Exploration of the role of tumor mutation burden in clinical significance, immunotherapy response predictor and immune cell infiltration in colon cancer

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Abstract

Background:

Tumor mutation burden has become a powerful bio-marker to predict prognosis and immunotherapy responsiveness to patients in various cancers, but the role of TMB in colon cancer is still unclear.

Methods:

The transcriptome profiling data of colon patients and the simple nucleotide variation data of colon cases were downloaded from the Cancer Genome Atlas (TCGA) database. The groups were divided into high TMB and low TMB group according to the median of TMB. Then we explored the relationship between immune checkpoints, immune cells and TMB, respectively.

Results:

Mutation profiles of 399 colon cancer samples were analyzed in TCGA database. The senior (age ≥ 65) had a strong relationship with higher-TMB level (p = 0.001). Low-TMB group correlated with advanced N stage (P = 0.001), M stage (P = 0.001), and pathologic stage (P = 0.001). High-TMB group had significantly higher mRNA level of PD-L1, TIGIT, HAVCR2, and LAG3 than low-TMB group, which indicated high-TMB referred to better immunotherapy responsiveness in colon cancer. And high-TMB level correlated with higher fractions of CD8 T cells (p = 0.021), higher CD4 memory T cells (p = 0.039), follicular helper T cells (p = 0.002) and M1 macrophages (p = 0.001), while the low-TMB groups correlated with higher regulator T cells (p = 0.002). So high-TMB correlated with stronger immune cell infiltration.

Conclusions:

The high TMB referred to better clinical pathologic features, better immunotherapy responsiveness and stronger immune cells infiltration in colon cancer. Hence TMB may be a very promising bio-marker to predict prognosis and immunotherapy responsiveness to patients in colon cancer.

Introduction

Colorectal cancer (CRC) is the second prevalent cancer and the fourth leading cause of cancer-related lethality globally. Traditionally, surgical resection, chemotherapy, has obvious limitations in recurrence and distant metastasis. Recently, immunotherapy seems to be the potential and effective treatment in various cancers, including colon cancer. However, only about 20% of patients with cancer have good response to immunotherapy. Because of the scarcity of bio-mark applied to predict the efficacy of immunotherapy in colon cancer, there is a compelling need to find out who could be the candidates to receive immunotherapy.
Researchers find the phenomenon CRC tumor of microsatellite instability (MSI) phenotype express more neo-antigen and are enriched with more immune cells, which activate antitumor responses. Hence CRC patients with microsatellite stability (MSS) significantly correlate with poorer immune cell infiltration and worse prognosis than CRC patients with MSI\textsuperscript{12}. MSI in cancers is characterized by a tumor mutation burden (TMB), defined as the number of DNA damages, including somatic single variant (SNV), insertions, deletions and frameshift mutations\textsuperscript{12,13}. Previous studies demonstrated TMB was strong relative to immune checkpoint inhibitors (ICI) and immune cell infiltration in various cancers\textsuperscript{14-18}, which are heavily associated with response to immunotherapy and tumor behavior\textsuperscript{19-21}.

Given the importance of TMB and less effective immunotherapy in CRC, it is urgent to explore the role of tumor mutation burden in clinical significance, immunotherapy response predictor, and immune cell infiltration in colon cancer.

**Materials And Methods**

**Getting transcriptome profiling data, simple nucleotide variation and clinical data**

Our data was based on the Cancer Genome Atlas (TCGA) database via the GDC data portal (https://portal.gdc.cancer.gov/). We downloaded the transcriptome profiling, simple nucleotide variation and clinical data of colon cancer in the Data Category as the data source, and choose “VarScan2” software from the “Masked Somatic Mutation”. Then, we got 471 tumor samples from the transcriptome profiling and the analysis result of simple nucleotide variation which was Genecloud, interaction, summary and warterfall maps by the “maftools” package. Besides, the clinical data was related to the case of colon cancer, comparing with age, gender, TNM grouping and stage.

**Analysis of TMB**

We got the data of simple nucleotide variation from TCGA database and use “VarScan2” software to get each gene exact value of TMB (total number of mutations per megabase in colon cancer). We divided TMB into high and low expression group by median. Then using “limma” package, we explored the relationship between TMB and clinical basal information, such as age, gender, TNM grouping and stage.

**Immune checkpoints and TMB**

Immune checkpoints are the predictor of the efficacy of immunotherapy. In this study, we tied to explore the association between TMB and the mRNA level of immune checkpoints (PDL-1, TIGIT, LAG3, HAVCR2).

