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ORIGINAL RESEARCH

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Tumour mutational burden and immune-cell infiltration in cervical squamous cell carcinoma

Shanquan Yan[1], Dan Liu[1], Xitong Yang[2], Guangming Wang[2,*]

[1]School of Clinical Medicine, Dali University, 671000 Dali, Yunnan, China
[2]Genetic Testing Center, The First Afliated Hospital of Dali University, 671000 Dali, Yunnan, China

***Correspondence**
wgm1991@dali.edu.cn
(Guangming Wang)

Abstract

Background: Cervical carcinoma is one of the most common gynaecological malignancies worldwide and severely affects the health of women; cervical squamous cell carcinoma is the most prevalent form. The aim of this study was to assess the tumour mutational burden (TMB) and immune infiltration in cervical squamous cell carcinoma. **Methods**: Cervical carcinoma-related data were downloaded from The Cancer Genome Atlas (TCGA). The patients were divided into low- and high- TMB groups based on the median TMB score. Differentially expressed genes (DEGs) were identified based on the TMB score. Bioinformatics tools were used to analyse the immune infiltration in cervical squamous cell carcinoma and patient survival. **Results**: Single nucleotide polymorphisms were more familiar than deletions or insertions, and C>T was the primary single nucleotide variant. Moreover, TTN, PIK3CA, and MUC16 were the three main genes with mutations. The survival curve was not clinically significant, but a correlation was observed between high TMB levels and tumour grade. 69 DEGs were identified, which primarily consisted of the FC receptor, Ras, and MAPK signalling pathways. Furthermore, a significant difference was observed in the extent of infiltration of $CD4^+$ T cells, regulatory T cells, and natural killer cells. **Conclusions**: We observed a relationship between high TMB standards and the development of cervical squamous cell carcinoma. However, we could not present an immune prognostic model for cervical squamous cell carcinoma.

Keywords

tumour mutational burden; cervical squamous cell carcinoma; immune infiltration; bioinformatics

1. Introduction

In recent years, cervical carcinoma has become one of the primary gynaecological malignancies worldwide, whose incidence is the second among gynaecological tumours [1]. With the increase in health awareness, the incidence of the carcinoma in developed countries has dropped significantly, but the incidence and mortality of cervical cancer in developing countries remain high [2]. There were 604,000 new cases of cervical cancer worldwide in 2020, with a crude incidence rate of 15.6/100,000, and a world age-standardised incidence rate of 13.3/100,000, with 342,000 related deaths and a crude death rate of 8.8/100,000; the mortality rate was 7.3 per 100,000 [3]. Numerous high-risk human papillomavirus (HPV) infections are short-lived, and only few of these develop into cervical cancer [4]. Vaccination and screening are considered primary and secondary methods, respectively, to prevent cervical cancer. Although cervical vaccination has markedly increased [5], the rate of malignant transformation continues to rise, which seriously affects patients' lives. Currently, the optimal treatment for the disease at the early stages is surgery, followed by radiotherapy and chemotherapy [6].

TMB is closely related to tumorigenesis. When mutations occur in the protein-coding area, the tumour cells can produce new antigens, which promote the proliferation of tumour cells and make them cancerous [7]. Therefore, the higher the TMB, the more types and quantities of neoantigens are produced by tumour cells. TMB influences the course of cancer development, for example it can activate the immune system to launch an anti-tumour immune response thereby increasing the probability of killing tumour cells [8]. High TMB is reportedly correlative with survival, which is poor in skin cutaneous melanoma [9]. Simultaneously, it is also associated with extended survival and enhanced immune infiltration in uterine corpus endometrial carcinoma [10]. Furthermore, TMB may play a potential role in cancer therapy. TMB promotes the release of microalveolar cells that may produce cell free immunogens to be used by dendritic cells, based on cancer therapy [11–13]. Whether TMB or other features can be used as prognostic indicators in the disease remains ambiguous. Therefore, in this study, our goal is to explore the potential relationship between the TMB and immune infiltration in cervical squamous cell carcinoma.

TABLE 1. Clinical information of patients.

Variables	Number
Gender	
Female	255 (100%)
T	
Tis	1 (0.39%)
T1	111 (43.53%)
T2	59 (23.14%)
T3	19 (7.45%)
T4	9 (3.53%)
TX	15 (5.88%)
Unknown	41 (16.08%)
M	
M0	102 (40.00%)
M1	8 (3.14%)
MX	99 (38.82%)
Unknown	46 (18.04%)
N	
N0	106 (41.57%)
N1	51 (20.00%)
NX	57 (22.35%)
Unknown	41 (16.08%)
Grade	
Grade 1&2	122 (47.84%)
Grade 3&4	105 (41.18%)
GX	20 (7.84%)
Unknown	8 (3.14%)

2. Materials and Methods

2.1 Data Collection and Arrangement

Three types of data were obtained from the TCGA-CESC tumour data set (Cervical squamous cell carcinoma and endocervical adenocarcinoma, CESC), including 257 cases of cervical squamous cell carcinoma, 255 clinical cases, and 288 mutation cases. The clinical data included information related to patient age, sex, survival duration, survival status, tumour grade, and tumour node metastasis (TNM) staging. Mutation data were obtained from the 'simple nucleotide variation' option of TCGA-CESC; the data type was selected as 'masked somatic mutation'. Perl (Strawberry version) and R software (Version 3.6.3, University of Auckland, New Zealand) were used to process these data. The 'maftools' [14] R package was used to generate the visual mutation annotation format files 'input.maf'. The data underwent a series of transformations and sorting for subsequent operations. Due to the retrospective nature of the study, ethical approval was abandoned, and these data were provided through informed consent prior to downloading from TCGA.

