A Signature of Immune-related Gene Pairs (IRGPs) for Risk Stratification and Prognosis of Oral Cancer Patients

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Abstract

Background: With low response to present immunotherapy, it is imperative to identify new immune-related biomarkers for more effective immunotherapies for oral cancer.

Methods:RNA profile for 390 oral cancer patients and 32 normal samples were downloaded from the Cancer Genome Atlas (TCGA) database and differentially expressed genes (DEGs) were analyzed. Immune genesets from Immportrepository were overlapped with DEGs. After implementing univariate Cox analysisand the least absolute shrinkage and selection operator (LASSO) Cox regression analysis, key immune-related gene pairs (IRGPs) among the overlappedDEGs for predicting the survival riskwere obtained. Then, the cutoff of risk score was calculated by receiver operating characteristic (ROC) curve to stratify oral cancer patients into high and low-risk groups. Multivariate Cox analysis was used to analyze independent prognostic indicator fororal cancer.Besides, infiltration of immune cells, functional annotation and mutation analysis of IRGPs were conducted.Biological function correlated with IRGPs were enriched by Gene Set Enrichment Analysis (GSEA) method.

Results: We identified698 differentially expressed genes (DEGs)in response to oral cancer. 17IRGPs among the DEGs were identified and integrated into a risk score model. Patients in high-risk group have significantly worse prognosis than those in low-risk group. Meanwhile, IRGPs model was identified as an independent prognostic factor for oral cancer. Different infiltration pattern of immune cells were found between high- and low-risk groups that more types of T and B cells were enriched in low-risk group. More immune-related signaling pathways were highly enriched in low-risk group. And Tenascin C (TNC) was the most frequently mutated genes. We have developed a novel 17-IRGPs signature for risk stratification and prognostic predication of oral cancer.

Conclusion: Our study provides a foundation for improved immunotherapy and prognosis and is beneficial to the individualized management of oral cancer patients.

Introduction

As one of the most common fatal cancer, oral cancer is estimated to lead to 53,260 new cases and 10,750 deaths in 2020 [1]. Several factors have been reported to be the main causes for oral cancer, such as smoking, pan chewing, drinking and human papillomavirus (HPV) persistent infection[2]. With high rate of early occurrence and metastasis, the prognosis of oral cancer was poor with a low 5-year survival rate at about 50% [3]. Despite great improvements have been made for treatments of oral cancer, the survival outcome is still disappointing. Over the past decade, immunotherapy has brought great breakthrough in treatment of cancers.

To strengthen immunotherapy, the researchers have been focusing on the investigation of tumor microenvironment (TME), which is mainly composed of cytokines and various cells, including immune cells, tumor-associated fibroblasts and extracellular matrix components[4]. TME plays key roles of various biological behaviors of cancer cells, such as mediating survival, apoptosis, invasion,

angiogenesis and metastasis[5]. Of note, by regulating the crosstalk of tumor cells and stroma, TME is highly involved in immune evasion and suppression of tumor cells, which is critical to tumor initiation, progression and the response to different cancer therapies[5]. Accumulating evidence has implicated some immune cells and immune-related genes (IRGs) important to the progression of oral cancer, such as myeloid-derived cells, TGFβ and CCL3 [4, 6-8]. Considering the importance of immune system in oral cancer, investigational and Food and Drug Administration (FDA) has approved some Programmed death-1 (PD-1) inhibitors to treat advanced oral cancer[9]. PD-1 is a receptor expressed on T and B cell surface, which would inhibit activity of these lymphocytes by binding to its ligands[9]. However, the therapeutic effects were poor with a low response rate at about 20%. Therefore, to improve diagnosis and prognosis of patients with oral cancer, identifying new immune-related biomarkers is imperative for more effective immunotherapies.

