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Primary research

Keywords: immune microenvironment, APC, MSS/pMMR, colon cancer

Posted Date: August 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-56379/v1

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ABSTRACT

Background Immunotherapy is currently the most advanced anti-tumor treatment approach. The efficacy of anti-tumor immunotherapy is closely related to the tumor immune microenvironment, including immune cells, infiltration of immune factors, and expression of immune checkpoints. At present, the biomarkers for predicting the efficacy of colon cancer immunotherapy do not cover all colon cancer patients suitable for immunotherapy. In this study, TCGA database was used to identify tumor genotypes suitable for anti-tumor immunotherapy. **Methods** We downloaded 390 samples with RNA-sequencing data and somatic mutation status data from TCGA. We applied the ESTIMATE, Package limma, the CIBERSORT and some other algorithms to analyze the cellular immune microenvironment. And validated with immunohistochemistry in tumor tissues of colon cancer patients. **Results** We found that some of the MSS/pMMR populations, that were initially considered unsuitable for immunotherapy, might actually be suitable. In APC-wt/MSS colon cancer, the expression of PD-1, PD-L1, CTLA4 and CYT（GZMA and PRF1）were increased. Based on calculations done by ESTIMATE and CIBERSORT algorithms, the ImmunoScore and the proportion of CT8+ T cell infiltration is increased in these patients. Enrichment analysis was done to screen signaling pathways involved in immune response, extracellular matrix, and cell adhesion. Tumors from 42 colon cancer patients, including 22 APC-mt/MSS and 20 APC-wt/MSS, were immunohistochemically evaluated for expression of CD8 and PD-L1. And APC-wt/MSS tumors showed significantly higher expression of CD8 and PD-L1 than APC-mt/MSS tumor. **Conclusions** Based on the results, we found that some colon cancers of APC-wt/MSS are classified by Tumor Immune Microenvironment types (TIMTs) TMIT I. So that we speculate that APC-wt/MSS colon cancer patients could benefit from anti-tumor immunotherapy.

KEYWORDS immune microenvironment, APC, MSS/pMMR, colon cancer

Background

Tumor immune microenvironment, known as the tumor "seventh major marker" [1],

is composed of innate and adaptive immune cells, cytokines, and cell surface molecules. These immune components constitute a complex regulatory network and play an important role in tumor genesis and development. Wherein, the development of immune checkpoint pathways is a major mechanism by which tumors evade immune surveillance [2]. Immune checkpoints refer to inhibitory pathways in the immune system that are essential for maintaining self-tolerance and minimizing chronic autoimmune inflammation [3]. Use of immune checkpoint inhibitors is one of the treatment methods that reactivates anti-tumor immunity. Currently, the approval of anti-tumor immunotherapy at clinical brake sites of the immune response include agents directed against CTLA-4 (ipilimumab), and programmed cell death protein 1 (PD-1; nivolumab, pembrolizumab) or PD-1 ligand 1 (PD-L1; atezolizumab, durvalumab, avelumab) [4-7].

Despite these advances, only a few patients with advanced/metastatic cancer respond to immune checkpoint inhibitors, thereby exposing the remaining patients to potentially ineffective, toxic, and expensive treatments. Therefore, biomarkers are needed to predict the response and guide clinical treatment decisions. For example, in colon cancer, NCCN guidelines currently approve immunotherapy only for MSI-H/dMMR patients. These patients comprise less than 15% for the sporadic colon cancer patient population [8-10]. At present, the overall clinical response rate of colon cancer to immunotherapy is higher than this rate [11]. For colon cancer patients with MSS/pMMR, other biomarkers are needed to predict the efficacy of immunotherapy.

There are many molecular types associated with colon cancer. About 70% of sporadic colon cancers are caused by inactivation of the tumor suppressor gene for biallelic APC, resulting in abnormal activation of the WNT/ β -catenin signaling pathway [12]. Most APC-mutation (APC-mt) cancers are assumed to have developed through the classic adenoma-cancer pathway. Other major routes for colorectal cancer development account for another 15% to 20% of the cases [13]. Typical manifestations of this pathway are precursor sessile adenoma, wild-type APC (APC-wt), BRAF mutation, characteristic CpG island methylation phenotype, poor

differentiation, and mucosa histology [14]. Here re report on studies of the effect of APC status on the immune microenvironment of MSS colon cancer, aiming to identify its TIMT and determine whether it might be a potential predictor for immunotherapy efficacy in these patients.

RESULTS

Molecular features and clinicopathological assessment of CRC patients

There are 462 COAD patient data in TCGA database, including 390 cases with SNP and transcriptome data. The median age at diagnosis is 68 years. There are 52 patients with MSI/dMMR, and 338 with MSS/pMMR, including 261 with APC-mt and 77 with APC-wt. There are 152 patients with KRAS-mt and 186 patients with KRAS-wt. There are 194 patients with TP53-mt and 144 patients with TP53-wt. There were 45 patients with KRAS-/P53-/APC+/MSS, 55 patients with KRAS+/P53-/APC+/MSS, 71 patients with KRAS+/P53+/APC+/MSS, 11 patients with KRAS+/P53+/APC-/MSS, 15 patients with KRAS+/P53-/APC-/MSS, 90 patients with KRAS-/P53+/APC+/MSS, 22 patients with KRAS-/P53+/APC-/MSS, and 29 patients with KRAS-/P53-/APC-/MSS.

Of the 390 colon cancer patients with SNP and transcriptome data, 14.6% (57/ 390) were in stage I, 23.3% (91/390) were in stage II, 34.9% (136/ 390) were in stage III, and 13.9% (54/390) were in stage IV. The 52 MSI/dMMR colon cancer patients accounted for 2.3% (9/390) in stage I, 4.1% (16/390) in stage II, 5.4% (21/390) in stage III, and 1.5% (6/390) in stage IV. MSS/pMMR colon cancer patients in the database are mainly in stage II, while MSI/dMMR colon cancer patients are mainly in stage III. APC mutations were detected in 75% (293 of 390) of the tumors. In the APC-mt cases, the frequencies of KRAS and TP53 mutations were 48.0% and 60%, respectively. In the APC-wt cases, the KRAS and TP53 mutation rates were 33% and 42%, respectively. Moreover, the BRAF mutation rate was significantly increased by 36% in this group compared to the APC-mt group.

We found that APC mutations were strongly and negatively correlated with BRAF mutations and positively correlated with KRAS and TP53 mutations ($P \leq 0.05$).

Comparison of gene expression profile and different gene subtypes in colon cancer based on ImmuneScores, StromalScores, and tumor mutation burden (TMB)

To reveal the relationship between MSS/pMMR colon cancer overall gene expression profile and APC mutation, we compared the Affymetrix microarray data of all 338 colon cancer cases obtained from TCGA database. The heat map in Figure 2A shows the different gene expression profiles of APC-wt/MSS and APC-mt/MSS cases. In the APC gene mutation group, 379 genes were down-regulated and 117 genes were up-regulated (fold change > 1.5, $P < 0.05$; Supplementary Table 1). In MSS/pMMR colon cancer, the expression of immune checkpoint genes such as PD-1, PD-L1, and CTLA4 in the APC-mt/MSS group was significantly lower than in the APC-wt/MSS group. Compared with KRAS-wt/MSS group, the expression of CTLA4 and PD-L1 in the KRAS-mt/MSS group was down-regulated. The difference, however, was smaller than that between the APC-wt and APC-mt groups. The expression of PD-1, PD-L1, and CTLA4 did not differ between TP53-wt/MSS and TP53-mt/MSS groups. In addition, the ImmuneScore and StromalScore calculated by the ESTIMATE were significantly lower in the APC-mt/MSS group than in the APC-wt/MSS group. The ImmuneScore and StromalScore calculated for the KRAS-mt/MSS group were also lower than those in the KRAS-wt/MSS group. The range of the calculated scores was, however, smaller than that between the APC groups. The TP53-mt/MSS group had a lower ImmuneScore than that of the KRAS-wt/MSS group, but the difference was not so obvious. Compared with APC-wt/MSS group, the TMB was significantly lower in the APC-mt/MSS group than in the APC-wt/MSS group. Mutations in KRAS or TP53 did not affect the TMB. These results suggest that the proportion of immune-related components and expression of immune checkpoint are higher in the APC-wt/MSS colon cancer tumor microenvironment. The presence of APC-wt/MSS, combined with TMB, is consistent with this increase. It can, therefore, be presumed that APC-wt/MSS colon cancer could become a beneficiary of immune checkpoint inhibitors. A follow-up research will need to focus on further exploration in this direction.

