Identification of An Immune-related Signature in Predicting Prognosis of Oral Squamous Cell Carcinoma Patients

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Research

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Abstract

Background: Immunotherapy is one of the most promising treatment strategies in cancer, including oral squamous cell carcinoma (OSCC). This study aims to identify an immune-related signature to predict clinical outcomes of OSCC patients.

Methods: Gene transcriptome data of OSCC tumour and normal tissues and corresponding clinical information were downloaded from The Cancer Genome Atlas (TCGA). Tumor Immune Estimation Resource algorithm (ESTIMATE) was used to calculate the immune/stromal-related scores. The immune/stromal scores and associated clinical characteristics of OSCC patients were evaluated. Univariate Cox proportional hazards regression analyses, least absolute shrinkage, and selection operator (LASSO) and receiver operating characteristic (ROC) curve analyses were performed to assess the prognostic prediction capacity. Gene Set Enrichment Analysis (GSEA) and Gene Ontology (GO) function annotation were used to analysis the functions of TME related genes.

Results: 11 predictor genes were identified in the immune-related signature and overall survival (OS) in the high-risk group significantly shorter than the low-risk group. ROC analysis showed the TME related signature has well ability of predicting the total OS of OSCC patients. What's more, GSEA and GO function annotation proved that immunity and immune-related pathways are mainly enriched in the high-risk group.

Conclusions: We identified an immune-related signature that was closely correlated with the prognosis and immune response to OSCC patients. This signature may have important implications for improving the clinical survival rate of OSCC patients and provide a potential strategy for cancer immunotherapy.

Background

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies arising in the oral cavity worldwide, and the incidence of OSCC has been increasing by at least 1% annually, especially in China [1, 2]. In recent years, common treatment options for OSCC patients include: surgery, adjuvant chemotherapy, radiotherapy, immunotherapy, etc. These treatment methods have reduced the mortality rate of OSCC patients to a certain extent [3]. However, despite the rapid progress in the treatment of OSCC, its mortality and incidence are still rising due to the tumour heterogeneity [4]. Therefore, in order to improve the survival rate of OSCC patients, the development of new predictive biomarkers to accurately predict the prognosis of patients is of great significance for improving the quality of life of OSCC patients.

Immunotherapy is one of the recent breakthroughs in cancer therapy and becoming a new promising approach in cancer treatment, including OSCC [5]. However, due to tumor heterogeneity and multifaceted immunosuppressive signals within the tumor microenvironment (TME), the efficacy of cancer immunotherapy is limited [6, 7]. TME refers to the cellular environment where tumor cells reside, and is composed of tumor cells, immune cells, fibroblasts, stromal cells, endothelial cells, and other components [8, 9]. TME plays a regulatory role in tumorigenesis and in the interactions between cancer cells and

surrounding components (such as cancer-associated fibroblasts, tumor-associated macrophages and tumor-associated lymphocyte), which promotes tumor development and metastasis [10, 11]. Immune and stromal cells are the most abundant cell types and their degree of infiltration in the TME has a clinical prognostic value in many cancer types [12]. Therefore, a deeper understanding of the cell components (including immune cells and stromal cells) and the immunosuppressive status of the TME is critical to improving the efficacy of immunotherapy in cancers [13]. The algorithm of Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data (i.e., ESTIMATE) is used to estimate the levels of infiltrating stromal and immune cells by calculating stromal/immune/ESTIMATE scores [14, 15]. Recently, immune-related signatures based on ESTIMATE scores have been developed and used to predict prognosis in certain cancers [16], but rarely been reported in OSCC.

In this study, the stromal and immune scores of 319 patients from the TCGA OSCC database were determined using the ESTIMATE algorithm. A total of 562 upregulated and 31 downregulated immune-related genes were identified. Moreover, functional enrichment analysis showed that the immune-related genes mainly played a critical role in immune response, activation/proliferation of immune-related cells, and chemokine activity. Finally, a prognostic 11 genes model (*AC103563.1*, *CCL22*, *FLT3*, *GALR2*, *IGKV1D.8*, *IGLV1.36*, *IGLV4.60*, *IL10*, *LINC00861*, *LINC01508*, and *MS4A2*) was constructed and confirmed to be significantly associated with the overall survival (OS) of OSCC patients. In conclusion, we screened the immune-related signature and explored the relationship between the screened immune-related signature and the prognosis of OSCC patients, and aim to use this signature as a potential prognostic biomarker and immune therapeutic target for OSCC patients.

