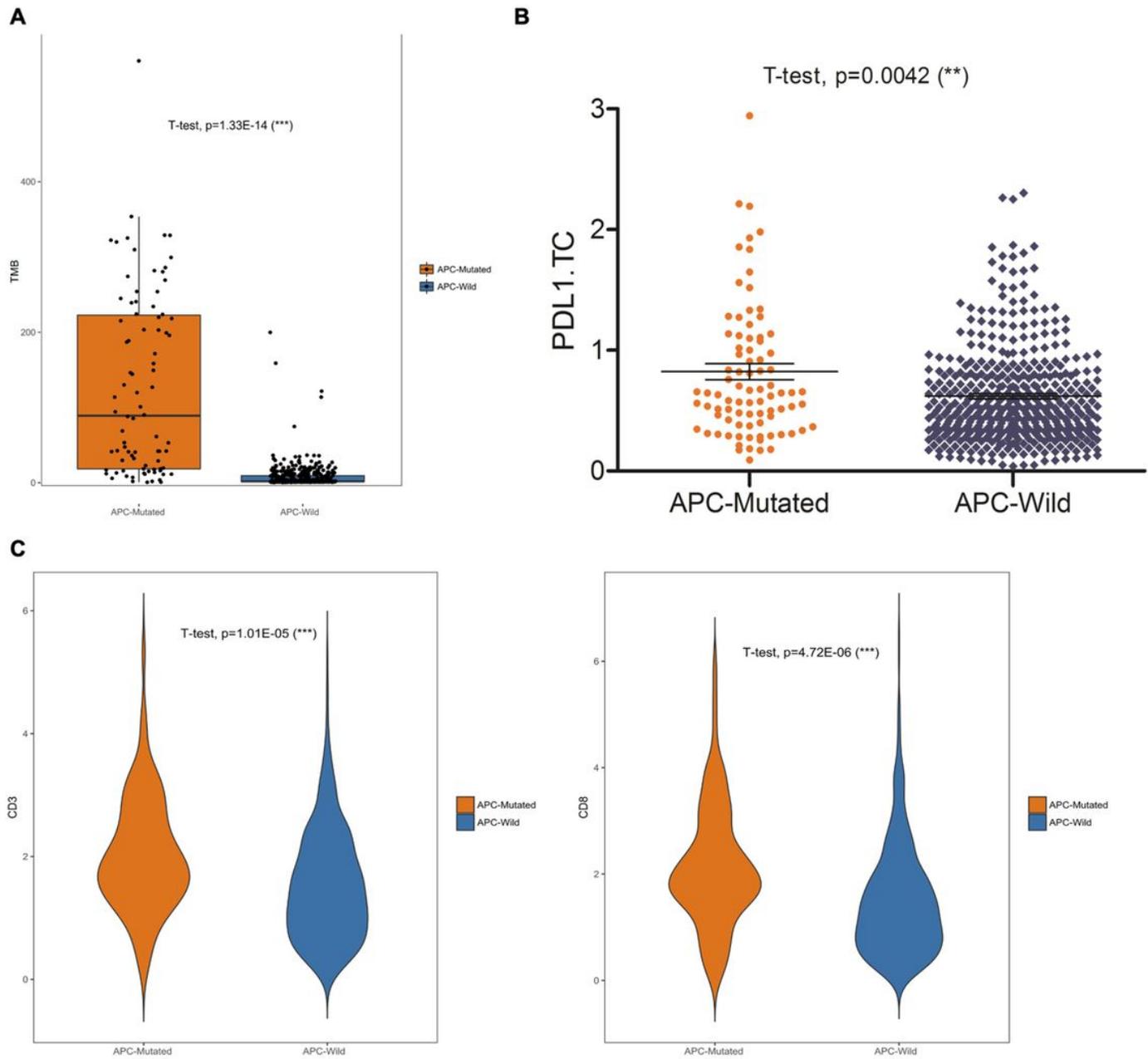


Figure 4

Analysis of TCGA. A. Relationship between APC and TMB B. Relationship between APC and PD-L1 Expression. C. Relationship between APC and CD3+ and CD8+ T cell infiltration

