Exploration of the relationships between tumor mutation burden with immune cell infiltration in cervical squamous cell carcinoma

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Abstract

Background: In various malignant tumors, whether tumor mutational burden (TMB) was related to improving survival outcomes or promoting immunotherapy remained controversial. We aimed to study the prognosis of tumor mutation burden in cervical squamous cell carcinoma (CSCC) and its potential association with tumor-infiltrating immune cells.

Methods: Transcriptome and somatic mutation profiles of CSCC were downloaded from the TCGA database. Somatic mutations were analyzed by "maftools" and visualized in waterfall plot. Then, TMB was calculated and we classified the samples into high-TMB and low-TMB groups based on the median data. Survival analysis and Wilcoxon test were used to investigate the prognostic value of TMB and its association with clinical variables. Differentially expressed genes (DEG) were identified in 2 TMN groups, and functional analysis was performed to find out significant biological pathways. Finally, we used the CIBERSORT algorithm to estimate the association between TMB and immune infiltration, and used the R package "vioplot" to visually draw the violin chart for each immune cell.

Results: The data from TCGA were analyzed, and higher TMB levels conferred high overall survival time, associated with higher T staging ($p = 0.048$) and older age ($p = 0.029$). Then, the differential analysis determined 122 DEGs. And pathway research showed that the enrichment of TMB-related signature correlated with several cancer-related signaling pathways, including PI3K-Akt signaling pathway, Rap1 signaling pathway, MAPK signaling pathway and Ras signaling pathway. Through CIBERSORT package and Wilcoxon rank-sum test, T cells CD8 ($p = 0.008$), T cells CD4 memory activation ($p = 0.002$) and T cells follicular helper cells ($p = 0.028$) showed higher levels of infiltration in the high TMB group. However, T cells CD4 memory resting ($p = 0.003$) and T cells regulation (Tregs) ($p = 0.033$) showed lower levels of infiltration in the high TMB group.

Conclusions: In summary, high TMB may inhibit the development of CSCC through anti-tumor immune cells. And our study might have some merits in elucidating the potential mechanism of TMB and immune infiltrates in CSCC and providing guidance of immunotherapy for endometrial cancer.

Background

Cervical cancer is one of the most common malignancies in females with over 527000 new cases and 265000 deaths worldwide per year^{1,2}. As the most common histological types of cervical cancer, the squamous cell type accounts for >85% of cervical cancers³. Nowadays, treatments for cervical squamous cell carcinoma (CSCC) include surgery, chemotherapy, target therapy, immunotherapy and so on. Though most of the early CSCC can be cured by surgery, the main treatment of locally advanced CSCC is cured by radiotherapy and chemotherapy at the same time. However, the treatment of advanced and recurrent CSCC is limited, the 5-year survival rate is less than 20%. Recently, immune checkpoint blockade has been studied in metastatic and persistent cervical cancer with promising outcomes^{4,5}.

However, there are still not very specific biomarkers to evaluate the effectiveness of immunotherapy in CSCC.

Tumor mutation burden, defined as the total number of somatic coding errors, base substitutions, and indel mutations per million bases⁶, can effectively estimate overall mutational load and neoantigenic load⁷. TMB is an emerging characteristic of cancer and is associated with microsatellite instability^{8,9}. TMB increase in the human cancer genome is attributed to endogenous factors and environmental damage¹⁰. Research has revealed that tumors with a higher TMB may contain newer antigens for activating immune cells, so TMB is closely related to tumor immunotherapy¹¹. Therefore, TMB is an emerging biomarker for the prediction of tumor behavior and response to immunotherapy¹².

With the development of sequencing technology, many valuable biological information resources about TMB and cancer immunotherapy response can be obtained from many public databases such as the Cancer Genome Atlas (TCGA) database, Gene Expression Omnibus (GEO) database, and so on. Wang's study showed that the prognostic role of TMB and the relationship between TMB and immune infiltrate varied from different types of cancers¹³. However, few related studies have focused on TMB with immune infiltration in CSCC, and no accepted opinions have been obtained. Therefore, we conducted this study to investigate the prognostic role of TMB and its potential association with immune infiltration in CSCC.