Composition of immune cells in MSS/pMMR colon cancer with different genetic subtypes

We studied the proportion of immune cells infiltrating between different genetic subtypes in the colon cancer cohort retrieved from TCGA. All 338 MSS/pMMR colon cancer samples met CIBERSORT requirements at $P < 0.05$. The proportion of CD8+ T cells is significantly lower, and the proportion of M0 macrophages is significantly higher in APC-mt/MSS colon cancer in comparison to APC-wt/MSS. However, neither mutations in KRAS nor TP53 could affect the proportion of infiltrating immune cell types in MSS/pMMR colon cancer. Considering the results shown in Figure 3, APC-wt/MSS has a higher percentage of immune-related components infiltration in the tumor microenvironment compared to APC-mt/MSS colon cancer. Furthermore, through the application of univariate and multiple Cox regression analyses, we demonstrate that immune cell infiltration and APC gene status in CRC patients are related to PD-1 expression. In addition, assessment of TILs in the tumor microenvironment is challenging, immune cell cytolytic activity (CYT) might be used to assess TILs including CD8+ CTL and other immune cells (e.g., natural killer T cells). And CYT was measured by the mRNA expression levels of granzyme A (GZMA) and perforin 1 (PRF1) [15]. Figure3J and K showed that GZMA and PRF1 were significantly higher expression in APC-wt/MSS than APC-mt/MSS colon cancer. The immune-promoting lymphocyte infiltration ratio, such as CD8+ T cells, and the expression ratio of PD-1, the immune checkpoints, have significantly increased. It is speculated that APC-wt colon cancer is more likely to be a "hot tumor," and is more likely to benefit from anti-tumor immunotherapy.

Relationship between immune status and APC mutations in CRC patients

We divided the CRC samples in the cohort retrieved from TCGA into APC-wt (77 samples) and APC-mt (261 samples) groups and performed gene set enrichment analysis (GSEA). The results show that APC-wt colon cancer was significantly enriched in 115 KEGG pathways ($P < 0.05$; Supplementary Table S2-S3). These include pathways related to immune signaling, such as natural killer cell-mediated cytotoxicity, leukocyte transendothelial migration, NOD-like receptor signaling,

TOLL-like receptor signaling, TGF-β signaling, and other immune-related signaling pathways (Figure 4A). We then performed Gene Ontology (GO) analysis of the immune-related DEGs in APC-wt/MSS colon cancer. Circular plot of GO pathways was enriched by processes regulating leukocyte and T cell activation, and leukocyte cell−cell adhesion. These findings again indicate that APC mutations play a role in the immune response of colon cancer.

Calculation and validation of the ImmunoScore, and evaluation of its prognostic ability in the CRC cohort retrieved from TCGA

We have identified 65 overlapping genes (shown in Table S4) among the DEGs (496 genes related to APC status, shown in Supplementary Table S1) and the DEGs related to immunophenotypes (1297 genes shown in Supplementary Table S5). Using Lasso and Cox regression analyses, the eight genes with the highest prognostic value were identified (Figure 5A, B). We then selected these genes to establish an immune scoring model, which was assessed for its ability to predict the prognosis of CRC patients. The formula of the immune scoring model is described in the Materials and methods section. Next, we divided the CRC patients into a high score and low score groups based on an optimal cutoff value of the immune score obtained by the survminer R package. The results show that patients with a high score had a worse OS than those with a low score (Figure 5C). Figure 3b shows that the area under the ROC curve (AUC) of the 5-years OS prognostic model is 0.614. Figure 3c shows the immune score distribution and selected gene expression data.

Immunohistochemical expression of markers for TIMTs

The tumors of all 42 patients were immunopositive for markers of TIMT. 8/22 APC-mt/MSS colon cancer (36.3%) was immunopositive for CD8, and 12/20 APC-wt/MSS colon cancer (60%) was immunopositive for CD8. 5/22 APC-mt/MSS colon cancer (22.5%) was immunopositive for PD-L1, and 11/20 APC-wt/MSS colon cancer (55%) was immunopositive for PD-L1. In addition to the positive rate, the degrees of positive expression for CD8 and PD-L1, APC-wt/MSS is significantly higher than APC-mt/MSS with immunostaining. TIMTs divide tumors into four categories based on the presence or absence of TILs and PD-L1 expression levels (type I: TILs+ and

PD- L1+; type II: TILs- and PD-L1-; type III: TILs+ and PD- L1-; type IV: TILs- and PD-L1+). The 42 patients, containing APC-mt/MSS and APC-wt/MSS two groups, were divided into one of the above four types according to expression patterns of markers CD8 and PD-L1. 2/22 APC-mt/MSS colon cancer (9.1%) was immunopositive for both CD8 and PD-L1 (TIMT I). 6/22 APC-mt/MSS colon cancer (27.2%) was immunopositive for only CD8 (TIMT III). 3/22 APC-mt/MSS colon cancer (13.6%) was immunopositive for only PD-L1 (TIMT IV). 11/22 APC-mt/MSS colon cancer (50%) was immunonagtive for either CD8 or PD-L1 (TIMT II). 10/20 APC-wt/MSS colon cancer (50%) was immunopositive for both CD8 and PD-L1 (TIMT I). 2/20 APC-wt/MSS colon cancer (10%) was immunopositive for only CD8 (TIMT III). 1/20 APC-wt/MSS colon cancer (5%) was immunopositive for only PD-L1 (TIMT IV). 7/20 APC-wt/MSS colon cancer (35%) was immunonagtive for either CD8 or PD-L1 (TIMT II). Therefore, APC-wt/MSS mainly includes TIMT I type colon cancer, which was consistent with the statistical result in the TCGA database. No difference in clinical characteristics between the two groups. (Table 1)

Characteristics	APC-mt/MSS Patients	APC-wt/MSS Patients
Patients (n)	22	20
Age (years), median (range)	67.5（42-89）	66.4（43-89）
>60 years (n)	14	12
⩽60 years (n)	8	8
Sex (Male/Female) (n/n)	10/12	9/11
KPS score (%), median (range)	80（30-100）	80（20-100）
⩾60% (n (%))	18	17
<60% (n (%))	4	3
Stage I (n)	0	0
Stage II (n)	4	3
Stage III (n)	10	10
Stage IV (n)	8	7

Table 1. Clinical characteristics of patients with APC-mt/MSS and APC-wt/MSS

colon cancer

DISCUSSION

In the current work, we tried to identify relevant genes in TCGA database that could help clarify the status of the tumor immune microenvironment. We calculated TMB, immune scores, and immune checkpoints by comparing the commonly mutated genotypes in colon cancer. We then screened them as biomarkers that might predict the efficacy of immunotherapy for MSS/pMMR colon cancer. Further analysis of the infiltrating immune cell types revealed a higher proportion of CT8+ T cells, immune components infiltration, and immune checkpoint expression in APC-wt/MSS colon cancer. A four-tiered classification for tumor microenvironment immune type (TMIT) has been proposed to describe the patient's immune status and to determine immunotherapy-responsive subgroups [16-18]. The four TMIT types are defined as follows: Type I, PD-L1-positive with TIL(CD8/CYT-positive) (adaptive immune resistance); Type II, PD-L1-negative with no TIL (immune ignorance); Type III, PD-L1-positive with no TIL (intrinsic induction); and Type IV, PD-L1-negative with TIL (possible role of other suppressors in producing immune tolerance) [19-21]. It seems that APC-wt/MSS colon cancer is TMIT1, in which immunotherapy is expected to be more effective.