Discussion

In the present study, differentially expressed immune-related genes in oral squamous carcinoma cancer (OSCC) samples were identified and were used to construct an immune-related signature, which perform well in predicting outcomes of OSCC patients respectively.

OSCC ranks as the eighth most common form of oral malignancy, with an estimated 500,000 new cases being reported annually, and OSCC is associated with high grade, rapid progression, poor treatment effects, and bad outcomes [17]. Clinical treatment of OSCC is often a combination of surgical resection with chemotherapy, however, the effect of these treatments is very limited and the 5-year survival rate of OSCC patients remains lower than 50% [18, 19]. There are several ways to predict the prognosis of OSCC patients, including but not limited to the following: 1) TNM stages ; 2) tumor grade ; 3) the number of lymph node metastasis ; 4) the expression level of key proteins, such as epidermal growth factor receptor, and P53 [20–22]. However, there is still lack of a unified and effective standard to determine the prognosis of OSCC patients.

TME plays a vital role in OSCC progression and consists of tumor cells and other components, such as immune cells, stromal scores, tumor-associated macrophages (TAMs), and carcinoma-associated fibroblasts (CAFs) [23, 24]. Moreover, tumor development, progression, and responses to

immunotherapies are regulated by cytokines, immune infiltration, TAMs, and CAFs within the TME [25, 26]. Therefore, in this study, we developed an immune-related signature to predict OSCC prognosis, which may help to improve the response rate to immunotherapy. ESTIMATE is an algorithm that has been used to calculate the immune and stromal scores in several cancers, including pancreatic ductal adenocarcinoma [27], gastric cancer [28] and colorectal cancer [29].

In this study, the stromal, immune, and ESTIMATE scores were calculated using the ESTIMATE algorithm in the TCGA-OSCC cohort. Then immune cells and immune-related genes of TME were associated with clinical indexes and outcomes of OSCC. We have several novel findings: 1) we found that many OSCC clinical indexes (such as TNM stages, grade stages, and male) were associated with the stromal, immune, and ESTIMATE scores, but the correlation was not significant (Fig. 1). However, high stromal, immune, and ESTIMATE scores correlated with better outcomes and longer progression-free survival in OSCC patients, indicating that the TME components, especially the stromal and immune cells affected the clinical outcomes of OSCC patients (Fig. 2). 2) DEGs in OSCC tumor and para-tumor tissue were identified. We classified OSCC patients into high- and low-score groups using the median stromal, immune, and ESTIMATE scores. The GO terms showed that DEGs in the high scores group regulated the T cell receptor complex and immunoglobulin complex (Fig. 3A). Besides, GO and KEGG pathway enrichment data indicated that DEGs in the high scores group also regulated the T cell receptor signaling pathway and viral myocarditis (Fig. 3B). 3) LASSO and Random forest (RF) algorithm analysis identified the 11 prognostic TME-related genes (AC103563.1, CCL22, FLT3, GALR2, IGKV1D.8, IGLV1.36, IGLV4.60, IL10, LINC00861, LINC01508, and MS4A2). In particular, high levels of GALR2, LINC01508, IGKV1D.8, and IGLV1.36 were found to be negatively correlated with the OS of OSCC patients, while high levels of AC103563.1, CCL22, FLT3, IGLV4.60, IL10, LINC00861, and MS4A2 were positively correlated with the OS in OSCC patients, suggesting that these immune-related genes may have high clinical relevance.