Methods

Data Source and Mutation Analysis

We downloaded the CSCC data from TCGA database via the GDC data portal (<https://portal.gdc.cancer.gov/>). From the four subtypes of data files, we selected the "Masked Somatic Mutation" data and processed it based on the VarScan2 software¹⁴. We prepared the Mutation Annotation Format (MAF) of somatic variants and implemented the "maftools" R package which provides multiple of analysis modules to perform the visualization process¹⁵. Moreover, the clinical data (fuptime, fustat, age, grade, stage, TMN) and RNA-seq gene expression profiles were downloaded from TCGA database. All in all, there are 289 samples for mutation information, 255 samples for clinical information, and 253 samples for RNA-seq data. As for all the data in our study was downloaded from public databases, there was no ethical conflict to declare.

TMB Value Estimation and Prognostic Analysis

We used a Perl script to extract the somatic mutation information, after which TMB value can be estimated by dividing the number of somatic mutations by the total length of exons. Then, we utilized R soft to combine the patient's TMB information and clinical information, including survival time and survival status. And we divided the CSCC samples into low- and high-TMB groups based on the median data. Then, we merged the TMB data with corresponding survival information via the id number of samples. Kaplan-Meier analysis was conducted to compare the survival difference between low- and

high-TMB groups, and the P of log-rank test was calculated. In addition, we further evaluated the correlation between TMB levels and clinical characteristics, in which Wilcoxon rank-sum test was used to compare the two clinical variables, while Kruskal-Wallis test was used when it comes to three or more groups.

Functional Analysis of TMB-Related DE Genes

According to the aforementioned, we divided RNA-seq gene expression samples into low- TMB and high-TMB groups according to the median TMB value. Then, we derived DEG through the R package "limma"¹⁶ to analyze the relationship between TMB and gene expression. We normalized the TCGA CSCC gene expression data based on -2 logarithmic transformation and used Wilcox rank-sum test for DE gene analysis. The p-value ($p < 0.05$) was considered statistically significant for DE mRNA. Finally, all differentially expressed genes were output with $p < 0.05$ and $|\log FC| > 0.5$. The R package "pheatmap" was used to generate heatmaps and clustering.

After obtaining the Entrez-ID of each DEG through the "org.Hs.eg.db" R package, we performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses and visualized the results, using the "clusterProfiler", "enrichplot" and "ggplot2" R packages¹⁷.

Relationship Between the Tumor Mutation Burden and Immune Infiltration

CIBERSORT, a deconvolution algorithm developed by Bindea G and others, can estimate the cell composition of complex tissues based on standardized gene expression data. This method energizes the abundance of specific cell types. High resolution is a key advantage of CIBERSORT, which also lists 22 immune cell types and applies the characteristics of more than 500 marker genes to quantify the relative fraction of each cell type¹⁸. The method was well-verified and successfully evaluated by flow cytometry and used to determine the immune cell landscape in several malignancies, such as the breast, liver, colorectal¹⁹⁻²¹. Here, the transcriptome data of TCGA CSCC were calibrated with the "limma" software package, and then the immune cell content matrix was obtained by CIBERSORT's deconvolution algorithm, where p value < 0.05 was the cutoff value. Then, we used the R package "vioplot" to visually draw the violin chart for each immune cell, and then we used the Wilcox rank-sum test to calculate whether the content of each tumor cell in samples between high- and low- TMB groups was statistically significant. Finally, we also used the "survival" R software package to analyze the percentage of and the Kaplan-Meier survival curve of 22 types of tumor-infiltrating immune cell components in CSCC.