First, we found on TCGA database 390 colon cancer samples with transcriptome and gene sequencing. Among them, 338 were of MSI/dMMR colon cancer, which comprised of 261 APC-mt and 77 APC-wt. We thus divided the cases into APC-mt/MSS and APC-wt/MSS groups. We then compared the expression level of PD-L1, PD-1, CTLA4, and CYT between the groups. The ImmunoScores and StromaScore, calculated by ESTIMATE, were compared between the two groups. CIBERSORT was used to calculate the ratio of infiltrating immune cells between the two groups. The sequencing results of the two groups were used to calculate the TMB for comparison. We further analyzed and compared the 496 differentially-expressed genes between the APC-wt/MSS and APC-mt/MSS groups, and found that many of them are related to the tumor microenvironment, as was shown by the GO and KEGG terminology

analyses. Out of these, we performed Cox regression analysis on the 65 immune-related DEGs, and found eight genes that are associated with survival. On these eight genes, we performed the Lasso analysis to achieve risk stratification, and conducted a survival analysis for the high- and low-risk groups (Figure 7). In addition, we selected 42 patients' colon cancer tissues, including 22 cases of APC-mt/MSS and 20 APC-wt/MSS, to performed immunohistochemistry to further confirm that APC-wt/MSS contained more TIMIT type I tumors.

APC is a protein that assists in inactivating β-catenin in the Wnt pathway [22]. When it is mutated, it can cause abnormal activation of the Wnt pathway. The Wnt pathway mainly involves the regulation of tumor stem cells [23]. A primary mechanism for the tumor's escape from the surveillance of the immune system is related to the minimization of antigenicity [24]. This is achieved by down-regulating key components of the cellular antigen processing and presentation mechanisms [25]. Studies have confirmed that down-regulation of the antigen processing and presentation mechanisms provides cancer stem cells with a way to evade T cell-mediated immune responses [26]. Other studies have confirmed that by mediating the functional inhibition of anti-tumor T cells, expression of the bifunctional ligand CD80 regulates the immune avoidance of cancer stem cells in different tumor types [27]. Theoretically, these mechanisms provide support to the conclusions of this study.

Conclusions

In summary, from analysis of the data retrieved from TCGA database, using immune scores based on the ESTIMATE and CIBERSORT algorithms, we found that APC-wt/MSS colon cancer is more likely to be a TIMT I tumor and these findings have been partially confirmed in the tissues of colon cancer patients. Therefore, we speculate patients with this type of tumor could potentially benefit from immunotherapy.

Legend

Figure 1. Genomic landscape and clinicopathological findings in CRC samples, based on APC status in the cohort retrieved from TCGA (The Cancer Genome Atlas). (A) Frequency and type of

mutations in the top 30 CRC-associated genes. Genes were sorted according to the frequency of mutations. (B) Interactions among mutations in the top 25 genes in CRC. (C, D) Summary of frequency and classification of mutations in the top 10 CRC-associated genes, presenting top gene mutations cloud panorama. (E, F) The pie chart indicates the stage of the tumor and the common oncogene mutation ratio. (G, H) Summary of frequency and classification of mutations in the top 10 genes in APC-mt/MSS CRC and APC-wt/MSS CRC, respectively.

Figure 2. ImmuneScore, stromalScore, and TMB are associated with APC mutation status. (A) Heatmap of the differentially expressed genes (DEGs) of APC-wt/MSS vs. APC-mt/MSS ($P < 0.05$, fold change > 1.5). (B-D) Expression of CTLA4, PD-1, and PD-L1 in MSS/pMMR colon cancer with different gene subtypes. From top to bottom, mutations in APC, KRAS, and TP53. (E) Distribution of TMB for APC, KRAS, and TP53 combined gene profile. (F) Distribution of TMB in MSS/pMMR colon cancer with different gene subtypes. From left to right, mutations in APC, KRAS, and TP53. A box-plot showing that there is a significant association between APC mutation status and TMB ($n = 338$, $P = 0.0011$). (G) Distribution of ESTIMATE score for APC, KRAS, and TP53 combined gene profile. (H) Distribution of ESTIMATE score for MSS/pMMR colon cancer with different genetic subtypes. From left to right, mutations in APC, KRAS, and TP53. A box-plot showing that there is a significant association between APC and KRAS mutation status and ESTIMATE score ($n = 338$, $P = 6.708e{-}07$ and $P = 0.004$, respectively). (I) Distribution of ImmuneScore for APC, KRAS, and TP53 combined gene profile. (J) Distribution of ImmuneScore of MSS/pMMR colon cancer with different genetic subtypes. From left to right, mutations in APC, KRAS, and TP53. A box-plot showing that there is a significant association between APC, KRAS, and TP53 mutation status and ImmuneScore ($n = 338$, $P = 9.331e{-}09$, $P = 0.008$, and $P = 0.003$, respectively). (K) Distribution of StromalScore for APC, KRAS, and TP53 combined gene profile. (L) Distribution of StromalScore for MSS/pMMR colon cancer with different genetic subtypes. From left to right, mutations in APC, KRAS, and TP53. A box-plot showing that there is a significant association between APC and KRAS mutation status and StromalScore ($n = 338$, $P = 2.38e{-}04$ and $P = 0.009$, respectively).

Figure 3. Composition of infiltrated immune cells in association with different genetic subtypes in the cohort retrieved from TCGA. The CIBERSORT tool deemed all samples eligible at $P < 0.05$. Twenty different immune cells were filtered and analyzed in the cohort retrieved from TCGA. (A)

Fractions of immune cells in the 338 MSS/pMMR colon cancer samples from TCGA. (B) Interaction among the 20 different immune cells in MSS/pMMR colon cancer. (C-D) Comparisons of immune cells between APC-mt/MSS and APC-wt/MSS colon cancer tissues from TCGA. (E-F) Comparisons of immune cells between KRAS-mt/MSS and KRAS-wt/MSS colon cancer tissues from TCGA. (G-H) Comparisons of immune cells between TP53-mt/MSS and TP53-wt/MSS colon cancer tissues from TCGA. (I) Forest plots showing an association between different immune cell subsets and PD-1 expression in the cohort retrieved from TCGA. Figure3J and K showed that GZMA and PRF1 were significantly higher expression in APC-wt/MSS than APC-mt/MSS colon cancer. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4. Genomic landscape and gene set enrichment analysis of the CRC samples, based on APC status in the cohort retrieved from TCGA (The Cancer Genome Atlas). (A) The immunity and cancer pathways that are significantly enriched in APC-wt CRC patients, compared with those in APC-mt CRC patients. (B-C) Gene Ontology (GO) analysis of the immune-related DEGs. Left: Circular plot of GO pathways enrich in APC-wt/MSS samples. Right: GO pathways cluster distribution. (D-E) GO analysis of the immune-related DEGs. Immune-related DEGs in the significantly enriched immunologic and cancer biological processes. (F-G) Kyoto encyclopedia of genes and genomes (KEGG) analysis of immune-related DEGs.