2.2 Computation of the TMB Value

The calculation of the TMB score was based on the number of mutations per megabase of the exon coding area of the assessed gene, which is the distribution density of non-synonymous mutations in the protein-coding area; it can be computed in R software by dividing the total amount of non-synonymous mutation sites in the protein-coding region by the total extent of the protein-coding area. TMB can be further divided into the following three levels considering the median TMB score: 1, low TMB, 1–5 mutations/Mb; 2, intermediate TMB, 6–19 mutations/Mb; and 3, high TMB, >20 mutations/Mb [13, 15].

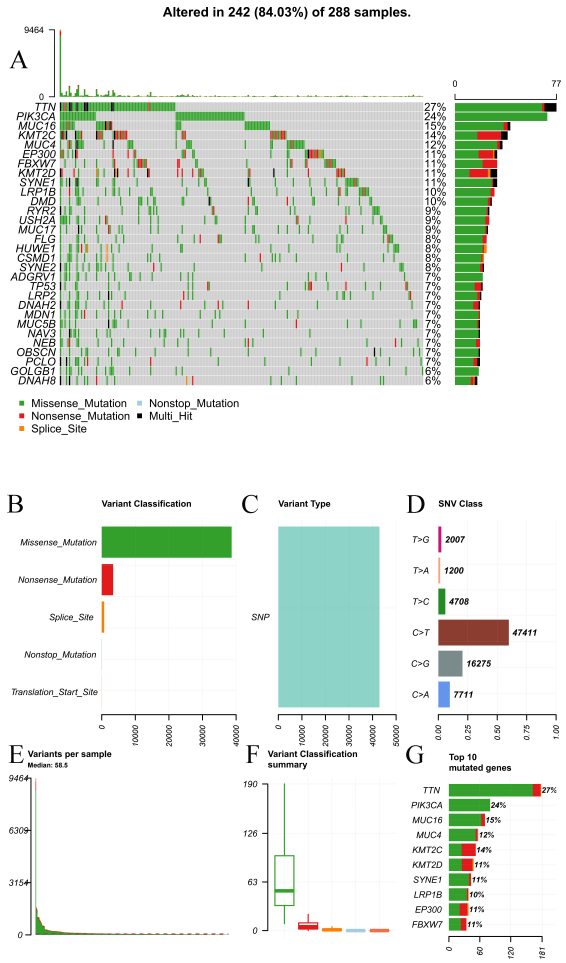

FIGURE 1. The landscape of mutations in the cervical squamous cell carcinoma of patients. (A) Waterfall figure of the top 30 mutated genes. (B,C) Description of the statistical analysis of mutation types in different categories. (D) Different colours reflecting different types of mutations. (E,F) The mutation in per patients. (G) The top 10 mutated genes in cervical squamous cell carcinoma.

2.3 Bioinformatics Analysis

2.3.1 The Relationship between TMB and Clinical Information

The cervical squamous cell carcinoma samples were divided into two groups, high and low, according to the TMB. The R package 'limma' was used to extract a text file named 'Clinical' after sorting the clinical data. The Kaplan–Meier analysis was used to assess the survival analysis of cervical squamous cell carcinoma. The statistical graphs show the relationship between TMB and the clinical information.

2.3.2 DEGs and Functional Enrichment Analysis

The DEGs were selected with a filter using |log2 FC| >1.0 and false discovery rate <0.05 as the condition. There were