Results

Landscape of Genome-wide Mutation Files in CSCC

We obtained somatic mutation profiles of 289 CSCC patients from the "VarScan2" process in the TCGA database. About 255 samples (88.24%) had somatic mutations, and the mutation information of each

gene in all samples was shown in the waterfall chart (Fig. 1). As for the top 10 mutant genes shown in Fig. 1 and Fig. 2F, we found that the gene TTN had the most frequent mutations, accounting for 29%, followed by PIK3CA (27%), KMT2C (18%), MUC4 (18%), MUC16 (16%), KMT2D (13%), DMD (12%), FBXW7 (12%), FLG (12%) and EP300 (11%). Gene mutations were usually divided into 9 types, as shown in Fig. 2A. Among these mutations, missense mutation was the most common form. Single nucleotide polymorphism (SNP) was a more common type of mutation than insertion or deletion (Fig. 2B), and C > T mutation was more common than other types of SNP (Fig. 2C). Then, we counted the number of variants for each sample and displayed the variant types with different colors in the boxplot (Fig. 2D and 2E). The co-occurring or mutually exclusive expression of mutant genes was shown in Fig. 2G. At the same time, the Genecloud graph in **Fig. S1** showed the mutation frequency of other genes.

In addition, the obtained transcriptome profile consists of 253 CSCC samples. The clinical information of 255 CSCC patients was shown in Table 1, in which the mean age of patients was 48.80 ± 14.05 years old and they were all female.

Table 1
Clinical baseline of 255 CSCC
patients included in study from
TCGA cohort

Variables	Number(%)
Futime	1002.09 ± 1179.92
Fustat	
Alive	59(23.14)
Dead	196(76.86)
Age,y	48.80 ± 14.05
Gender	
Female	255(100)
Male	0
Grade	
G1	12(4.71)
G2	110(43.14)
G3	104(40.78)
G4	1(0.39)
Unknow	28(10.98)
Stage	unknow
AJCC-T	
T1	111(43.53)
T2	59(23.14)
T3	19(7.45)
T4	9(3.53)
TX	57(22.35)
AJCC-M	
M0	102(40.00)
M1	8(3.14)
MX	145(56.86)
AJCC-N	

Variables	Number(%)
N0	106(41.57)
N1	51(20.00)
NX	98(38.43)
TMB level	
Low level	118(46.28)
High level	122(47.84)
Undetected	15(5.88)

The relationship between TMB and survival and clinical characteristics

We used the Kaplan-Meier method to explore whether there was a correlation between TMB and the prognosis of CSCC patients. Although the overall survival time of the high- TMB group was not statistically different from that of the low TMB group ($p = 0.556$), the high- TMB group had a higher overall survival time in the interval of 5–10 years (Fig. 3A). Next, we analyzed the relationship between TMB and clinical characteristics and found that the TMB of patients over 65 years of age was significantly higher than that of patients under 65 years of age ($p = 0.029$, Fig. 3B), and patients with a higher T stage had higher TMB values ($p = 0.048$, Fig. 3C).

DEGs in two TMB groups and functional analysis

The LIMMA software package was used to analyze the differential genetics between the low- TMB group and the high- TMB group, and the cut-off values were $|\log FC| > 0.05$ and $P < 0.05$. The smaller the p-value, the greater the possibility of differential gene expression. Finally, we determine 122 DEGs and drew the heatmap of all DEGs with the R-pheatmap package (Fig. 4A). In biological process group, tissue migration, ameboidal-type cell migration, and cell-substrate adhesion were enriched. And in the CC category, TMB-related genes participated in the collagen-containing extracellular matrix, basement membrane, interstitial matrix, and platelet alpha granule. And in the MF group, these differential genes were mainly involved in heparin-binding, sulfur compound binding, glycosaminoglycan binding (Fig. 4B). Pathways research also supported the results that the enrichment of TMB-related signature correlated with several cancer-related signaling pathways, including PI3K-Akt signaling pathway, Rap1 signaling pathway, MAPK signaling pathway, and Ras signaling pathway (Fig. 4C).

