Journal of Dental Sciences 18 (2023) 57–64

Available online at www.sciencedirect.com

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Original Article

Methylation related genes are associated with prognosis of patients with head and neck squamous cell carcinoma via altering tumor immune microenvironment

Xudong Tian [a,b†], Congyu Shi [a,b†], Shan Liu [a,b], Chengzhi Zhao [a,c], Xiaoyi Wang [a,b], Yubin Cao [a,c*]

[a] *State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Sichuan University, Chengdu, China*
[b] *Department of Head and Neck Oncology, West China College of Stomatology, Sichuan University, Chengdu, China*
[c] *Department of Oral and Maxillofacial Surgery, West China College of Stomatology, Sichuan University, Chengdu, China*

Received 1 July 2022; Final revision received 12 July 2022
Available online 5 August 2022

KEYWORDS
Epigenomics;
Methylation;
Computational biology;
Prognosis;
Immune checkpoint molecule;
Head and neck cancer

Abstract *Background/purpose:* Analysis of methylomes may enable prognostic stratification in patients with head and neck squamous cell carcinoma (HNSCC). This study aimed to identify methylation-related differentially expressed genes (mrDEGs), and to assess their efficacy in predicting patients' survival, tumor immune microenvironment alterations and immune checkpoints in patients with HNSCC.
Materials and methods: The methylome and transcriptome data of 528 HNSCC and 50 normal samples from TCGA database were used as training cohort. We identified mrDEGs and constituted a risk score model using Kaplan-Meier analysis and multivariate Cox regression. The prognostic efficacy of the risk score was validated in GSE65858 and GSE41613. We determined the enrichment of previously defined biological processes of mrDEGs. We separated the HNSCC patients into low-risk and high-risk groups and compared their immune cell infiltration and immune checkpoints' expressions.
Results: The risk score model was constituted by nine prognostic mrDEGs, including *LIMD2*, *SYCP2*, *EPHX3*, *UCLH1*, *STC2*, *PRAME*, *SLC7A4*, *PLOD2*, and *ACADL*. The risk score was a significant prognostic factor both in training ($P < 0.001$) and validation dataset (GSE65858: $P = 0.008$; GSE41613 $= 0.015$). The prognostic mrDEGs were enriched in multiple immune-

* Corresponding author. State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Department of Head and Neck Oncology, West China College of Stomatology, Sichuan University, No. 14, 3rd Section of Ren Min Nan Rd, Chengdu, 610041, China.,
E-mail addresses: yubin.cao@scu.edu.cn, yubin.cao@qq.com (Y. Cao).
† These two authors contributed equally to this work.

https://doi.org/10.1016/j.jds.2022.07.010

associated pathways. Effector immune cells were increased in low-risk patients, including *CD8*+ T cells, activated *CD4*+ T cells, and plasma cells, whereas tumor associated M2 macrophages were recruited in the high-risk group. Expressions of immune checkpoints were generally higher in low-risk patients, including *CTLA-4*, *PD-1* and *LAG3*.
Conclusion: The mrDEGs can stratify HNSCC patients' prognosis, which correlates with alterations in tumor immune infiltrations and immune checkpoints.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the common malignant cancers worldwide. Nearly 300,000 people die of HNSCC each year.[1] Over 63,000 new cases of HNSCC were reported in the United Stated per year, accounting for 3.6% of all sites.[2] Due to radical ablation of lesions, survivors suffered severe pain, changed voice, unfavorable swallowing, decreased social activities, and a lower quality of life.[3] It is urgent to further explore the etiological mechanism of HNSCC and provide novel treatment targets.

Over recent years it has been proved that epigenetic mis-programming is a core component of cancer initiation and progression.[4] DNA methylation remains the primary marker of epigenetics that can be measured reliably and genome-widely in large samples.[5] Analysis of methylomes enabled prognostic stratification in patients with other cancers.[6,7] However, studies rarely evaluated methylation biomarkers by analyzing methylome profiles in HNSCC patients.