In the present study, we performed differentially expressed genes and the least absolute shrinkage and selection operator (LASSO) Cox regression analyses to screen out IRG pairs (IRGPs) associated with prognosis of oral cancer. Then 17 IRGPs were obtained and integrated into a model for division of risk groups and prognostic predication of oral cancer patients.

Materials And Methods

Data collection

Our study was based on the Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) database. RNA profile from 390 oral cancer and 32 normal patients were downloaded.

Differentially expressed genes (DEGs) in response to oral cancer

DEGs were analyzed by using “edge” package in R. DEGs between oral cancer and normal samples were screened out with the criterions: $|\log_2 \text{FC (fold-change)}| > 1$ and $P < 0.05$.

Screening forIRGs

Immport database was used to download IRGs and 2,498 IRGs were obtained. The intersection between 2,498 IRGs and DEGs were analyzed with Venn diagram. Subsequently, to overcome technical bias for gene expression, we analyzed the IRGs in pairs. In IRGPs, the ratio between the expression of two IRGs in one patient was set to 1 when the expression level of latter gene was lower than that of the former. If not, the ratio was set to 0. When the ratios of IRGP were 0 or 1 in more than 80% of the samples, the IRGP was removed. And the remaining IRGPs were selected as candidates.

LASSO Cox Regression analysis of IRGPs

“Caret” package in R was applied to randomly divide the 387 oral cancer samples with survival informationinto two groups training and test sets at a ratio of 1:1.Univariate Cox analysis was performed to examinehow IRGPs was correlated with the survival of oral cancer. IRGPs significantly associated with

the prognosis (P<0.01) were used to construct LASSO Cox regression model. LASSO regression analysis shrinks the subset that reduces the complexity of the model to increase the prediction accuracy and interpretability. We performed 10-fold cross-validation in LASSO model. Then we calculated the risk score of an individual patient based on the formula as follows: Risk Score= α1×ratioIRGP1+α2×ratioIRGP2+…+αn×ratioIRGPn (αwas the coefficient from LASSO analysis, and ratioIRGP was the relative expression of the IRGP).Subsequently, receiver operating characteristic (ROC) curve was plot using “pROC” package in R to identify discrimination threshold that distinguishes high-risk patients with low-risk patients. Multivariate Cox analysis was applied to assess the prognostic value of the IRGP model.

Immune cell infiltration in oral cancer

The enrichment of immune cells in the two risk groups were evaluated by using CIBERSORT in R. CIBERSORTestimates the percentage of 22 types of immune cells with deconvolution method. The radar chart was developed to describe the relative abundance of the immune cells in high- and low-risk groups. When P value <0.05, the results were seen as significant.

Gene set enrichment analysis (GSEA)

GSEA analysis was utilized to analyze the biological function of IRGPs including Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. When P value <0.05, the results were seen as significant.

Mutation analysis

Mutation analysis was conducted using the IRGs in the model. The “maftoools” package in R was installed tovisualize Mutation Annotation Format (MAF), which contained the mutation data in high-risk group.

Results

Identification of immune-related gene pairs in oral cancer

Total 390 oral cancer and 32 normal samples from TCGA database were used to identify DEGs.Total 698 DEGs including 257 up-regulated genes and 441 down-regulated genes were identified between oral cancer and normal samples with the criterions $|\log_2 FC| > 1$ and $P < 0.05$. The distribution of DEGs were visualized by volcano plot (Fig. 1A). Subsequently, 2,498 IRGs were downloaded from Immport database. And 84 immune-related DEGs were obtained by comparing the 698 DEGs with 2,498 IRGs (Fig. 1B). Then to minimize the bias of gene expression from different platforms, the IRGs were analyzed in pairs. And 1,490 IRGPs were established with the criterions described in Materials and Methods.

Construction of immune-related gene pairs model

The 387 tumor samples with survival information were randomly divided into training (n=194) and test (n=193) sets. After univariate Cox analysis in training set, 49 IRGPs were significantly associated with the prognosis of oral cancer (P<0.01). LASSO Cox regression analysis was performed using the 49 IRGPs. And the predication accuracy was evaluated by 10-fold cross-validation (Fig.2A). 17 IRGPs were obtained and integrated into the prediction model (Table 1).