Immune cell fractions in groups of high- and low-TMB

We used the newly established CIBERSORT method to estimate 22 immune cells per patient and generated a box plot, where the different colors reflect the various cell subgroups (Fig. 5A). We also

showed the distribution of 22 immune cell fractions in the two groups by heatmap plot (Fig. 5B). In addition, we also evaluated the correlation between the TIICs in CSCC. It was illustrated in Fig. 5C that the fractions of some kinds of immune cells were mutually correlated in the TCGA cohort. Specifically, the two most relevant TIICs were T cells CD4 memory resting and T cells CD8, with an R-value of 0.6. At the same time, there was the highest positive correlation between the M0 of macrophages and activated mast cells ($R = 0.35$). In addition, a vioplot showing the expression levels of 22 TIICs subgroups in high- and low- TMB was shown in Fig. 5D. As a result, T cells CD8 ($p = 0.008$), T cells CD4 memory activation ($p = 0.002$) and T cells follicular helper cells ($p = 0.028$) showed higher levels of infiltration in the high TMB group. However, T cells CD4 memory resting ($p = 0.003$) and T cells regulation (Tregs) ($p = 0.033$) showed lower levels of infiltration in the high TMB group. Finally, in order to evaluate the prognostic value of TIICs for CSCC patients, a Kaplan-Meier survival curve was generated based on TCGA data. We found that T cells CD4 memory activation ($p < 0.001$), NK cells resting ($p = 0.013$) and T cells CD8 ($p = 0.013$) were associated with a good prognosis, while T cells CD4 memory resting ($p = 0.002$), Mast cells activation ($p = 0.004$), NK cells activation ($p = 0.016$), and eosinophils ($p = 0.044$) were associated with poor prognosis of CSCC patients (Fig. S2).

Discussion

Cervical cancer is the highest incidence of female cancer in the world, and cervical squamous cell carcinoma is the most common histological types of cervical cancer. It can be seen that the incidence of cervical cancer has been increasing and younger in recent years, seriously threatening the health of women worldwide. It is estimated that the overall survival rates were 81–96%, 65–87%, 35–50%, and 15–20% for patients diagnosed at clinical stage I-IV, respectively^{22,23}. Besides, poor prognosis and a high recurrence rate still remain a challenge in the world especially in developing countries²⁴. Therefore, it is important to find more reliable and effective biomarkers to improve early diagnosis and new treatment methods to improve the survival rate of patients with CSCC.

In recent years, tumor immunotherapy has begun to receive widespread attention. Among them, immune checkpoint inhibitor therapy is a popular research object and has been applied to a variety of tumor treatments, but not all patients can benefit from it. In order to find out which patients can benefit, combined with predictive efficacy markers to predict efficacy before immunotherapy, including PD-1, PD-L1, CTLA-4, and TMB²⁵, in which TMB is a promising biomarker in recent years. And the TMB form genome-sequencing detected is a new biomarker for predicting immune checkpoint blockade (ICB) response, which has shown its effectiveness in several tumors, such as breast cancer²⁶, lung cancer²⁷, and so on. The reason that why TMB has become a marker of immunotherapy stems from the biological mechanism of somatic mutation and immune response. Somatic mutations in tumors include synonymous mutations and non-synonymous mutations. Non-synonymous mutations produce abnormal proteins by changing the amino acid sequence. However, the immunogenicity of abnormal proteins in tumors is the basis of tumor immune response. If abnormal proteins are finally recognized by immune cells, they will become neoantigens, and subsequent immune responses can develop²⁸. That is to say,

when the TMB of the tumor sample is high, the mutations that produce immunogenic neoantigens in the mutations will also increase. The immune system is easier to recognize and remove tumor cells, and the survival rate of patients will be relatively improved. However, the prognostic role of CSCC and the interaction of immunotherapy have not been explored. We are investigating the prognostic role of TMB and the potential connection with CSCC immune infiltration in this study.