DNA methylation may be related to the tumor microenvironment.[8] Infiltrating immune cells within the local tumor microenvironment contribute to proliferation, cell death resistance, invasion, metastasis and blood vessel formation in the dynamic process of malignancy.[9] Altered signaling pathways in tumor cells contribute to producing a suppressive microenvironment, mainly composed of inhibitory cells (such as active T cells), for tumor growth.[10] The DNA methylation may participate in alterations of infiltrating immune cells, reflecting a specific immune response to the cancer cell presence.[11] Tumor immune microenvironment (TIME) are associated with therapy responsiveness of immune-checkpoint blockade.[12]

Therefore, this study aims to perform a genome-wide integrated transcriptome and methylome analysis to identify methylation-related differentially expressed genes and assess their efficacy in predicting patients' survival, TIME alterations, and responses to immunotherapy and chemotherapy in HNSCC patients.

Materials and methods

Data acquisition and preprocessing

We downloaded DNA methylation (528 HNSCC samples and 50 normal samples), RNA-sequencing (500 HNSCC samples and 40 normal samples) and clinical information data of The Cancer Genome Atlas Program (TCGA) HNSCC cohort from UCSC-Xena (https://xenabrowser.net/) on August 28th, 2020. The GSE65858 (with 270 HNSCC specimens) and GSE41613 (with 97 oral squamous cell carcinoma specimens) dataset from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo) comprises 96 HNSCC specimens with gene expression profiles, and the associated clinical characteristics were included as the validation set.

Data preprocessing

All data were normalized in the R computing environment (version 4.1.0) (https://www.r-project.org) using the limma package. Methylation data were in the form of β value, representing the ratio of the methylation probe data vs total probe intensities. The average DNA methylation value for all CpG sites correlated with a gene was calculated using the MethylMix and DESeq2 packages. Data were utilized according to the data access policy of TCGA and GEO.

Development of risk score model

Kaplan-Meier analysis was utilized to evaluate the relationship between mrDEGs and the survival time of HNSCC patients using glmnet, survival, and survminer packages. We adopted the multivariate Cox regression after primary filtration to further narrow the scope of the candidate prognostic mrDEGs. The risk score for each patient was calculated based on the risk score formula: risk score $= \exp(\text{gene}_1) \times \beta_1 + \exp(\text{gene}_2) \times \beta_2 + \ldots \exp(\text{gene}_n) \times \beta_n$. Herein, $\exp(\text{gene}_n)$ means expression of a mdDEG, β_n means the gene' coefficient in the linear model.

Testing and validation of risk score model

Samples were stratified into high-risk and low-risk groups by setting the median value of risk scores as the cutoff value. The overall survival (OS), disease-specific survival (DSS), and progression-free survival (PFS) of these two groups were calculated by the Kaplan-Meier method with the log-rank test. The expressions of mrDEGs in this risk score model were visualized by pheatmap package. Multivariate Cox regression and Receiver operating characteristic (ROC) curve were used to determine whether the risk score was

independent prognostic factors for HNSCC patients after considering other clinical features, including age, gender and stage. In the validation analysis, we verified the Kaplan-Meier plot by using GSE65858 and GSE41613.

Relationship between risk model and tumor immune microenvironment

Gene set enrichment analysis (GSEA) was performed using R package "clusterprofiler" to determine the enrichment of previously defined biological processes of mrDEGs using RNA-sequencing data from TCGA-HNSCC cohort. The collection of annotated gene sets in Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb/index.jsp) was chosen as the reference gene sets in R, and the P value < 0.001 was set as the cutoff. Cibersort (https://cibersort.stanford.edu) was used to provide an estimation of the abundances of member cell types in a mixed cell population and analyze the fraction of immune cells in high-risk and low-risk samples with HNSCC.[13] We compared the counts of low-risk and high-risk groups. We also compared the gene expressions of immune checkpoints, including *CTLA4*, *LGALS9*, *LAG3*, *HAVCR2*, *TIGIT*, *PD-1*, and *CD274*.