Figure 5. Immune-related DEGs and construction of the ImmunoScore model. (A-B) Lasso coefficient profiles of 13 genes were related to prognosis. The optimal values of the penalty parameter λ were determined by ten-fold cross-validation. (C-E) Patients were stratified based on low or high ImmunoScore (low or high score). Kaplan-Meier curves, heatmap, and time-dependent ROC curve in the cohort retrieved from TCGA. (* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

Figure 6. Histology (H&E: hematoxylin and eosin) and immunohistochemistry showing four different expression patterns of CD8 and PD-L1 in the representative case. We identifed patients with 22 APC-mt/MSS (left) and 20 APC-wt/MSS (right) colon cancer. APC-wt/MSS is significantly higher than APC-mt/MSS in the positive rate and the degrees of positive expression for CD8 and PD-L1 with immunostaining.

Figure 7. Work flow of the current work

MATERIALS AND METHODS

Database

The somatic mutation status data (identified by VarScan2), gene expression data, and corresponding clinical information of CRC were downloaded from The Cancer Genome Atlas (TCGA) website (https://portal.gdc.cancer.gov/repository). 390 samples with RNA-sequencing data and somatic mutation status data were subjected to further study.

Immune scores and stromal scores

Immune scores and stromal scores were calculated by applying the ESTIMATE algorithm.

Identification of differentially expressed genes (DEGs)

Package limma was used to perform data analysis. Cutoffs were set as fold change > 1.5 and adj. $p < 0.05$ to screen for differentially expressed genes (DEGs).

Overall survival curve

Kaplan-Meier plots were used to illustrate the relationship between gene expression levels of DEGs and patients' overall survival. The relationship was tested by log-rank test.

Enrichment analysis of DEGs

Functional enrichment analysis of DEGs was performed to identify GO categories by their molecular functions (MF), biological processes (BP), or cellular components (CC). Pathway enrichment was analyzed with reference from KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. False discovery rate (FDR) < 0.05 was used as the cut-off.

Estimation of Immune Cell Type Fractions

The CIBERSORT was used to quantify the proportion of immune cells in CRC samples from microarray data. The normalized gene expression data was analyzed using the CIBERSORT algorithm, and there were 1,000 permutations. The CIBERSORT p value reflects the statistical significance of the results; the recommended threshold is <0.05.

Construction and Validation of an Immunoscore Prognostic Model

Using the univariate Cox proportional hazards regression model, we calculated the risk proportion of DEGs in the GEO cohort. We analyzed DEGs with $p < 0.05$ and used LASSO to screen out the most useful prognostic genes in DEGs. Establish an immune score model to predict the patient's survival formula: immune score = gene

Xi's S Cox coefficient × gene Xi scale expression value

Immunohistochemistry

Immunohistochemical expression of TIMT markers (CD8 and PD-L1) were investigated in all patients. Each tumor sample was fxed in formalin and embedded in paraffn. The blocks were sliced into 5 μm-thick sections,which were deparaffnized in Histo-Clear (Cosmo Bio), hydrated in a graded series of alcohols, and subjected to heat-activated antigen retrieval. After blocking endogenous peroxidase activity, the tissue was incubated with CD8 (rabbit monoclonal antibody; ab237709; abcam; ready to use) and PD-L1(rabbit monoclonal antibody; ab237726; abcam) antibodies for 4 hours at room temperature. Subsequently, the sections were washed and incubated with biotinylated secondary antibody for 30 minutes at room temperature. The reaction complexes were visualized with diaminobenzidine and counterstained with hematoxylin.

Statistical Analysis

The two normally distributed variables used the unpaired t test to estimate the statistical significance of. Use the survminer package to evaluate the best cut-off value based on the association between the overall survival and immune score of each data set. Univariate Cox proportional hazards regression model was used to calculate the hazard ratio of univariate analysis. In order to select the most useful prognostic genes, we applied the LASSO Cox regression algorithm to the genes related to the prognosis. Receiver operating characteristic (ROC) was used to describe the sensitivity and specificity of survival prediction based on immune score, and timeROC R package was used to quantify the area under the curve (AUC). Subgroup survival curves were generated by Kaplan-Meier method, and Log-rank test showed statistically significant differences. Multivariate Cox regression analysis determined independent prognostic factors; only patients with comprehensive clinical data were included. All statistical analyses were performed using R software. All statistical tests are two-tailed tests, $p < 0.05$ is considered statistically significant.

Ethics approval and consent to participate

Declarations

Ethics approval and consent to participate

Ethics approved by Bioethics Committee. The approval number is 2020-P2-154-01.

Consent for publication

Not applicable.

Availability of data and materials

The data generated during this study are available from the corresponding author upon reasonable request. The data that support the findings of this study are available in The Cancer Genome Atlas (TCGA) website.These data were derived from the following resources available in the public domain: https://portal.gdc.cancer.gov/repository.

Competing interests

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

Conceptualization, H.L., H.C.; Methodology, T.M, K.S.; Investigation, H.L.; Funding Acquisition, B.C.; Resources, B.C.; Supervision, H.L. and B.C..

ACKNOWLEDGEMENTS

The authors thank Dr. Bing Cao for editing the manuscript.

FUNDING

This work was supported by the start-up fund from the Digestive Medical Coordinated Development Center of Beijing Hospitals Authority [No：XXT01 to B.C.], the The capital health research and development of special [2018-2-2022 to B.C.], the Beijing key clinical specialty [2018-2020 to B.C.], the pilot project of clinical collaboration with traditional Chinese medicine and western medicine in major refractory disease—esophageal cancer [to B.C.]