According to the Kaplan-Meier survival curve, the 5–10 year survival status of the patients in the high-TMB group is better than low-TMB group. This is consistent with a study conducted by Goodman et al., who found that melanoma and non-small cell lung cancer patients with High-TMB revealed better progression-free survival (PFS) than that with Low-TMB²⁹. Park came to a similar conclusion that high TMB in patients with HER2-positive refractory metastatic breast cancer may be a prognostic marker for good overall survival⁵. Moreover, in this study, we also found that the higher the T stage, the higher the TMB value. Finally, our research also found that older patients have higher TMB levels, which is consistent with the significant trend of TMB increasing with age³⁰.

At the same time, we explored the potentially functional pathways of DEGs in the two TMB groups through KEGG and GO analysis. GO functional annotation analysis showed that DEGs were mainly involved in the regulation of extracellular matrix and cell migration. Further analysis of the KEGG pathway showed that these DEGs were associated with CSCC mutations, including PI3K-Akt, Rap1, MAPK, and Ras signaling pathways. A series of genetic changes induced by HPV in CSCC activates four major upstream pathways (GFR, Notch receptor, RAS isoforms, and p110α) to stimulate host cell survival, proliferation, and carcinogenesis through the PI3K/AKT/mTOR pathway. In particular, the mutations of PIK3CA E542K and E545K promote glycolysis and proliferation of CSCC in vitro and vivo³¹. Currently, only preclinical trials of PI3K inhibitor LY294002 have revealed it significantly radiosensitized CSCC cell lines in vitro and vivo^{32,33}, and the terminated clinical trials of AKT inhibitor GSK2141795 has tried to display a novel treatment approach to patients of CSCC³⁴.

On the other hand, high TMB levels were significantly associated with a higher proportion of T cells CD8, T cells CD4 memory activation, and T cells follicular helper. In the low TMB subgroup, the ratio of T cells CD4 memory resting and T cells regulation (Tregs) were elevated. Many cancers are characterized by immune cell infiltration³⁵. Other studies have also found that TMB levels have an impact on immune cell infiltration signatures of cutaneous melanoma and clear cell renal cell carcinoma^{6,36}. These findings indicate that TMB may affect the tumor microenvironment, and TMB-related genes may lead to variations in the tumor microenvironment.

Finally, the analysis of the prognosis of immune cells showed that T cells CD4 memory activation, NK cells resting, and T cells CD8 were associated with a good prognosis, while T cells CD4 memory resting, mast cells activation, NK cells activation, and Eosinophils were associated with poor prognosis in patients with CSCC. Traditionally, immunity mediated by activated memory T cells is considered anti-tumor because patients with higher levels of active memory T cells are predicted to have longer-term disease-free survival. This is consistent with our research results.

However, current research still has some limitations that must be considered. Firstly, this is a retrospective analysis based on publicly available data, some of which may lack clinical information of the patients. Secondly, no experimental verification was performed. Therefore, in further research, a large clinical cohort is needed to verify the impact of TMB on the prognosis, the accuracy of the risk scoring model, and the nomogram. In addition, basic experiments are needed to verify the relationship between TMB and immune infiltration.

Conclusions

Our group was the first to study the association between TMB and TILs in CSCC based on large-scale mutation data in public databases. In the current research results, we innovatively explained that TMB is not only related to survival in CSCC, but also related to TILs. In addition, we also studied the relationship between TILs and CSCC survival. It was found that T cells CD4 memory, NK cells resting, T cells CD8, mast cells activation, NK cells activation, and eosinophils can be used as potential immunotherapy targets. In conclusion, our study may have some advantages in elucidating the underlying mechanisms of TMB and TILs in CSCC and providing evidence of high TMB as an indicator of CSCC immunotherapy.