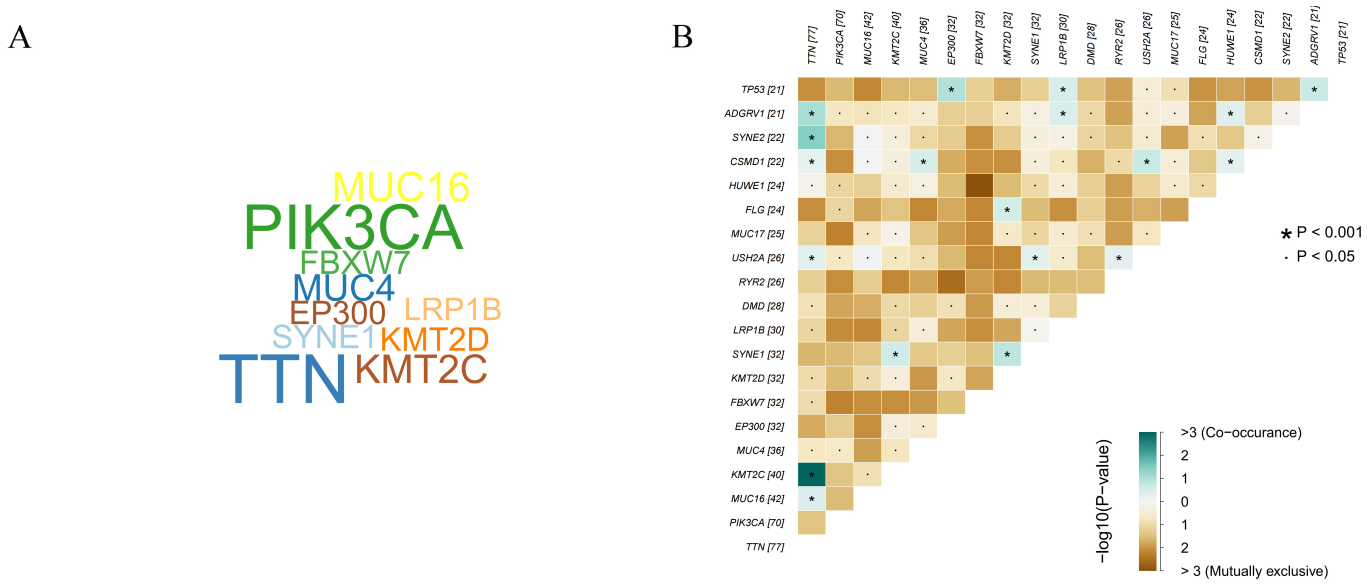

FIGURE 2. Summary of the mutation information with statistical significance (*$p < 0.001$, $p < 0.05$). (A) Gene cloud plot of the mutation frequencies. (B) The coincident and exclusive relationships across mutated genes; green and brown show positive and negative relationships, respectively.

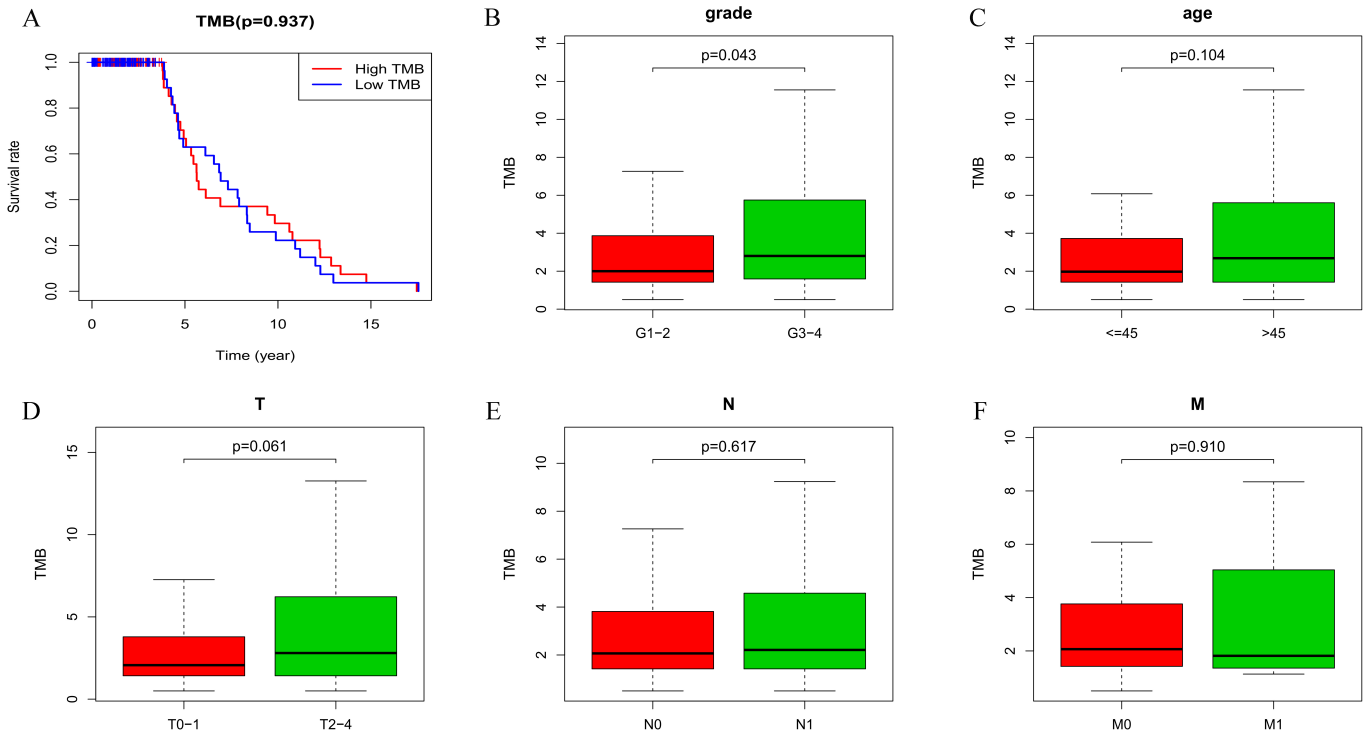

FIGURE 3. The relationship between TMB and clinical information in cervical squamous cell carcinoma. (A) The survival curves showing the survival differences in two groups. (B, C, D, E, F) Wilcox test based on the age, grade, and TNM of patients. TMB score correlated with tumour grade ($p = 0.043$).