Indeed, the 11 immune-related genes have been implicated in many other cancers. For instance, CCL22 (C-C motif chemokine 22) controls T cell immunity by recruiting regulatory T cells to the tumor tissue and promoting regulatory T cell communication with dendritic cells in TME [30]. Higher infiltration rates of CCL22⁺ cells are reported to be associated with poor outcomes in cervical cancer patients [31]. Moreover, studies have shown the efficacy of flt3-L and CD40-L combination immunotherapy on prostate tumor growth in TME [32]. Besides, FLT3 is a potential targeted protein and has been reported as an immune-related prognostic gene in lung adenocarcinoma [33], indicating the clinical value of FLT3. Furthermore, Galanin receptor 2 (GALR2) is a G protein-coupled receptor that induces tumor growth and proliferation in SCCHN (squamous cell carcinoma of the head and neck) [34], and GALR2 has been identified as a novel target and biomarker for prostate, colon, and breast cancer screening [35]. In addition, IL10 is an important immune regulatory cytokine in TME [36], and a recent study has shown that IL10 promotes cancer cell metastasis and proliferation via immunosuppression, and also has a predictive value in clear cell renal cell carcinoma [37]. Recently, a study has shown that MS4A2 (the membrane-spanning 4 domains, superfamily A, number 2) gene is located on chromosome 11q13, a region that is linked to asthma-related phenotypes, and it plays an important role in the regulation of human mast cell

proliferation and survival [38]. However, very few studies report the role of MAS4A2 in TME. Other immune-related genes are relatively novel and scattered evidence has reported for their roles in human diseases. For example, IGLV1-36 gene is a potentially therapeutic option for POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes) syndrome [39]. Also, LINC01508, a long non-coding RNAs, the downregulation of LINC01508 contributes to cisplatin resistance in ovarian cancer [40]. Additionally, another recent study has shown that the LINC00861/miR513b5p axis inhibits cancer cell progression through the PTEN/AKT/mTOR signaling pathway in cervical cancer cells [41]. Besides, LINC00861 is also used as a biomarker to predict survival in patients with ovarian cancer [42], early diagnosis of Parkinson's disease [43] and breast cancer [44]. However, few studies have reported the role of AC103563.1, IGKV1D.8, and IGLV4.60 in TME (especially in OSCC), which will be our future directions.

There are some limitations to this study. Firstly, the OSCC immune-related signature has not been verified using the GEO and Array Express databases. Secondly, the OSCC TME related signature has not been verified by biological experiments both *in vivo* and *in vitro*. In our ongoing project, these immune-related genes and their potential mechanisms in OSCC are being verified. Thirdly, due to the limited samples in the OSCC subgroups, the prognostic value of the immune-related signature in some OSCC subgroups did not show any statistical significance. Therefore, large cohort of OSCC patient samples are needed for the future validations.

Conclusion

In summary, we propose immune-related signature in OSCC, which can be used as an independent prognostic biomarker to stratify risk subgroups based on OSCC patients. The 11 immune-related gene signatures have a predominant performance in predicting prognosis of OSCC patients, and it is possible to achieve more personalized and precise tumor immunotherapy in the future.

Methods

OSCC datasets acquisition and handing

The RNA-sequence dataset (TCGA, OSCC, n =319) was obtained from the National Cancer Institute GDC Data Portal (<https://portal.gdc.cancer.gov/>). Patients with complete follow-up data and survival status information were included in this analysis. Immune and stromal scores were calculated using the ESTIMATE algorithm [14].

Screening of differentially expressed genes (DEGs)

The package “limma” in R (version 4.0.2) was used to screen for differentially expressed genes (DEGs) between OSCC tissue and normal tissues. $FDR < 0.05$ and $|\log_2\text{FoldChange}| > 1$ were considered as

thresholds values for the identification of DEGs. Heatmap was used to visualize the expression profiles of the DEGs. Venn diagrams were used to show the overlaps among the DEGs [45].

Functional enrichment analyses and functional annotation of DEGs

Gene Ontology (GO), including biological processes (BPs), molecular functions (MFs), and cellular components (CCs) were determined using the R package “ggplot2”, “clusterProfiler” and “enrichplot”. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for enrichment analysis of pathways [46]. The search tool for retrieval of Interacting Genes/Proteins (STRING) (<https://string-db.org>) was used to predict the functional interaction of proteins. Cytoscape software was used to analyze the interaction scores of the PPI network nodes, and scores > 0.95 were considered the key PPI nodes [47].