**Gene Set Enrichment Analysis (GSEA) and TMB**

Via “org.Hs.eg.db” package, we can know the symbol ID to analyze. Then GSEA analysis was performed between high- TMB group and low-TMB group by Molecular Signatures Database v7.1 (http://software.broadinstitute.org/gsea/msigdb/).
Immune cells and TMB

By removing extreme low expression genes, we used CIBERSORT to get the immune cells via "limma" and "preprocessCore" packages. Obviously, we get Histogram of immune cell content (p=0.05), after that we also compared different immune cells in high- TMB group and low-TMB group by "vioplot" and "limma" packages.

Statistical analysis

We also used the “Limma” package, which is data analysis, linear models and differential expression for microarray data. We conducted the association between TMB and immune checkpoints via Student Test in Graphpad 8.0. Wilcoxon test was applied to compare data between different groups. All P-values were two tailed, and P<0.05 was considered statistically significant. Last, all statistical analysis was run on R x64(Version 3.9.6).

Results

Landscape of mutation profiles in colon cancer

We loaded the results of whole-exome sequencing of 471 patients with colon cancer and the simple nucleotide variation data of 433 colon cases from the Cancer Genome Atlas (TCGA) database, and mutation profiles of 399 colon cancer samples were analyzed in TCGA database. Then we used the “maftools” package to visualize and classify the mutation profiles. In summary, missense mutation was the most fraction, SNP occurred most frequently, and C>T tranversion accounted for the most common type of SNV in colon cancer. We also showed the number of altered bases per sample and the mutation type (Figure 1A). Furthermore, we found out the top 10 mutated genes in colon cancer, including APC (75%), TP53 (55%), TTN (49%), KRAS(43%), SYNE1(29%), PIK3CA(28%), MUC16(28%), FAT(23%), ZFHX4(21%), RYR2(21%). Meanwhile, the frequencies of all mutated genes were presented by genecloud in Figure S1 and the main interactions across them were shown in Figure S2. At last we exhibited mutation information of the top 30 mutated genes in each sample (Figure 1B).

TMB correlated with clinical relevance and immune checkpoint inhibitor

After calculating the frequencies of mutations per million bases as the TMB for 399 patients with colon cancer, we divided the patients into high-TMB group and low-TMB group by the cutoff value, which is the median number of TMB. Interestingly, the senior (age≥65) had a strong relationship with higher-TMB level(p=0.001) (Figure 2A), which made it hard to analysis the overall survival between high-TMB group and low-TMB group with Kaplan-Meier plotter. Obviously, low-TMB group correlated with advanced N stage (P<0.001), M stage (P<0.001), and pathologic stage(P<0.001), which indicated low-TMB group had a poorer survival for patients with colon cancer (Figure 2D-F). There were no significant differences in association of TMB with gender and T stage (Figure 2B-C).
High-TMB group had significantly higher CD274 (P=0.001), HAVCR2 (P=0.001), and LAG3 (P=0.001), TIGIT (P=0.001) than low-TMB group in Figure 3. Hence high-TMB group might be more sensitive to immune checkpoint inhibitor.

**Association of TMB and immune cell infiltration**

We conducted GSEA analysis between high-TMB group and low-TMB group, and we found the top items (|NES|<2, FDR<0.05, p<0.01), including antigen processing and presentation (Figure 4A), natural killer cell mediated cytotoxicity (Figure 4B), autoimmune thyroid disease (Figure 4C), graft versus host disease (Figure 4D). Because the results of GSEA had a correlation with immune signature, we performed immune cells analysis. We presented 22 specific immune fractions in each colon cancer sample in Figure 5A. And CIBERSORT algorithm was performed to calculate the 22 specific immune fractions between high-TMB group and low-TMB group in Figure 5B. We found high-TMB level correlated with higher fractions of CD8T cells (p=0.021), higher CD4 memory T cells (p=0.039), follicular helper T cells (p=0.002) and M1 macrophages (p=0.001), while the low-TMB groups correlated with higher regulator T cells (p=0.002). So compared with the low-TMB group, the high TMB referred to stronger immune cells infiltration in colon cancer.

**Discussion**

Tumor genesis and development follows a complex and multistep process, involving somatic mutations in genome\(^\text{22-24}\). Somatic mutations lead to a change in amino acids which may increase the number of neo-antigens theoretically, which could activate the immune system and strengthen the immune cell infiltration. The change in immune microenvironment could affect immunotherapy response and have an impact of prognosis in patients with different cancers\(^\text{25-28}\). Based on these, TMB is becoming a hot biomarker for various cancers\(^\text{29-36}\). However, the role of TMB in colon cancer is still undefined\(^\text{14,34,36,37}\). In this study, we firstly and comprehensively explored the role of tumor mutation burden in clinical significance, immunotherapy response predictor, and immune cell infiltration in colon cancer using TCGA database.