three bioinformatics tools within the enrichment analysis, Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and gene set enrichment analysis (GSEA, Version4.1.0). These files were obtained by further sorting the tumour data downloaded from TCGA and the tumour-related DEGs with the 'limma' R package. The 'heatmap', 'colorspace', 'ggplot2'etc. were fixed according to the commands. For the R scripts analysed by GO and KEGG, the *p*-value filter was set as '$p = 1$' along with the corrected q-value filter condition 'q = 1'. All screening criteria were set and the enrichment analysis was conducted.

2.3.3 Immune Infiltration Analysis

Cibersort is a computational method for quantifying immune-cell subtypes based on gene data [16], and the data were processed with the 'Cibersort' algorithm. The composition proportion of immune cell subtypes in each sample was analysed and the sum of various immune cells was obtained as 1

TABLE 2. The top 20 DEGs in cervical squamous cell carcinoma.

Gene	Low-TMB	High-TMB	LogFC	*p*-value	FDR
CPA3	5.073644835	2.382856347	–1.090330552	1.89E-05	0.009948596
IGF1	0.871965916	0.159465227	–2.451029877	0.000156768	0.022653721
SLCO2A1	10.32155256	4.589559339	–1.169232449	0.000494925	0.03449363
IGKV3-15	84.07503656	41.64950782	–1.013378054	2.43E-05	0.010363454
IGHV4-34	45.58919629	17.7890542	–1.357702168	0.000409294	0.032073591
IGKV3-7	3.41960965	1.670061465	–1.033930449	0.000556908	0.036411417
IGHV3-25	0.344196486	0.139800144	–1.299866522	0.000380278	0.031277137
IGKV1-12	3.205285154	1.23563019	–1.375205684	0.000285487	0.028192233
IGLV1-40	154.1244679	74.44330794	–1.049881845	0.00045499	0.033488572
IGHV1-67	1.765604307	0.598165318	–1.561545884	8.57E-05	0.017296443
IGHV1-46	37.26222931	11.73508081	–1.666886214	6.56E-05	0.015264769
IGKV1OR2-108	4.779969807	1.981456925	–1.270439901	0.000893381	0.045773587
ARHGAP42	1.36372581	0.624129322	–1.127636708	0.000498396	0.034582541
IGLV3-13	0.358385426	0.152484451	–1.232849837	0.000865699	0.045452088
SOX17	6.260948993	1.997105727	–1.648470637	0.000288169	0.028192233
IGKV1D-33	0.918152986	0.128221798	–2.840093016	0.000241834	0.027242749
SOX8	0.506559047	0.087299593	–2.536683612	5.81E-05	0.014984115
MS4A2	0.314544862	0.145307206	–1.114159548	0.00014159	0.021818015
IGKV3D-20	12.1927462	4.957945107	–1.298208901	0.000288062	0.028192233
SLC51B	0.408494603	0.132813514	–1.62091507	0.000241092	0.027242749

[17]. The tumour immune infiltration graph was obtained with the filter '$p = 0.05$'.

2.4 Statistical Analysis

We used the "survival" R package to execute the Cox regression model. The Wilcoxon rank sum method and Kruskak-Wallis test were applied to non-parametric statistical hypothesis methods which are mainly used for comparison between two groups. Statistically, $p < 0.05$ was considered significant.

3. Results

3.1 Mutations in Cervical Cancer

The mutation data were downloaded from TCGA and processed to obtain a visual analysis graph of the cervical cancer mutation data. The results revealed that approximately 84.03% (242) of the 288 patient samples harboured mutations (Fig. 1A, Table 1). Missense mutations and single nucleotide variants (SNVs) were the major mutation types (Fig. 1B,C,D) and the C > T transversion was the most common SNV (Fig. 1D). The number of mutant bases in individual patients, were also measured and are shown Fig. 1E, and F. *TTN, PIK3CA, MUC16, MUC4, KMT2C, KMT2D, SYNE1, KRP1B, EP300,* and *FBXW7* were the top ten mutated genes in cervical squamous cell carcinoma (Fig. 1G). *TTN* (27%), *PIK3CA* (24%), and *MUC16* (15%) were the three major mutant genes. The mutation frequency of the genes is reflected in the gene cloud plot (Fig. 2A). Furthermore, we analysed the coincident and exclusive relationships among the mutated genes (Fig. 2B).