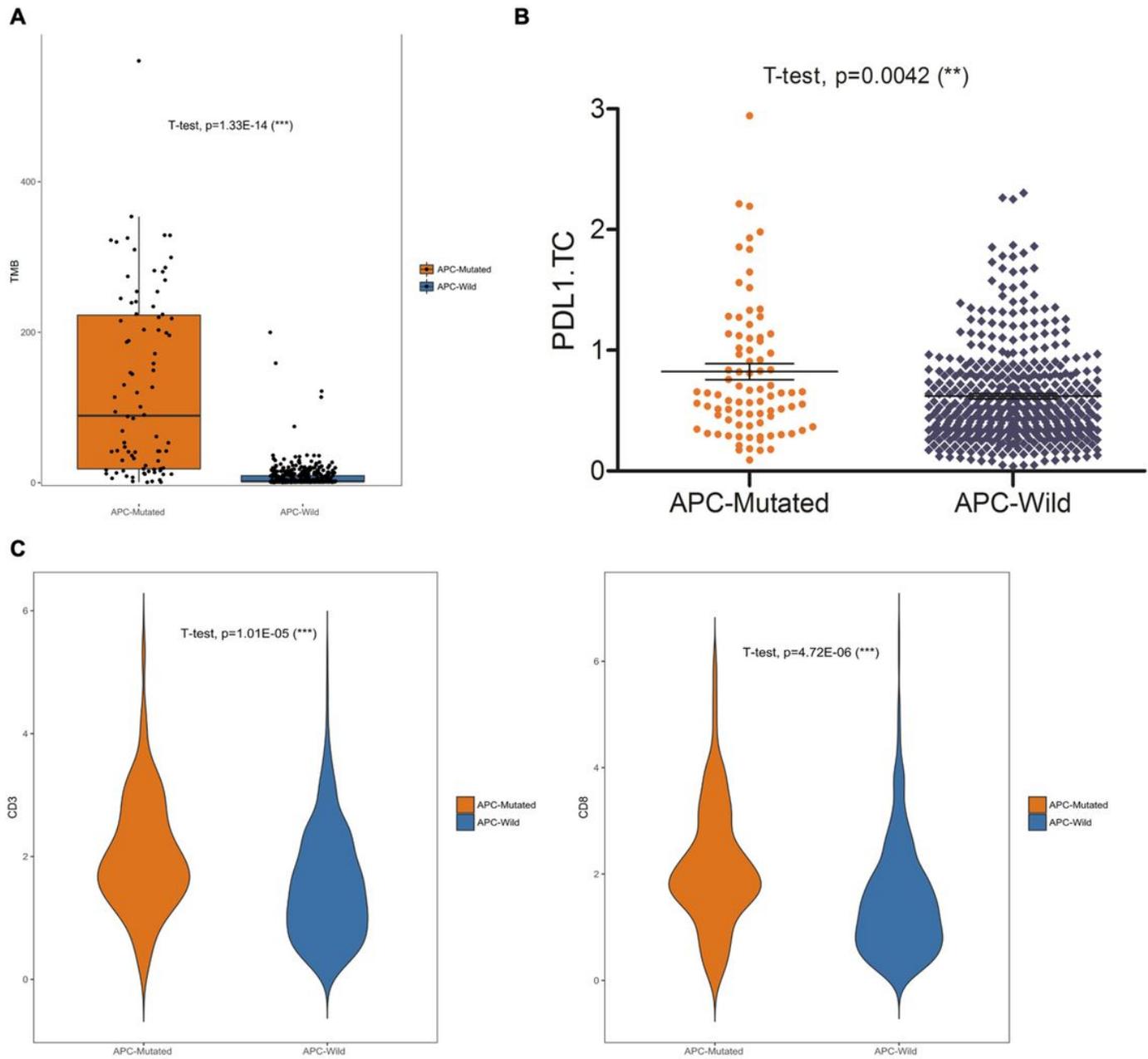


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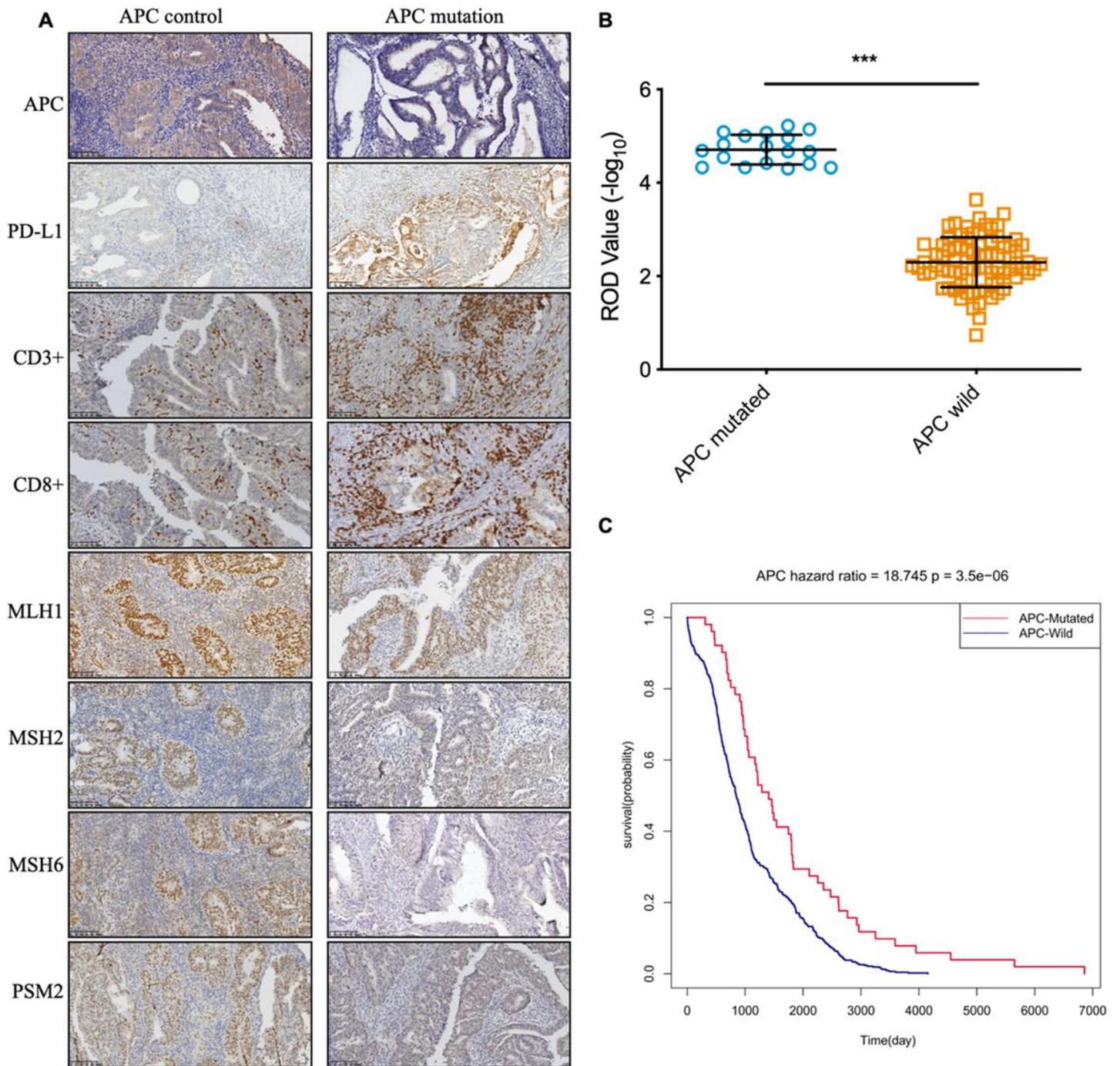


Figure 5

A. Immunohistochemistry staining of the indicated proteins in APC control samples and APC mutation samples. B. APC are weakly expressed in human endometrial cancer ($p=6.17624e-27$). C. Prognostic value of APC expression in endometrial cancer patients from TCGA (Kaplan-Meier was used to estimate the survival rate).