Then the risk score (prognostic index) was calculated as the sum of relative expression of IRGP multiplying its coefficient in the LASSO Cox regression model (Table 1). It is obvious that the coefficients of 6 IRGPs were negative, indicating the relative expression of the IRGPs were negatively associated with the survival of oral cancer patients. And coefficients of the other 11 IRGPs were positive, suggesting a positive correlation. The top 3 IRGPs most strongly and positively correlated with outcome of oral cancer were TNFRSF12A|TNC, PLAU|DEFB1 and TAP|IGHG2.And OASL|SPP1 was the most strongly and negatively correlated with the prognosis of oral cancer. One-year dependent ROC curve was plot to stratify the patients into high- and low-risk groups. Fig. 2B revealed that the optimal cutoff of risk score was identified as 1.016. The area under the curve (AUC) value for 1-year overall survival rate in training set was 0.774 (Fig. 2C). With the cutoff in both training and test cohorts, high-risk patients had significant lower overall survival rates than low-risk patients (Fig. 2D and E, P<0.01). And the number of deaths was increased with the increasing risk score in training and test sets (Fig. 2F and G).

Further, univariate and multivariate Cox analysis was applied to examine the prognostic value of IRGP model in oral cancer. As shown in Fig.3A and B, age and IRGP riskScore were independently associated with survival outcome of oral cancer in training set (P<0.05). And age, grade, node stage and IRGP riskScore could be independent prognostic factors (Fig.3C and D, P<0.05) in test set. Therefore, IRGP model can be seen as an independent factor for oral cancer prognosis.

Immune cell filtration in high- and low-risk groups

It is well accepted that the filtration of lymphocytes into tumor was highly associated with prognosis of cancer [10]. Next, the association of the 17 IRGPs with infiltrating immune cells in oral cancer was examined with CIBERSORT. Fig. 4A displayed that the abundance of 22 types of immune cells varied a lot in different patients. Moreover, we detected the strongest positive correlation between CD8 T cells and activated memory CD4 T cells (Fig. 4B). Naive B cells was also strongly and positively correlated with plasma cells (Fig. 4B). Besides, the strongest negative correlation was found between the CD8 T cells and M0 macrophages (Fig. 4B). And M0 macrophages was also negatively correlated with M1 macrophages and activated memory CD4 T cells(Fig. 4B). Among the 22 types of immune cells, 4 types of immune cells including M0 macrophages, activated mast cells, eosinophils and naïve CD4 T cells were highly enriched in high-risk group in comparison to low-risk group (Fig. 4C). Whereas, the low-risk group highly expressed 6 types of immune cells including naïve B cells, resting mast cells, plasma cells, activated memory CD4 T cells, CD8 T cells, and regulatory T cells (Tregs) compared to high-risk group (Fig. 4C). The radar chart described the relative abundance of the immune cells in the two risk groups (Fig. 4D).

Functional annotation of the IRGP model

GSEA method was used to discover biological function of the 17 IRGPs in oral cancer. GO analysis displayed that various immune-related GO terms including T cell activation involved in immune response, positive regulation of T cell proliferation and adaptive immune response were significantly enriched in low-risk group (Fig. 5A, Supplementary Table1). Similarly, KEGG pathway analysis revealed that several immune-related signaling pathways were significantly changed in response to the IRGP model. As shown in Fig. 4B, natural killer cell mediated cytotoxicity, cell adhesion molecules cams, T cell receptor signaling pathway, etc. were significantly enriched in low-risk group(Fig. 5B, Supplementary Table2). These results offered a guide for investigation of molecular mechanisms for how the 17-IRGPs signature affected the prognosis of oral cancer patients.