3.2 TMB and Clinical Information

In the present study, 257 patients with cervical squamous cell carcinoma were divided into two groups according to the TMB scores; 121 cases were assigned to the high-TMB group, 118 cases to the low-TMB group, and the remaining cases were assigned to the median group. The clinical data was collated and R software was used to perform survival analysis on the collated data. The results are displayed in Fig. 3A, and the survival curve was not statistically significant ($p = 0.937$). Moreover, we found that TMB was correlated with the clinical information. The relationship between TMB and tumour grade was clinically significant (Fig. 3C, $p = 0.043$), however, no association was observed between TMB and age (Fig. 3B) or TNM (Fig. 3D,E,F) staging.

3.3 DEGs and Functional Enrichment Analysis

We identified 69 DEGs based on the TMB score, and the top 20 DEGs are listed in Table 2. A heat map was obtained by analysing the data (Fig. 4A) and the 40 most significant DEGs were selected. Venn plot analysis identified immune genes related to TMB (Fig. 4B). Additionally, we conducted GO, KEGG, and GSEA to investigate the most familiar biological processes and pathways where all DEGs were included. These DEGs mainly participated in the FC receptor signalling (Fig. 4C, Table 3) by GO. The pathway enrichment analysis of KEGG revealed that these DEGs were mainly included in breast cancer, the Ras signalling pathway, the MAPK signalling pathway, and Neuroactive ligand-receptor signalling

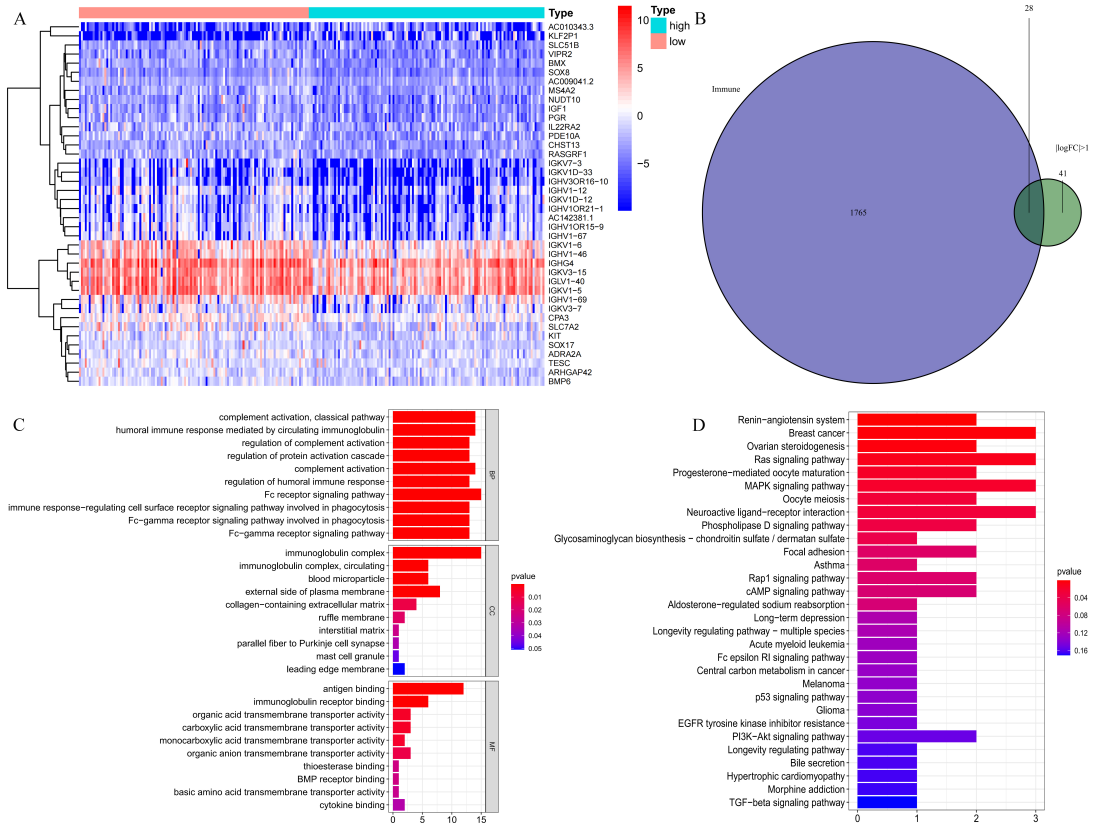

FIGURE 4. The functional enrichment analysis of DEGs. (A) The heatmap of the top 40 DEGs; red and blue show overexpression and reduced expression, respectively. (B) Venn plot. (C,D) Functional analysis of the top ten enriched pathways of GO and KEGG pathway analysis. BP, biological processes; CC, cell composition; MF, molecular function.