Construction of the TME-related prognostic signatures

OSCC patients (n = 319) were divided into the high-risk and low-risk groups based on immune and stromal scores estimated by ESTIMATE. The Cox proportional hazards regression model was used to predict the OS of OSCC patients. $P < 0.05$ was statistically significant. Univariate Cox proportional hazards regression analyses was used to analyze the survival-related DEGs. The survival-related DEGs were put into the Cox proportional hazards model survival analysis with least absolute shrinkage and selection operator (LASSO) penalty. Finally the TME-related prognostic signature was constructed by weighting the Cox regression coefficients to calculate risk score for each patient. Using the cut-off value with the median value of risk score, patients were classified as low-risk and high-risk.

Statistical analysis

Univariate and multivariate Cox proportional hazards regression analyses were performed using the R package “survival”. LASSO Cox survival was performed using the R package “glmnet” and 1000-fold cross-validation was used. The R package “time-ROC” and “time AUC” were used to determine the ROC curves and area under the curve (AUC) for the predictive ability of the TME-related signatures. Heatmap was used to visualize the expression profiles of the DEGs. The alluvial diagrams were analyzed using “ggalluvial” in R. P -value < 0.05 was considered to be statistically significant and R software version 3.6.2 was used to perform the statistical analysis.

Results

Immune and stromal scores were not correlate with clinical data and prognosis of OSCC

To explore the relationship between Immune and stromal scores and the clinical characteristics and prognosis of OSCC patients. In this study, a total of 19467 RNAs were extracted from the OSCC-TCGA RNA-seq database. The immune and stromal scores of 319 OSCC patients were determined using the ESTIMATE algorithm. ESTIMATE scores ranged between -2340.425 and 4674.735, and immune scores ranged between -392.987 and 2705.005, and stromal scores ranged between -1947.438 and 1969.731. To explore the relationship between the immune, stromal scores, and overall survival (OS) in OSCC patients, the patients were categorized into the high and a low score group based on the ESTIMATE, immune, and stromal scores. Kaplan–Meier survival curves were used to calculate the OS of OSCC patients, and we found that the ESTIMATE, immune, and stromal scores are positively correlated with the OS of OSCC patients ($P = 0.635$, $P = 0.373$, $P = 0.668$, respectively) (Fig. 1A, 1B and 1C). Furthermore, for the clinical data extracted from the TCGA-OSCC database, we evaluated the association of the ESTIMATE, immune and stromal scores, and T stage, and we found that ESTIMATE ($P = 0.272$), immune ($P=0.208$) and stromal ($P=0.163$) scores were not significantly related to T stage (Fig. 2A-C). Moreover, we also compared the immune, stromal, and ESTIMATE scores with the gender of OSCC patients, and we found that there was no significant difference between ESTIMATE (Fig. 2D) or stromal (Fig. 2E) scores with gender. However, male patients had lower immune scores compared with the females (Figure 2E). We also noticed that there were no significant differences in ESTIMATE, immune and stromal scores with tumor grade (Fig. 2G-I). However, ESTIMATE (Fig. 2G) and immune scores (Figure 2I) of high-grade tumors were higher than low-grade tumors. The above results indicate that immune and stromal scores are not significantly related to the clinical characteristics and prognosis of OSCC patients.

Identification of immune-related differentially expressed genes

To uncover TME-related differentially expressed genes (DEGs), we performed “limma” in the R package. As a result, a total of 1,589 DEGs were found to be differently expressed between low- and high-stromal score groups (Fig. 3A), while the heat map (Fig. 3B) showed that 1,625 genes were differentially expressed in OSCC patients based on the ESTIMATE, immune and stromal scores. Compared with the low-TME score group, there were 2,171 (82.83%) (Fig. 3C) upregulated genes and 450 (17.17%) (Fig. 3D) down-regulated genes in the high-immune score group. These data indicate that immune-related differentially expressed genes had been obtained and identified.