Mutation analysis

Considering the importance of tumor mutation burden in immunotherapy, the mutation data of the 17 IRGPs were analyzed inhigh-risk group. The waterfall plot revealed that missense mutation, nonsense mutation and splice site accounted for the majority of the mutation types (Fig. 6A and E). The variant type was concentrated entirely on single nucleotide polymorphisms (SNPs, Fig. 6B). In terms of SNPs, C>T transition represented the largest proportion (Fig. 6C). The maximum number of mutations in each sample was 3 and the median number was 1 (Fig. 6D). Moreover, Tenascin C (TNC) possessed the largest number of mutation with the highest mutation frequency (Fig. 6F and G).

Discussion

Oral cancer is a malignant tumor with suppressed immune surveillance. PD-L1 was highly expressed in oral cancer cells and involved in immune escape [11]. In 2017, nivolumab targeting PD-1 was approved to treat advanced oral cancer with metastasis or recurrence [12]. Two years later, pembrolizumab, another PD-1 antibody come into use for oral cancer [13, 14]. However, therapeutic effects of these PD-1 inhibitors would be mitigated in patients with certain characteristics, such as high expression of inhibitory T-cell receptors [4]. Thus, it is imperative to identify immune-related markers for more individualized immunotherapy of oral cancer.

To enhance the robustness of prediction, IRGPs were applied to be integrated to the prognostic signature. Herein, gene expression data were not required in this model, instead the relative expression of two genes were needed. The application of IRGPs brought about two advantages for prognostic prediction. Firstly, we did not have to perform standardization for gene expression from different platforms. Secondly, the effect of technical bias of different platforms was minimized on gene expression.

At first, 17 IRGPs associated with prognosis of oral cancer were screened out with DEG and LASSO analyses and integrated into a risk score model. The oral cancer patients were separated into high- and low-risk groups based on the critical risk score point. And a higher overall survival rate was detected in low-risk patients. Besides, the mortality risk increased with higher riskScore. Subsequently, univariate and multivariate Cox analysis was performed to assess the correlation between IRGP-model and clinical parameters including age, gender, stage, tumor and node status. The results revealed that IRGP-model

can be considered as an independent prediction factor for oral cancer prognosis.IRG-based model was widely applied in stratification for various cancers, such as low-grade glioma, ovarian cancer and melanoma [15-17]. Of note, multiple studies have developed prognostic models for oral cancer frequently based on IRGs. For example, Yao et al. integrated four IRGs and node status to develop a prognostic model for head and neck squamous cell carcinoma (HNSCC)[18]. Huang et al. stratified patients with oral squamous cell carcinoma into high- and low-risk groups based on 9 IRGs [19]. However, there is on study based on IRGPs for oral cancer. Zhang et al. constructed a 14-IRGPs signature for HNSCC with a relatively dismal predictive performance (AUC=0.7<0.75). In our study, the 17-IRGPs signature had a good predictive performance for oral cancer (AUC=0.774>0.75).

In the signature, the top 3 IRGPs strongest correlated with riskScore were TNFRSF12A|TNC (positive), PLAU|DEFB1 (positive) and OASL|SPP1 (negative). Given the negative correlation between riskScore and prognosis, TNFRSF12A, PLAU and SPP1 were correlated with prognosis negatively. Whereas, TNC, DEFB1 and OASLwere correlated with prognosis positively. Meanwhile, mutation analysis revealed that TNC was identified as the most frequently mutated gene in high-risk group. Accumulating studies have reported the association of these genes with prognosis in various cancers. Several studies have reported that the high expression of TNFRSF12A andPLAU were involved in worse outcome for HNSCC patients [18, 20]. Xu et al. found that SPP1 was increased and TNC was decreased in OSCC tissues[21]. The up-regulation of SPP1 was involved in worse outcome for HNSCC patients. And the level of TNC expression was decreased with higher stage [21]. However, Chi et al. identified the up-regulation of TNC and OASL in saliva samples from OSCC patients [22]. This is rational that the expression of genes are different in different tissues. Human beta-defensin-1 (DEFB1) has been reported to be a potential tumor suppressor in prostate and renal cancer [23, 24]. And recently, DEFB1 was confirmed to be positively and independently associated with OSCC survival [25]. In addition, the abnormal expression of most other genes have been also reported in various cancers, including oral cancer [26-28]. Collectively, most genes in the model have been investigated in various cancers. In our study, the integration of these 17 gene pairs suggested the important role of immune system in oral cancer.