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Figures

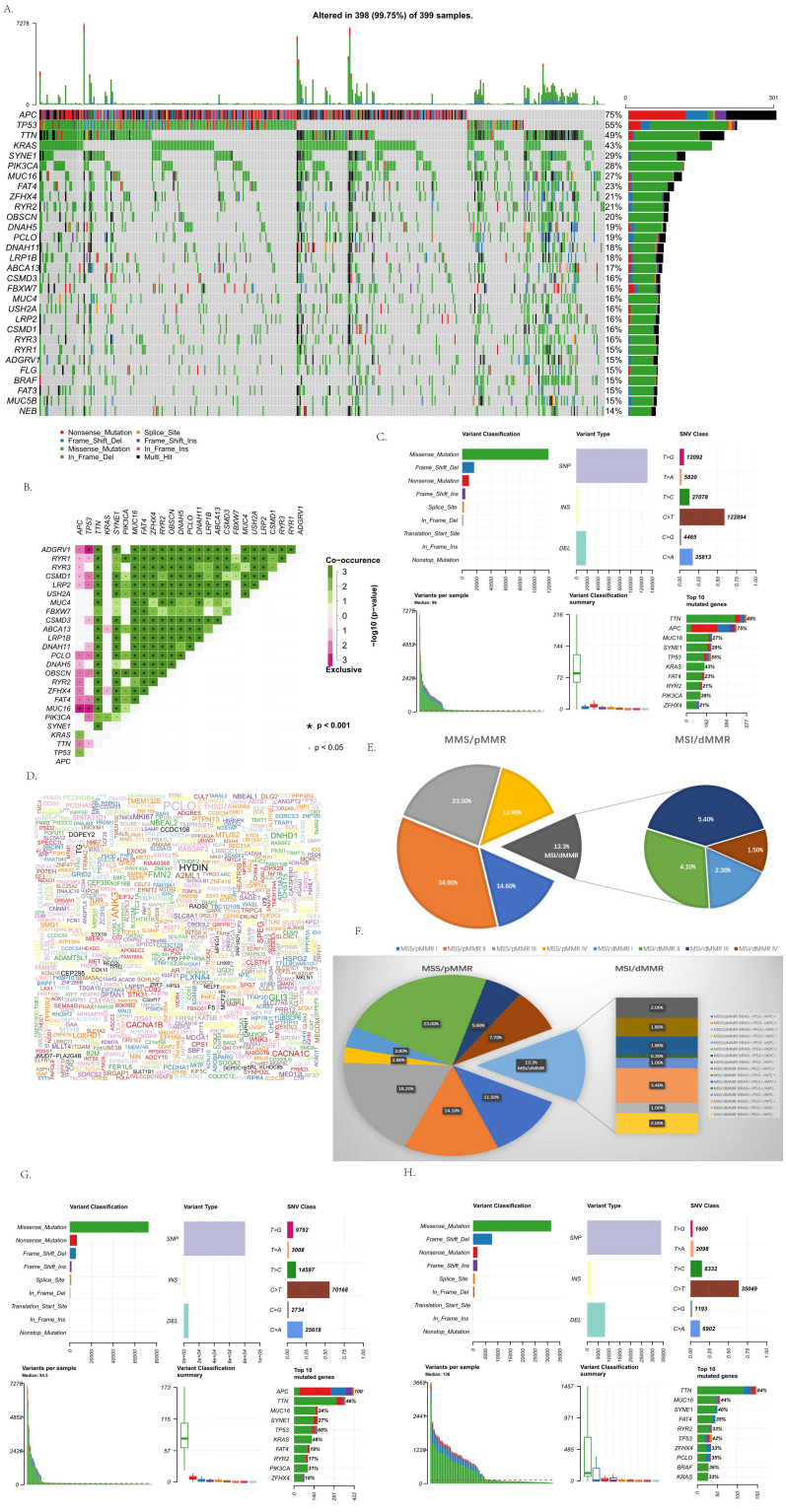

Figure 1

Genomic landscape and clinicopathological findings in CRC samples, based on APC status in the cohort retrieved from TCGA (The Cancer Genome Atlas). (A) Frequency and type of mutations in the top 30 CRC-associated genes. Genes were sorted according to the frequency of mutations. (B) Interactions among

mutations in the top 25 genes in CRC. (C, D) Summary of frequency and classification of mutations in the top 10 CRC-associated genes, presenting top gene mutations cloud panorama. (E, F) The pie chart indicates the stage of the tumor and the common oncogene mutation ratio. (G, H) Summary of frequency and classification of mutations in the top 10 genes in APC-mt/MSS CRC and APC-wt/MSS CRC, respectively.

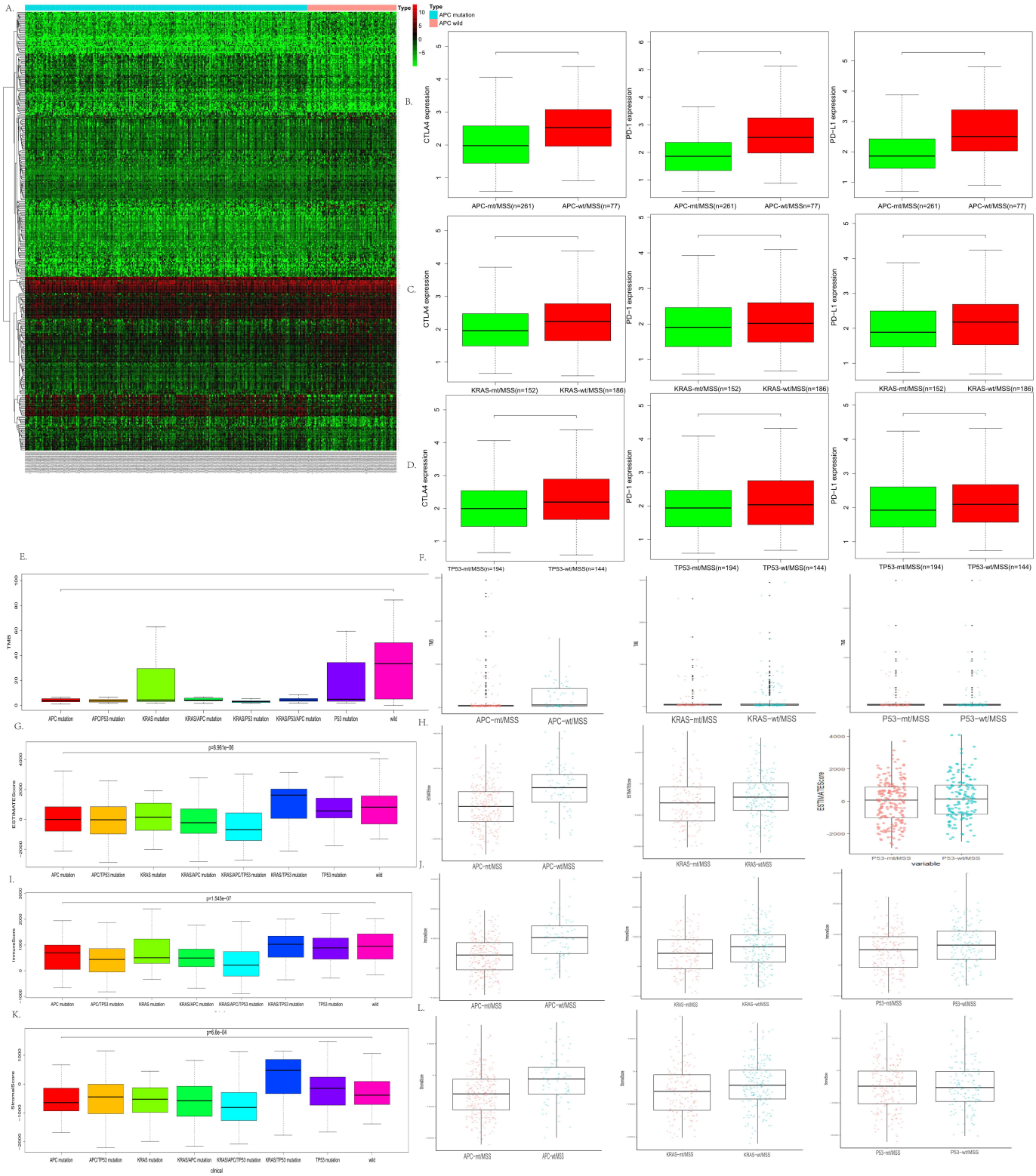

Figure 2

ImmuneScore, stromalScore, and TMB are associated with APC mutation status. (A) Heatmap of the differentially expressed genes (DEGs) of APC-wt/MSS vs. APC-mt/MSS ($P < 0.05$, fold change > 1.5). (B-D) Expression of CTLA4, PD-1, and PD-L1 in MSS/pMMR colon cancer with different gene subtypes. From top to bottom, mutations in APC, KRAS, and TP53. (E) Distribution of TMB for APC, KRAS, and TP53 combined gene profile. (F) Distribution of TMB in MSS/pMMR colon cancer with different gene subtypes. From left to right, mutations in APC, KRAS, and TP53. A box-plot showing that there is a significant association between APC mutation status and TMB ($n = 338$, $P = 0.0011$). (G) Distribution of ESTIMATE score for APC, KRAS, and TP53 combined gene profile. (H) Distribution of ESTIMATE score for MSS/pMMR colon cancer with different genetic subtypes. From left to right, mutations in APC, KRAS, and TP53. A box-plot showing that there is a significant association between APC and KRAS mutation status and ESTIMATE score ($n = 338$, $P = 6.708e{-}07$ and $P = 0.004$, respectively). (I) Distribution of ImmuneScore for APC, KRAS, and TP53 combined gene profile. (J) Distribution of ImmuneScore of MSS/pMMR colon cancer with different genetic subtypes. From left to right, mutations in APC, KRAS, and TP53. A box-plot showing that there is a significant association between APC, KRAS, and TP53 mutation status and ImmuneScore ($n = 338$, $P = 9.331e{-}09$, $P = 0.008$, and $P = 0.003$, respectively). (K) Distribution of StromalScore for APC, KRAS, and TP53 combined gene profile. (L) Distribution of StromalScore for MSS/pMMR colon cancer with different genetic subtypes. From left to right, mutations in APC, KRAS, and TP53. A box-plot showing that there is a significant association between APC and KRAS mutation status and StromalScore ($n = 338$, $P = 2.38e{-}04$ and $P = 0.009$, respectively).