Functional annotation of immune-related DEGs

To explore the biological functions of the above DEGs, GO term and KEGG pathway enrichment were performed using the R package “clusterProfiler”. As shown in Fig. 4A, the top 10 GO terms of the up and down-regulated DEGs are related to different biological process, cellular component, and molecular functions. Interestingly, the top 5 terms related to the biological process included lymphocyte activation, regulation of lymphocyte-mediated immunity, phagocytosis, complement activation, and B cell-mediated

immunity (Fig. 4A, top panel). In addition, the top 5 terms related to a cellular component included immunoglobulin complex, external side of plasma membrane, plasma membrane signaling receptor complex, T cell receptor, and immunoglobulin complex and circulating (Fig. 4A, middle panel). The top 5 terms related to molecular functions included antigen binding, immunoglobulin receptor binding, carbohydrate-binding, immune receptor activity, and cytokine receptor activity (Fig. 4A, bottom panel). On the other hand, the top 30 KEGG pathways for the DEGs related to the up and down-regulated DEGs included immune/inflammation related pathways, including B cell receptor signaling pathway, T cell receptor signaling pathway, intestinal immune network for IgA production, natural killer cell-mediated cytotoxicity, and C-type lectin receptor signaling pathway (Fig. 4B). The above data indicate that the signaling pathways and biological behaviors enriched by immune related DEGs are related to tumor microenvironment or immune functions.

Protein-Protein Interaction (PPI) analysis of the immune-related DEGs

To study the interaction between DEGs, and systematically conduct a comprehensive study on the crossover gene, all 167 intersection genes were uploaded to the STRING database to construct a PPI network. In our present study, the Search Tool Retrieval of Interacting Genes (STRING) database was used to draw the PPI network (Fig. 5). There was a total of 23 genes in the network with more than 20 interconnected nodes, including *SUCNR1*, *P2RY13*, *GPR83*, *GALR2*, *FPR3*, *CCR8*, *CCL13*, *C5AR1*, *ADORA3*, *P2RY12*, *CXCR3*, *CX3CR1*, *CCR4*, *ADRA2A*, *CCR1*, *FPR1*, *CCR2*, *CCR5*, *ITGB2*, *FCER1G*, *C3AR1*, *C3* and *FPR2* (Figure 6).

Construction of the risk score prognosis model

To analyze the survival-related immune-DGEs, Univariate Cox proportional hazards regression model survival analyses was performed. a total of 23 prognostic genes were identified as survival related DEGs: *AC093278.2*, *AC103563.1*, *AL365361.1*, *BTLA*, *CCL22*, *CCR4*, *CD5*, *CELF2*, *F5*, *FCRL3*, *FLT3*, *GALR2*, *IGKV1D-8*, *IGLV1-36*, *IGLV4-60*, *IL10*, *LINC00861*, *LINC01508*, *MS4A2*, *P2RY14*, *P2RY8*, *PPP1R16B* and *RUBCNL*(Fig. 7A). The least absolute shrinkage and selection operator (LASSO) was then used to exclude the over-fitting false positives data (Figure 7B-C). Finally, a prognostic model with 11 survival related DEGs was constructed for predicting the OS of OSCC patients. The risk scores were calculated for each patient as follows: risk score = $-0.0418 \times AC103563.1 - 0.0289 \times CCL22 - 0.2829 \times FLT3 + 0.03681 \times GALR2 + 0.0048 \times IGKV1D.8 + 0.0067 \times IGLV1.36 - 0.0139 \times IGLV4.60 - 0.0456 \times IL10 - 0.2040 \times LINC00861 + 0.1421 \times LINC01508 - 0.0245 \times MS4A2$. The median value was chosen as the cut-off value and the entire cohort was classified into two risk groups accordingly.