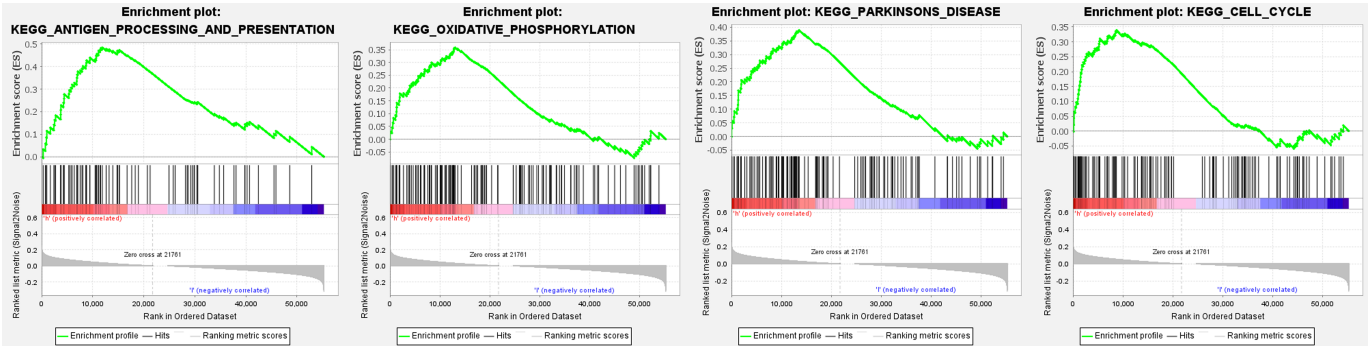

FIGURE 5. GSEA analysis of DEGs.

pathway (Fig. 4D, Table 4). GSEA revealed that a high-TMB can activate antigen processing and presentation, oxidative phosphorylation, Parkinson's disease, and cell cycle pathways enriched in cervical cancer (Fig. 5).

3.4 Immune Infiltration

In total, 257 patients were subjected to immune infiltration analysis, and the relative abundance of 22 immune cell types is summarised in Fig. 6A. When the high- and low-TMB groups were compared, the p-value of CD4 memory activated T cells ($p = 0.031$), natural killer cells ($p = 0.011$), and Treg cells ($p = 0.026$) were found to be significant in the high-TMB group (Fig. 6B).

4. Discussion

Approximately, 500,000 new cervical cancer cases are reported worldwide every year, accounting for 5% of all new cancer cases; more than 80% of these cases occur in developing countries [18]. Gene mutations can lead to the occurrence and development of tumours, and TMB is a form of expression of a gene mutation. TMB can indirectly reflect the ability and extent of the tumours to produce new antigens and predict the efficacy of immunotherapy for most tumours [19].

The immune system has three physiological functions as follows: immune defence, stability, and surveillance. Dunn *et al.* [20] reported that the interaction between the immune system and tumour could be grouped into three stages: (1) elimination, where the tumour cells are eliminated by the immune system

TABLE 3. GO pathway analysis.

GO term	Description	GeneRatio	BgRatio	*p*-value	p.adjust	q-value	Count
BP	complement activation, classical pathway	14/42	137/18670	2.95E-20	3.74E-17	2.95E-17	14
BP	humoral immune response mediated by circulating immunoglobulin	14/42	150/18670	1.10E-19	6.95E-17	5.48E-17	14
BP	regulation of complement activation	13/42	115/18670	2.01E-19	7.17E-17	5.65E-17	13
BP	regulation of protein activation cascade	13/42	116/18670	2.26E-19	7.17E-17	5.65E-17	13
BP	complement activation	14/42	175/18670	1.00E-18	2.55E-16	2.01E-16	14
BP	regulation of humoral immune response	13/42	134/18670	1.58E-18	3.34E-16	2.64E-16	13
CC	immunoglobulin complex	15/42	159/19717	1.65E-21	8.93E-20	7.83E-20	15
CC	immunoglobulin complex, circulating	6/42	72/19717	9.06E-09	2.45E-07	2.15E-07	6
CC	blood microparticle	6/42	147/19717	6.52E-07	1.17E-05	1.03E-05	6
CC	external side of plasma membrane	8/42	393/19717	1.51E-06	2.04E-05	1.79E-05	8
CC	collagen-containing extracellular matrix	4/42	406/19717	0.010699742	0.11555721	0.101365974	4
CC	ruffle membrane	2/42	94/19717	0.017108142	0.153973276	0.135064277	2
MF	antigen binding	12/39	160/17697	6.22E-16	6.72E-14	5.44E-14	12
MF	immunoglobulin receptor binding	6/39	76/17697	1.50E-08	8.08E-07	6.53E-07	6
MF	organic acid transmembrane transporter activity	3/39	153/17697	0.004610452	0.11713182	0.094755761	3
MF	carboxylic acid transmembrane transporter activity	3/39	153/17697	0.004610452	0.11713182	0.094755761	3
MF	monocarboxylic acid transmembrane transporter activity	2/39	50/17697	0.005422769	0.11713182	0.094755761	2
MF	organic anion transmembrane transporter activity	3/39	211/17697	0.011138743	0.199974648	0.161772864	3