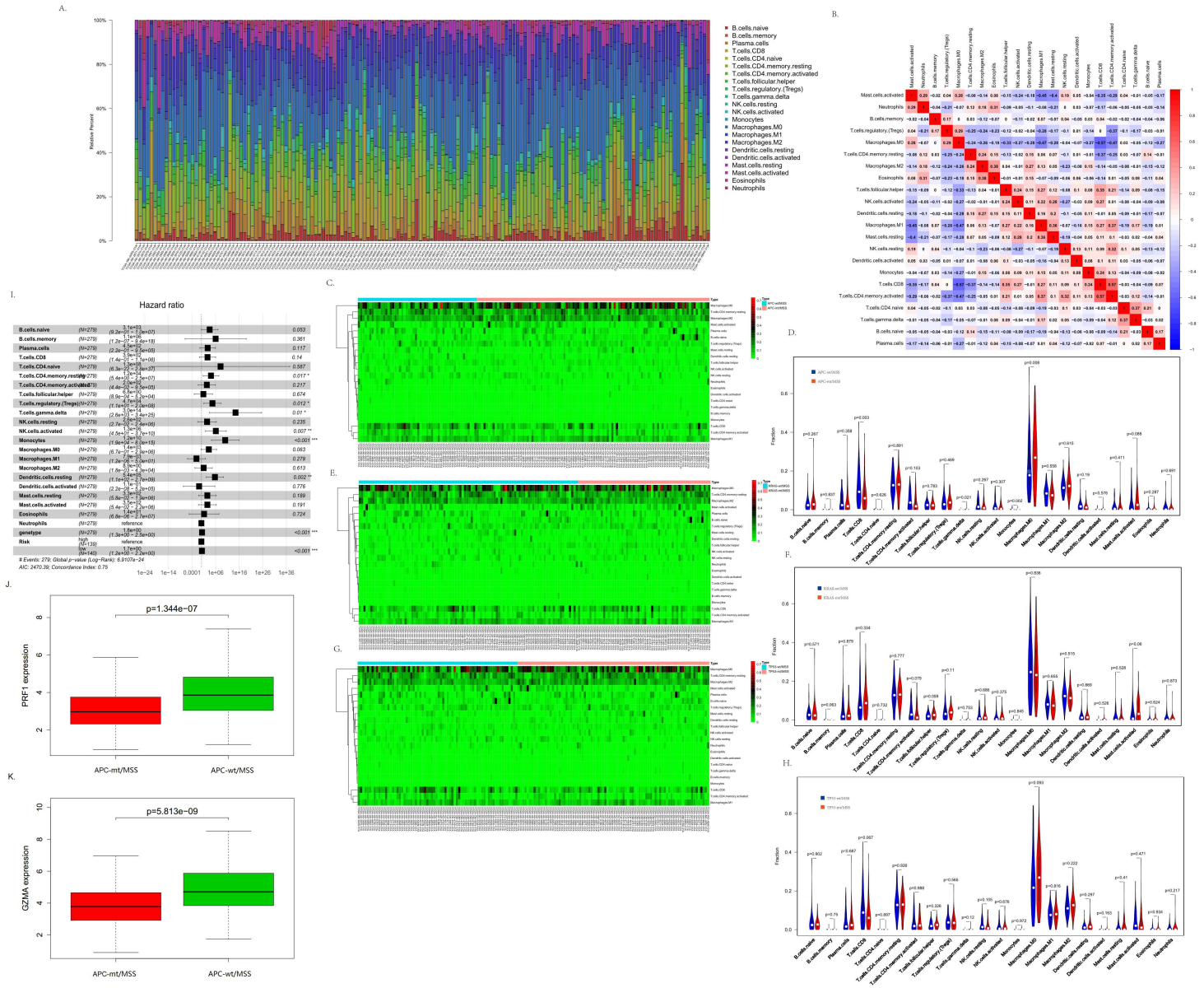

Figure 3

Composition of infiltrated immune cells in association with different genetic subtypes in the cohort retrieved from TCGA. The CIBERSORT tool deemed all samples eligible at P < 0.05. Twenty different immune cells were filtered and analyzed in the cohort retrieved from TCGA. (A) Fractions of immune cells in the 338 MSS/pMMR colon cancer samples from TCGA. (B) Interaction among the 20 different immune cells in MSS/pMMR colon cancer. (C-D) Comparisons of immune cells between APC-mt/MSS and APC-wt/MSS colon cancer tissues from TCGA. (E-F) Comparisons of immune cells between KRAS-mt/MSS and KRAS-wt/MSS colon cancer tissues from TCGA. (G-H) Comparisons of immune cells between TP53-mt/MSS and TP53-wt/MSS colon cancer tissues from TCGA. (I) Forest plots showing an association between different immune cell subsets and PD-1 expression in the cohort retrieved from TCGA. Figure3J and K showed that GZMA and PRF1 were significantly higher expression in APC-wt/MSS than APC-mt/MSS colon cancer. *P < 0.05, **P < 0.01, ***P < 0.001.