Considering the critical role of TME in cancers, we investigated the abundance of infiltrative immune cells in oral cancer. Among the 22 types of immune cells, 10 types were significantly different between high- and low-risk groups. Many studies have explored the prognostic values of immune cells. Song et al. has demonstrated that naïve B cells and regulatory T cells (Tregs) indicated a favorable survival in head and neck cancer [29].Whereas, eosinophils and activated mast cells indicated a poorer outcome [29]. Besides, Eosinophils have been reported to be involved in metastasis and negatively associated with cancer prognosis [29, 30]. Consistently, in our current study, after grouping based on our model, the abundance of naïve B cells and regulatory T cells (Tregs) was significantly enhanced in low-risk patients. And eosinophils and activated mast cells were highly expressed in high-risk group. Memory T cells are reported to play roles in eliminating tumor cells and activated memory CD4 T cells indicates an improved survival in several cancers [31, 32]. We also identified the high enrichment of activated memory CD4 T cells and CD8 T cellsin low-risk group. In addition, we detected the significant difference of mast cells, plasma cells and naïve CD4 T cells between high- and low-risk groups. Mast cells could influence tumor

progression by regulating inflammation[33]. Activated mast cells was found to be associated with poor prognosis of several cancer [33, 34]. Similarly, in our study, activated mast cells were enriched in high-risk group, whereas resting mast cells were highly expressed in low-risk group. The results were consistent with subsequent GSEA analysis that various immune-related GO terms and signaling pathways were enriched in low-risk group, including T cell activation involved in immune response, positive regulation of T cell proliferation, adaptive immune response, natural killer cell mediated cytotoxicity, cell adhesion molecules cams, T cell receptor signaling pathway, etc. These results demonstrated that the immune cells significantly enriched in low-risk group may play promising roles in improving the prognosis of oral cancer, which awaits further investigation.

Tumor mutation burden (TMB) has been investigated as a promising biomarker in various cancers [35, 36]. SNPs is a common type of gene variation, which are caused by point mutations. SNPs has been reported to be associated with tumorigensis and prognosis of cancers, including oral cancer [37, 38]. And TNC was identified to be the most frequent mutated gene among the 17 IRGPs in oral cancer. There is a possibility that the SNPs affected the immune cells infiltration in oral cancer based on a previous study [39]. To our best knowledge, this is the first study that constructed a IRGPs-based prognostic model for oral cancer and comprehensively analyzed infiltration of immune cells and TMB. However, there are several limitation in our study. Although the 17-IRGP signature were constructed and validated based on TCGA database, an individual database should be introduced to validate our model. Our study is retrospective, and needs to be corrected for clinical application.

Conclusion

In summary, we have developed a novel 17-IRGPs signature for risk stratification and prognostic predication of oral cancer with excellent predictive performance. High risk score was independently associated with worse prognosis of oral cancer. Meanwhile, tumors in low-risk group were infiltrated by more types of immune cells and associated with more immune-related pathways. And the model was an independent factor for oral cancer prognosis. Our study provides a foundation for improved immunotherapy and prognosis and is beneficial to the individualized managementof oral cancer patients.

Declarations

Acknowledgments

None.

Authors' contributions

Yanling Yu and Xuri Wang conceptualizated this study and drafted and wrote this manuscript. Jing Tian, Yanni Hou and Xinxin Zhang curateddata and performed analyses. Linhua Li provided the methodology. Peifu Cong and Lei Ji visualizated the figures and tables.