Abbreviations

TMB Tumor Mutational Burden (TMB)

CSCC Cervical Squamous Cell Carcinoma (CSCC)

DEG Differentially Expressed Genes (DEG)

TCGA The Cancer Genome Atlas (TCGA)

GEO Gene Expression Omnibus (GEO)

MAF Mutation Annotation Format (MAF)

GO Gene Ontology (GO)

KEGG Kyoto Encyclopedia of Genes and Genomes (KEGG)

ICB Immune Checkpoint Blockade (ICB)

PFS Progression-Free Survival (PFS)

Declarations

Acknowledgements

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Authors' contributions

Conception and design: BN. Development of methodology: BN, YXL. Acquisition of data: MW, YL, TZX. Analysis and interpretation of data: BN, YXL, MW. Writing, review, and/or revision of the manuscript: BN, MW, YCW. Study supervision: YCW. All authors read and approved the final manuscript.

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Availability of data and materials

The dataset analysed during the current study is available in the TCGA repository, <https://portal.gdc.cancer.gov/repository>.

Ethics approval and consent to participate

The TCGA dataset used and analysed in this study are unrestricted-access, which are available without any permission request through the TCGA portal (<https://portal.gdc.cancer.gov/repository>). We definitely followed the National Institutes of Health Genomic Data Sharing Policy as well as the National Cancer Institution Genomic Data Sharing Policy in this study.

Consent for publication

Not applicable.

Competing interests

No potential conflicts of interest were disclosed by the authors.

Footnotes

Publisher's Note

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Figures

Altered in 255 (88.24%) of 289 samples.

Figure 1

Landscape profile of somatic gene mutations in 289 CSCCs from TCGA database. Mutations of each genes in each samples were shown in waterfall plot. Each column presented each samples. Name of mutated genes was listed in the right, different forms of somatic mutation types were in different colors shown in the bottom and percent of gene mutation was shown in the left.

Figure 2

Summary of mutation profiles in CSCC. (A) Nine common variant mutation forms in all samples. Missense mutation accounted for the most compared with other types of variants. (B) Three variant types in all samples. SNP was more frequent than deletion or insertion. (C) Six subclasses of single nucleotide polymorphism (SNP) in all samples. C>T transition was the most frequent. (D-E) Summary of variant classification in all samples. (F) Top 10 mutated genes in all samples. (G) Co-occurrence or mutually exclusive interaction of mutated genes.

Figure 3

The relationship between TMB and Survival and clinical characters of age and T staging. (A) Kaplan-Meier survival curves for high-TMB subtypes (red line) and low-TMB subtypes (blue line). (B) Correlation analysis between TMB and age (with 65 years old as the cut-off point). (C) Correlation analysis between TMB and T staging.

Figure 4

Functional analysis of DEGs in 2 TMB groups. (A) The mutant genes with higher expression in the heatmap are shown in red, lower expression in green, and genes with the same expression level in black. The heatmap of the DEG of the left half (low-TMB subtypes) and the right half (high-TMB subtypes) of TMB. (B) Cellular composition, biological process and molecular function of GO enrichment analysis. (C) Enrichment analysis of KEGG pathway.

Figure 5

Comparisons of 22 important immune fractions between low- and high-TMB groups. (A) The specific 22 immune fractions represented by various colors in each sample were shown in barplot. (B) A heatmap of the 22 immune cell proportions based on TCGA data. Red indicates upregulation and green indicates downregulation. (C) Correlation matrix of 22 immune cell proportions calculated by CIBERSORT software. (D) Wilcoxon rank-sum test indicated that the infiltration levels of T cells CD8 , T cells CD4 memory activated and T cells follicular helper showed increased levels of infiltration in high-TMB group. And T cells CD4 memory resting and T cells regulatory (Tregs) displayed lower level of infiltration in high-TMB group.

Supplementary Files

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