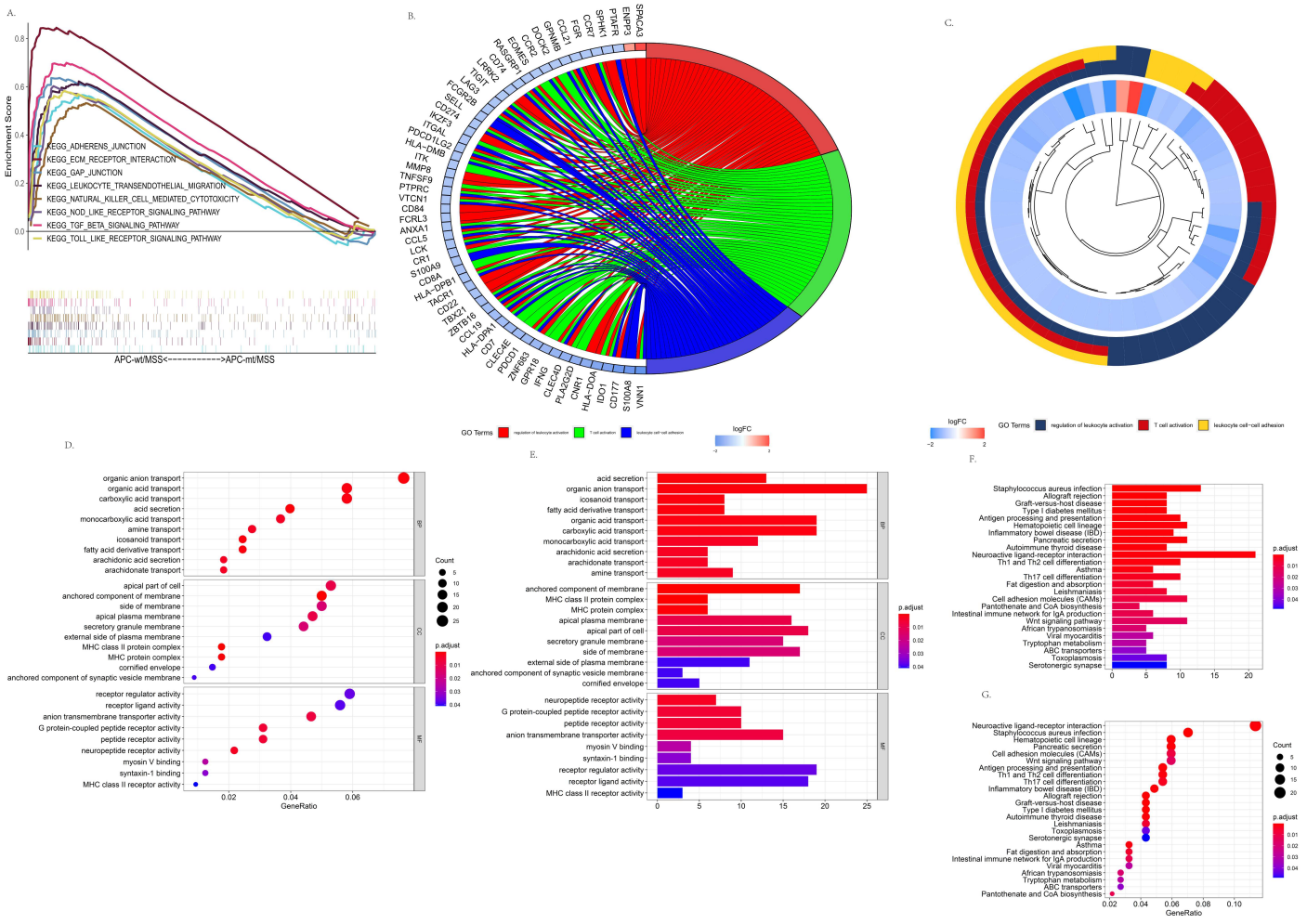

Figure 4

Genomic landscape and gene set enrichment analysis of the CRC samples, based on APC status in the cohort retrieved from TCGA (The Cancer Genome Atlas). (A) The immunity and cancer pathways that are significantly enriched in APC-wt CRC patients, compared with those in APC-mt CRC patients. (B-C) Gene Ontology (GO) analysis of the immune-related DEGs. Left: Circular plot of GO pathways enrich in APC-wt/MSS samples. Right: GO pathways cluster distribution. (D-E) GO analysis of the immune-related DEGs. Immune-related DEGs in the significantly enriched immunologic and cancer biological processes. (F-G) Kyoto encyclopedia of genes and genomes (KEGG) analysis of immune-related DEGs.

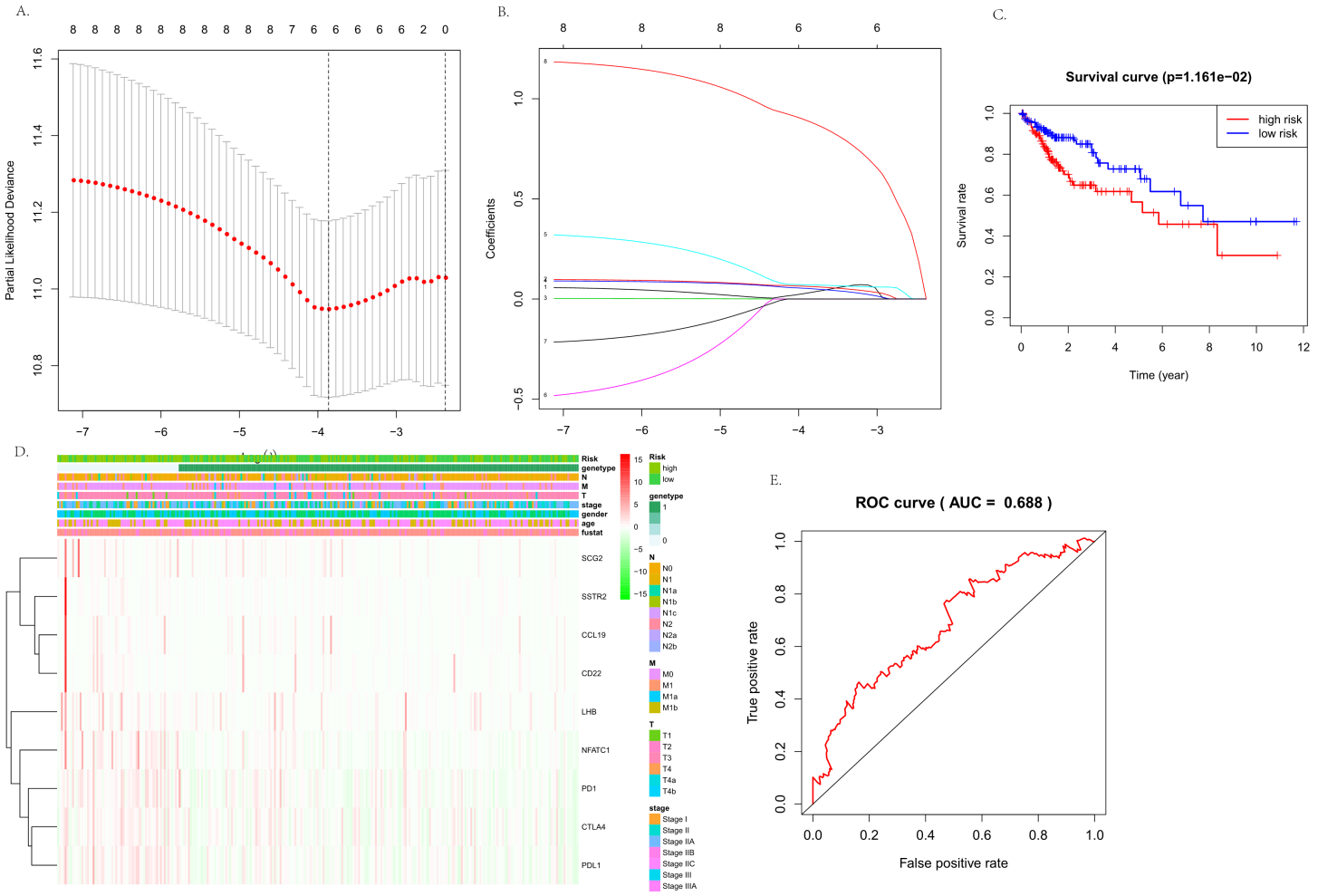

Figure 5

Immune-related DEGs and construction of the ImmunoScore model. (A-B) Lasso coefficient profiles of 13 genes were related to prognosis. The optimal values of the penalty parameter λ were determined by ten-fold cross-validation. (C-E) Patients were stratified based on low or high ImmunoScore (low or high score). Kaplan-Meier curves, heatmap, and time-dependent ROC curve in the cohort retrieved from TCGA. (* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