Survival analysis of immune-related model

To test the effect of the above model on the evaluation of OSCC patients in the future, we analyzed the outcome survival (OS) of the high-risk group and the low-risk group OSCC patients. The risk distribution, survival status, and gene expression pattern are shown in Fig. 8 A-C. The scatter plot (Fig. 8A) shows that most of the patients in the high-risk score group died and most of the patients in the low risk group survived during 15 years follow-up. The heat map (Fig. 8C) shows that 7 ARGs were highly expressed in the low risk group while 4 ARGs were highly expressed in the high-risk group. To determine the relationship between immune-related DEGs and OS in OSCC patients, we used the Kaplan-Meier method to plot the survival curves using data obtained from the OSCC-TCGA database. Kaplan-Meier plots show that patients in the high-risk score group have a significantly poorer OS than those in the low risk score group (Fig. 8D). Moreover, the AUC for 1-year, 2-year, and 3- year PFS were 0.64, 0.63, and 0.65 respectively (Fig. 8E). Thus, these data suggest that the 11 DEGs prognostic model performs well in predicting OS in OSCC patients.

Abbreviations

OSCC, oral squamous cell carcinoma; **TCGA**, The Cancer Genome Atlas; **TME**, tumor micro-environment, **BP**, biological processes; **CC**, cellular components; **DEGs**, differentially expressed genes; **GO**, gene ontology; **HR**, hazard ratio; **KEGG**, Kyoto Encyclopedia of Genes and Genomes; **LASSO**, Least Absolute Shrinkage and Selection Operator; **MF**, molecular functions; **OS**, overall survival; **PPI**, protein and protein interaction; **ROC**, receiver operating characteristic.

Declarations

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Ethics approval and consent to participate

Not necessary.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

Availability of data and materials

The raw data of this study are derived from the TCGA database (<https://portal.gdc.cancer.gov/>), which are publicly available databases.

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Authors' Contributions

Qilin Li and Xinyao Meng: Data curation; formal analysis; investigation; visualization; writing-original draft. Weimin Chen: Data curation; methodology; software; validation. Xin Chen and Jing Mao: Conceptualization; data curation; formal analysis; funding acquisition; writing review and editing.

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Figures

Figure 1

KM survival curve of OSCC patients based on their immune/stromal/ESTIMATE scores. The KM curve of the OS of OSCC patients in low and high groups (A) ESTIMATE scores, (B) immune scores, and (C) stromal scores.

Figure 2

The relationship between immune/stromal/ESTIMATE scores and clinical features of OSCC patients. Relationship between immune/stromal/ESTIMATE scores and OSCC TNM stages (A-C), patient genders (D-F), and tumor grades (G-I).

Figure 3

Venn diagram and heatmap of the gene expression based on the immune and stromal scores. Heatmap diagrams showing the differentially expressed genes (DEGs) based on the (A) immune scores and (B) stromal scores ($p < 0.05$). Venn diagrams reveal the number of (C) upregulated DEGs and (D) downregulated DEGs based on the immune and stromal score groups. In the heatmaps, red indicates higher levels of expressed genes, black indicates similar expression levels of genes, and green indicates lower levels of expressed genes.

(a)

(b)

Figure 4

Functional analysis of the DEGs. (A) Gene ontology (GO) enrichment analysis and (B) KEGG pathway analysis for DEGs.

Figure 6

The key nodes of PPI analysis. Key nodes and their number in the PPI network.

Figure 7

Univariate Cox and LASSO Cox survival analyses for the DEGs. (A) Univariate Cox survival analyses for the differentially expressed and survival-related DEGs. (B-C) LASSO Cox analysis identified 11 DEGs with the best prognostic value. 11 DEGs were identified as prognostic factors of OSCC patients. Protective factors mean the genes' HR < 1, while risk factors mean the genes' HR > 1 in OSCC.

Figure 8

Effect evaluation of the 11-gene signature. (A-C) The survival status, risk scores of each patient, and heatmap of TME-DEGs expression pattern (D) Kaplan-Meier survival curve for OS of patients in the low-risk group and high-risk group. (E) Prognostic value evaluation of the model using time-specific ROC curves and dynamic AUC lines. The time-dependent ROC curves are based on 1, 2, and 3 years of follow-up and the dynamic AUC lines of OSCC patients.