before the clinical diagnosis is confirmed (this stage can also be called immune surveillance); (2) balance, the stage in which the tumour cells with high immunogenicity are dead and those with low immunogenicity are viable; (3) escape, where these tumour cells escape the host's immune surveillance. Immune checkpoint blockade (ICB) is a novel approach used to treat cancer and can regulate tumour development; however, it can also cause the recurrence of metastatic disease [21]. ICB relieves the inhibitory effect of tumour cells on immune cells by blocking the interaction between tumour cells expressing immune checkpoint molecules and immune cells. For instance, regulating the activity of T cells could improve the anti-tumour immune response. Initially, ICB was proven to be effective for patients with metastatic melanoma [22] and has now been extended to other types and some early-stage cancers. Cervical cancer is currently one of the main tumours that endanger women's health, and its incidence is increasing every year [18]. If ICB is used in the early diagnosis and treatment of cervical cancer, the survival rate of patients may be markedly improved. Given the low survival rate of patients with advanced cervical squamous cell carcinoma, studies related to TMB may aid in the discovery of mutations in cancer cells, which is closely related to the treatment of tumours and is presumed to improve the survival rate of tumour patients. The higher the TMB, the more mutations, the easier it is for immune cells to be found and become the target of tumour immunotherapy, and the more likely it is to be effective in immunotherapy. However, the prognostic role and relevance to immunotherapy of TMB in cervical squamous cell carcinoma is unclear. This study aimed to explore the influence of TMB on the occurrence and development of cervical squamous cell carcinoma, and its potential connection with the related immune infiltration.

Here, we obtained the sample- and mutation-related data from TCGA. The information included clinical sample and sequencing data, including various omics data derived from genome, transcriptome, epigenetic, and proteome. Genetic mutations are crucial for tumour occurrence and treatment and determining patient prognosis.

We chose 69 DEGs on the basis of the TMB score. Functional enrichment analysis promotes the further study of these DEGs. In this study, we used three approaches (GO, KEGG, and GSEA) to conduct the enrichment analysis of DEGs. Based on our results, we inferred that DEGs were related to immune-cell infiltration. Moreover, immune cells consisting of B cells, $CD8^+$ T, $CD4^+$ T, NK, Treg, $\gamma\delta$ T, and other immune cells were observed in cervical SCC. Among these immune cells, $CD4^+$ T, NK, and Treg cells were significantly related to the high-TMB group ($p < 0.005$) and had clinical relevance according to the statistical analysis. Yao *et al.* [23] reported high $CD8^+$ T lymphocyte infiltration, low PD-1 expression, and long survival in metastatic renal cell carcinoma in 2018. Similar results were observed in severe ovarian cancer patients with few $CD8^+$ TILs, PD-L1 positive tumours, and the shortest median overall survival [24–26]. The risk of death from breast cancer is markedly reduced due to the presence of $CD8^+$ T cells [27]. Simultaneously, $CD8^+$ T cells can