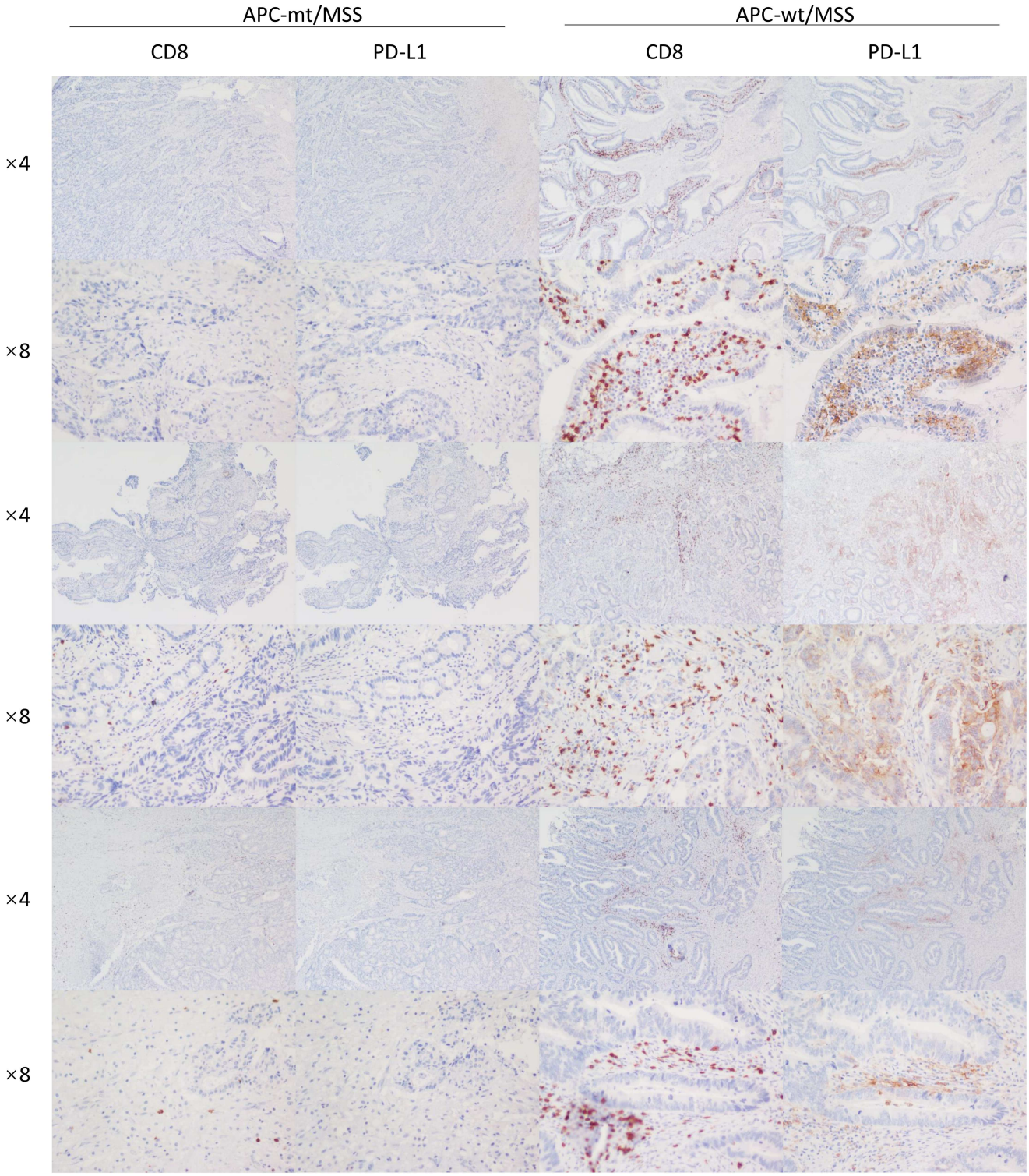

Figure 6

Histology (H&E: hematoxylin and eosin) and immunohistochemistry showing four different expression patterns of CD8 and PD-L1 in the representative case. We identifed patients with 22 APC-mt/MSS (left) and 20 APC-wt/MSS (right) colon cancer. APC-wt/MSS is significantly higher than APC-mt/MSS in the positive rate and the degrees of positive expression for CD8 and PD-L1 with immunostaining.

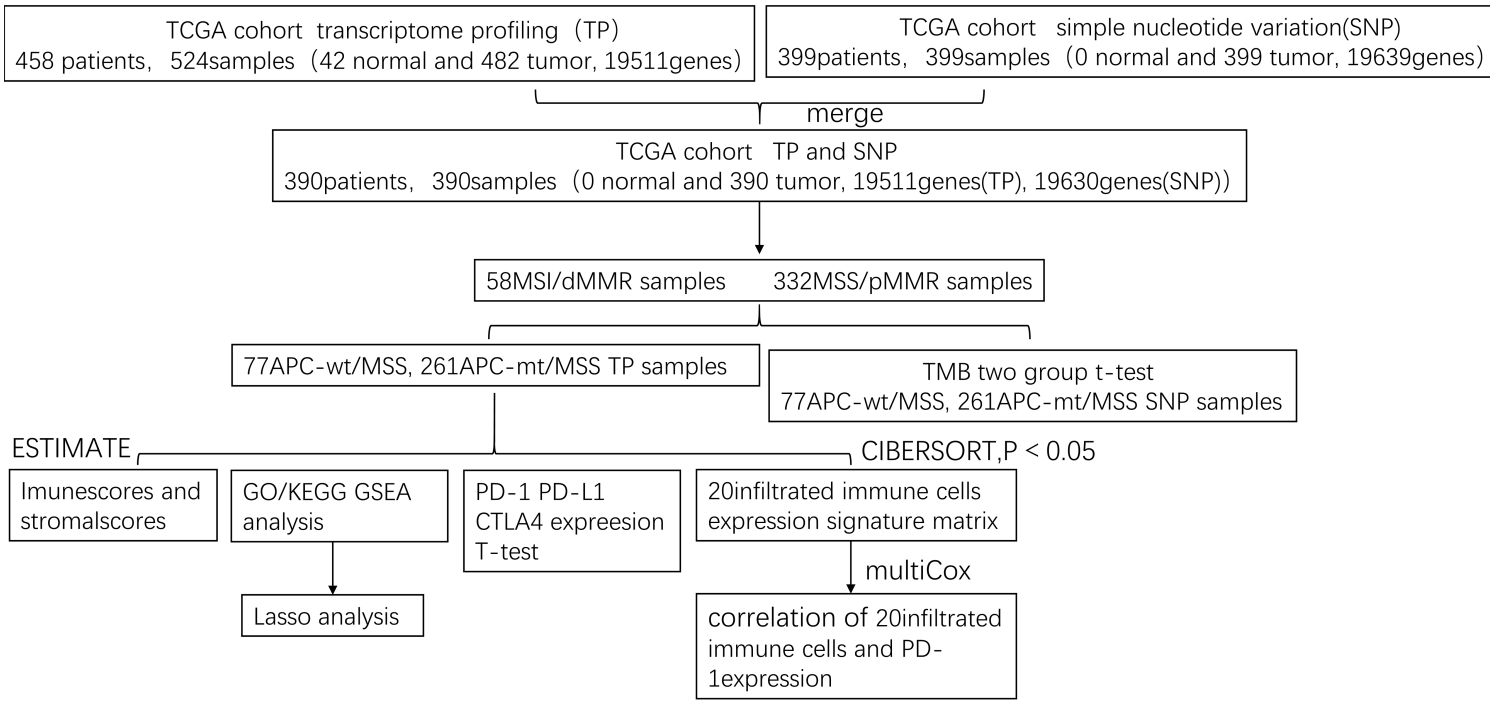

Figure 7

Work flow of the current work

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary1MSSpMMRAPCImmuneDiff.xls
- Supplement4MSSAPCIMMUNEEXP.xlsx
- Supplement3GESADOWN.xls
- Supplement2GESAHIGH.xls
- Supplement5tcgaImmuneExp.xlsx