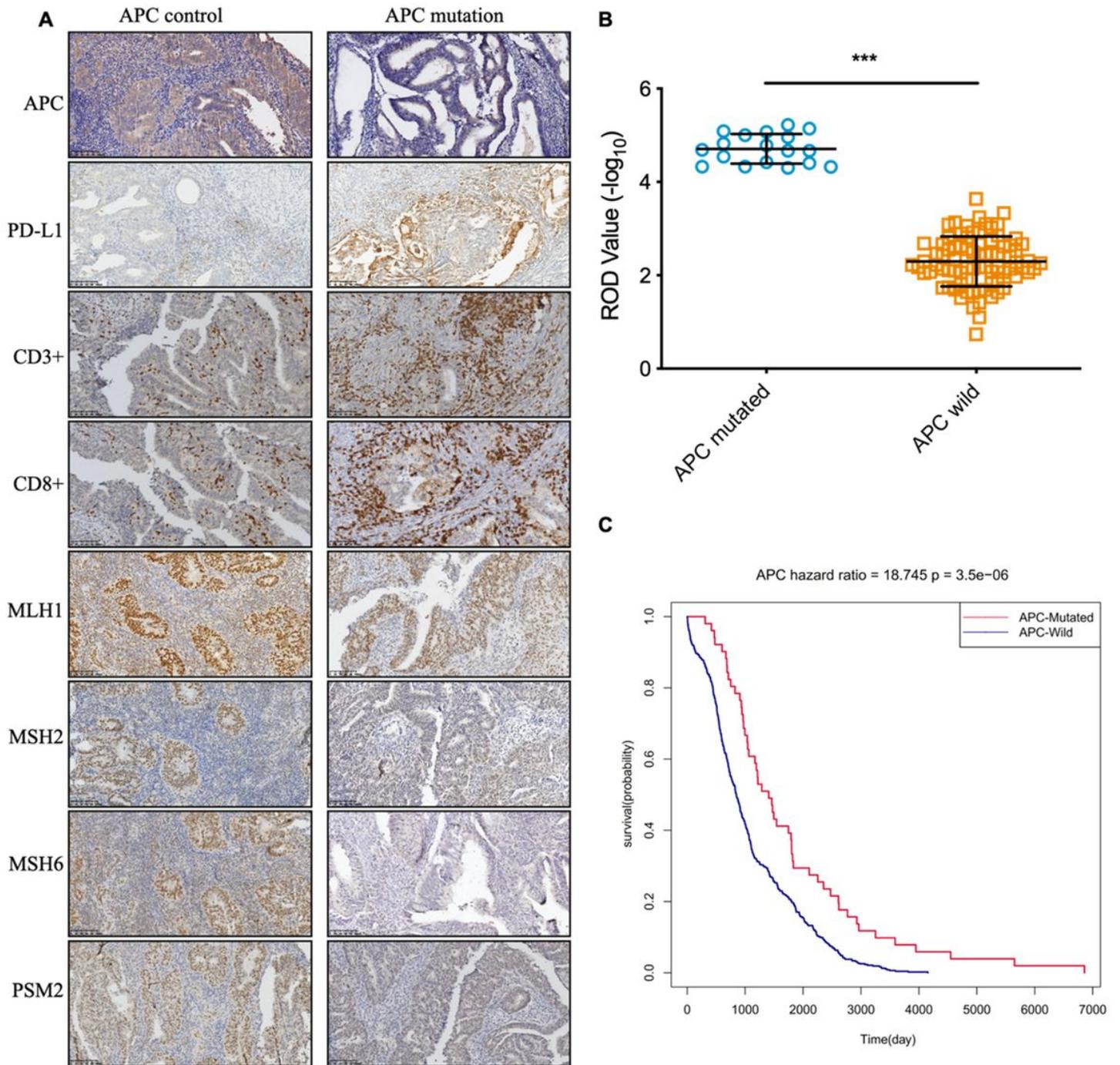


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