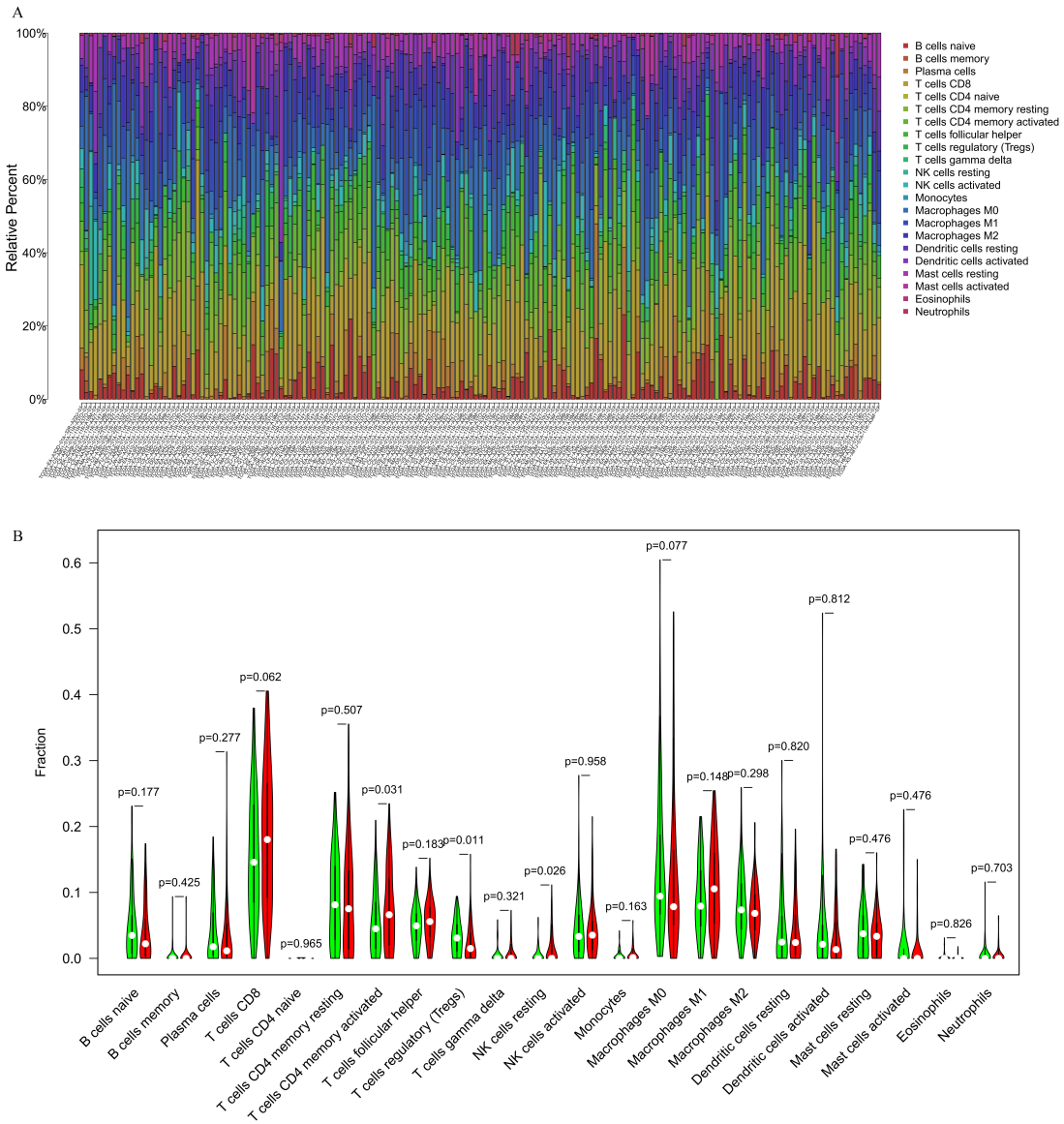

FIGURE 6. Immune infiltration. (A) Sum of each type of immune cell in cervical squamous cell carcinoma. Each bar graph shows the proportion of cells in each patient, 22 immune cells are represented in different colours. (B) Violin chart showing the difference of immune infiltration between high- and low-TMB groups; green is low, red is high, and $p < 0.05$ indicates a statistically significant difference.

prolong the survival of patients with liver and rectal cancers [28, 29]. These studies indicate that $CD8^+$ cells are related to the survival of tumour cells; however, further research is warranted to confirm this speculation.

Our study has certain limitations. First, a prognostic model of immune cells related to cervical squamous cell carcinoma samples could not be constructed. Second, experiments were not conducted to detect the interaction between immune genes and tumour cells. Third, the number of clinical specimens could not efficiently confirm the impact of TMB on patient prognosis and its possible immune link. Therefore, future studies should include numerous clinical specimens harbouring mutations to verify our findings. Finally, we could not successfully construct an immune prognostic model for cervical SCC. Nevertheless, our results reveal a correlation between high TMB levels and the development of cervical squamous cell carcinoma. We believe that our findings will promote the diagnosis and treatment of cervical squamous cell carcinoma in the future.

5. Conclusions

There is a connection between high TMB levels and the development of cervical squamous cell carcinoma; however, we could not present an immune prognostic model for cervical squamous cell carcinoma.

TABLE 4. KEGG pathway analysis.

Description	BgRatio	*p*-value	p.adjust	q-value
Renin-angiotensin system	23/8095	0.000904551	0.054273077	0.047607962
Breast cancer	147/8095	0.002762171	0.082865117	0.072688699
Ovarian steroidogenesis	51/8095	0.00441384	0.088276795	0.077435785
Ras signalling pathway	232/8095	0.009872238	0.148083569	0.129897867
Progesterone-mediated oocyte maturation	102/8095	0.016816793	0.18690266	0.163949702
MAPK signalling pathway	294/8095	0.018690266	0.18690266	0.163949702
Oocyte meiosis	131/8095	0.026890345	0.20692569	0.181513763
Neuroactive ligand-receptor interaction	341/8095	0.027590092	0.20692569	0.181513763
Phospholipase D signalling pathway	148/8095	0.03369055	0.224603668	0.197020762
Glycosaminoglycan biosynthesis – chondroitin sulfate/dermatan sulfate	20/8095	0.038841788	0.233050729	0.204430464
Focal adhesion	201/8095	0.05859528	0.282962423	0.248212652
Asthma	31/8095	0.059597283	0.282962423	0.248212652
Rap1 signalling pathway	210/8095	0.063320849	0.282962423	0.248212652
cAMP signalling pathway	219/8095	0.068175623	0.282962423	0.248212652
Aldosterone-regulated sodium reabsorption	37/8095	0.070740606	0.282962423	0.248212652
Long-term depression	60/8095	0.112320251	0.28525827	0.250226553
Longevity regulating pathway – multiple species	62/8095	0.115852203	0.28525827	0.250226553
Acute myeloid leukaemia	67/8095	0.12462453	0.28525827	0.250226553
Fc epsilon RI signalling pathway	68/8095	0.126369174	0.28525827	0.250226553
Central carbon metabolism in cancer	70/8095	0.129848689	0.28525827	0.250226553

AUTHOR CONTRIBUTIONS

SY, DL, XY, GW made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Due to the retrospective nature of the study, ethical approval was abandoned. The data used in this study were downloaded from the TCGA database (https://portal.gdc.cancer.gov/). The data used to support the findings of this study are available from corresponding websites upon request. The R software (https://www.r-project.org) was used for all statistical analyses.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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