We demonstrated that APC, TP53, PIK3CA and KRAS mutated high frequently in colon cancer, which were similar to previous studies\(^\text{38}\). And APC, P53 and KRAS mutations play a vital role in the colon tumorigenesis\(^\text{38}\). We firstly found the older were more likely to be with high TMB. Furthermore, we demonstrated low-TMB group correlated with advanced N stage, M stage, and pathologic stage, which indicated low-TMB group had a poorer survival than high-TMB group for patients with colon cancer. Lee et al also reported that TMB-high was an independent positive prognostic factor for patients with colorectal cancer who treated with curative surgery and adjuvant chemotherapy\(^\text{39}\). Similar results were drawn in breast cancer, non-small lung cancer and melanoma\(^\text{40,41}\).

Based on the relationship between TMB and colon oncology clinic, shown above, we conducted GSEA analysis between high-TMB group and low-TMB group, and we found the items, including antigen...
processing and presentation, natural killer cell mediated cytotoxicity, suspended on the top of list, which consolidated that TMB were strongly relative to the tumor immune microenvironment. Immune cells infiltration can affect tumor biological behavior, such its growth, invasion and migration\textsuperscript{15,16,27}. In our study, we found high-TMB level correlated with higher fractions of CD8T cells, CD4 memory T cells, follicular helper T cells and M1 macrophages, which are immunoreactive cells\textsuperscript{42}. And numerous previous studies linked high fractions of CD8T cells, CD4 memory T cells, and follicular helper T cells with better prognosis in colon cancer\textsuperscript{43,44}. M1 macrophages can secrete pro-inflammatory cytokines to prevent tumor growth, metastasis and angiogenesis\textsuperscript{45}. And Xiong Y reported M1 macrophages correlated with a favorable clinical outcome in CRC\textsuperscript{46}. Likewise, we found high TMB had a lower fraction of regulator T cells (Tregs) in colon cancer. Several studies demonstrated that Tregs negatively correlated with prognosis in CRC\textsuperscript{47,48}. Interestingly, Tregs may suppress the proliferation of immunoreactive T cells, such as CD8T cell \textsuperscript{49}.

Nowadays immunotherapy is becoming a potential treatment for cancers\textsuperscript{50-53}. However, only about 20% of patients with cancer have good response to immunotherapy\textsuperscript{11}. Researchers have been making great efforts to ICBs for cancers, and CD274 (PD-L1), PD-1, TIGIT, HAVCR2, VISTA and LAG3 et al. have become the popular ICBs to predict immunotherapy responsiveness. In our study we demonstrated high-TMB group had significantly higher PD-L1, TIGIT, HAVCR2, and LAG3 than low-TMB group. And many studies prove TMB can be applied to predict the efficacy to immunotherapy in various cancers\textsuperscript{10,19,43,50,54,55}, including colorectal cancer\textsuperscript{34}. Hence TMB may also be a reliable bio-predictor to immunotherapy responsiveness in patients with colon cancer.

There are still some limitations in this study. Firstly, TMB does not reflect the actual number of neo-antigens that can activate the immune system. Secondly, because of the significant difference of clinical and basic information, for instance the age, between high TMB and low TMB group, the relationship between TMB and overall survival is still unclear. Thirdly, present silicon analysis may be limited by the quality and quantity of samples.

Totally, the older were more likely to be with high TMB. Compared with the low-TMB group, the high TMB referred to better clinical pathologic features, better immunotherapy responsiveness and stronger immune cells infiltration in colon cancer. Hence TMB may be a very promising bio-marker to predict prognosis and immunotherapy responsiveness to patients in colon cancer.

### Declarations

#### CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

#### Data and Materials Availability Statement
The data and materials used to support the findings of this study are included within the article.

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**Figures**
Figure 1

Mutations in colon cancer. (A) Summary of the mutations information with statistical calculations. (B) Overview of the top 30 genes’ mutational frequencies and their types in all samples
Figure 2

TMB correlated with clinical relevance. The classification of T, N, M and stage are based on the American Joint Committee on Cancer staging system.
Figure 3

TMB correlated with immune checkpoint inhibitors, including (A) CD274, (B) HAVCR2, LAG3 (P<0.001), (D) TIGIT
Figure 4

GSEA analysis between high-TMB group and low-TMB group. And the top TMB-related crosstalk include antigen processing and presentation (A), natural killer cell mediated cytotoxicity (B), autoimmune thyroid disease (C), graft versus host disease (D). All P-value ≤ 0.01
Figure 5

the 22 specific immune fractions in colon cancer samples. (A) The details of 22 specific immune fractions in each colon cancer samples. (B) Comparisons of 22 important immune fractions between low- and high-TMB groups by CIBERSORT algorithm.

Supplementary Files

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

- S2interaction.pdf
• S1Genecloud.pdf