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Availability of data and materials

The data analyzed in this study are available from thecorresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no conflicts of interest.

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Tables

Table 1 Seventeen immune-related gene pairs were screened out by LASSO regression analysis.

Gene	Coefficient
FAM3D|FABP3	-0.123212435
APOD|PTGDS	0.102940062
APOD|IDO1	0.114162789
APOD|CCL19	0.175640735
PLA2G2A|FABP3	-0.154866389
PLAU|DEFB1	0.411294017
MMP9|SLURP1	0.118514628
SORT1|IGHG4	0.048939964
ULBP2|IDO1	0.02698727
TYMP|DES	-0.039797787
OASL|SPP1	-0.345276271
CLDN4|IGHG2	0.074023063
TNFRSF12A|TNC	0.467337778
RSAD2|TPM2	-0.049203572
STAT1|IGHG2	0.204728651
TAP1|IGHG2	0.303345321
CXCL11|IL1A	-0.22016231

Figures

Figure 1

Potential immune-related gene pairs (IRGPs) were identified. (A) Volcano plot of the gene expression in response to oral cancer. The down-regulated and up-regulated genes were marked by green and red dots, respectively. (B) The shared genes between 698 differentially expressed genes and 2,498 IRGs from Immport database were analyzed by Venn diagram.

Figure 2

Construction of immune-related gene pairs model. (A) Predication accuracy was evaluated by 10-fold cross-validation in LASSO model. (B) One-year dependent ROC curve was plotted to calculate the optimal cutoff of risk score (red point). (C) Area under the curve (AUC) value for 1-year overall survival rate was calculated in training set. (D, E) High-risk patients had lower overall survival rates than low-risk patients in both training (D) and test (E) sets. (F, G) the number of deaths was increased with the increasing risk score in training (F) and test (G) sets. $P<0.05$.

Figure 3

The prognostic value of IRGP model in oral cancer was examined by univariate and multivariate Cox analysis. (A, B) Age and IRGP riskScore were independently associated with survival outcome of oral cancer in training set. (C, D) Age, grade, node stage and IRGP riskScore could be independent prognostic factors. $P<0.05$.

Figure 4

Different infiltration pattern of immune cells were found between high- and low-risk groups. (A) The abundance of 22 types of immune cells varied a lot in different patients. (B) The correlation between the 22 types of immune cells was analyzed between high- and low-risk groups. (C) Comparison of the abundance of the immune cells between the two risk groups. (D) The relative abundance of the 22 types of immune cells in the two risk groups was described by radar chart.

Figure 5

Functional annotation of the IRGP model was analyzed by GSEA method. (A) Various immune-related GO terms including T cell activation involved in immune response, positive regulation of T cell proliferation and adaptive immune response were significantly enriched in low-risk group. (B) Several immune-related signaling pathways including natural killer cell mediated cytotoxicity, cell adhesion molecules cams, T cell receptor signaling pathway, etc. were significantly enriched in low-risk group. P<0.05.

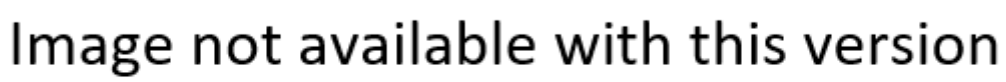

Figure 6

Mutation data of the 17 IRGPs from high-risk group were analyzed. (A) The mutation types were described by a waterfall plot. (B) The variant type was concentrated entirely on single nucleotide polymorphisms (SNPs). (C) C>T transition represented the largest proportion of SNPs. (D) The maximum number of mutations in each sample was 3 and the median was 1. (E) The number of each variation in each sample were displayed. (F, G) Top ten mutated genes were shown.

Supplementary Files

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

- SupplementaryTable2.xls
- supplementaryfiles.zip