Results

The flow chart of this study was shown in Fig. 1A. We identified 2104 downregulated differentially expressed genes (DEGs), and 1633 upregulated DEGs (Fig. 1B). A total of 1620 genes have significant methylation level alterations, in which 284 were downregulated genes and 286 were upregulated genes. 570 mrDEGs were identified by intersecting DEGs and genes with methylation alterations (Fig. 1C). To determine the prognostic role of these mrDEGs, univariate analysis was performed in the TCGA cohort with the cutoff of $P < 0.01$. 26 mrDEGs had significantly improved or decreased hazard ratio in the univariate analysis (Fig. 1D). Then nine mrDEGs were screened out in the multivariate regression.

We then built a predictive model using the 9 survival-relevant mrDEGs. The mrDEGs risk score for each patient could be calculated using the following formula: Risk score = - 0.02673exp(*LIMD2*) + 0.00126exp(*UCHL1*) + 0.006452exp(*STC2*) + 0.006518exp(*PRAME*) - 0.02482exp (*SYCP2*) + 0.013019exp(*SLC7A4*) - 0.00543exp(*EPHX3*) + 0.006065exp(*PLOD2*) + 0.060995exp(*ACADL*) (Fig. 2A). We divided the HNSCC patients into low-risk and high-risk groups by the median risk score (Fig. 2B). Low-risk patients yielded longer OS (P = 8.587e-10) (Fig. 2C), PFS (P = 1.755e-4) (Fig. 2D), and DSS (P = 7.799e-8) (Fig. 2E) than high-risk patients. The risk score was more effective in survival stratification than age, gender, histopathological grade, and clinical stage (Fig. 2F). The prognostic value of the mrDEGs was also validated in OS of GSE65858 (P = 8.151e-3) (Fig. 2G; Fig. S1) and GSE41613 (P = 1.474e-2) (Fig. 2H; Fig. S2). These results indicated that mrDEGs signature have considerable prognostic values for HNSCC patients.

To further characterize the potential signaling pathways involved in the influences on the risk score model, GSEA was performed to enrich the kyoto encyclopedia of genes and genomes (KEGG) pathways in genes relation value with the risk score. Many enriched pathways were associated with TIME, including leishmania infection, systemic lupus erythematosus, B cell receptor signaling, natural killer cell-mediated cytotoxicity, autoimmune thyroid disease, antigen processing and presentation, asthma, T cell receptor signaling, intestinal immune network for IgA production, graft versus host disease, allograft rejection, primary immunodeficiency ($P < 0.01$) (Fig. 3).

As we understood that the risk score model was associated with TIME, we next analyzed immune infiltrations in samples. The low-risk group had higher proportions of *CD8*+ T cells, activated *CD4*+ T cells, resting mast cells, T helper cells, plasma cells, B cells, regulatory T cells, monocytes, M1 macrophages, eosinophils, dendritic cells. The high-risk group had higher proportions of M0 macrophages, *CD4*+ T cells, activated mast cells, activated dendritic cells, NK cells, M2 macrophages (Fig. 4A; Fig. S3). This indicated that the risk score was related to the immune infiltration cell recruiting of HNSCC, which may contribute to its prognostic stratification. Then we explored the expression levels of immune checkpoints and found that *CTLA-4*, *LGALS9*, *LAG3*, *TIGIT*, *PD-1*, and *HAVCR2* were more expressed in the low-risk group (Fig. 4B; Fig. S4; Fig. S5), which indicated more benefits of low-risk patients from immunotherapy.

Discussion

Epigenetic changes, such as DNA methylation, can drive abnormal gene expression of crucial genes involved in cancer development and progression, including HNSCC.[14,15] Hypermethylation of tumor suppressor genes and hypomethylation of proto-oncogenes at the promotor sites were associated with carcinogenesis and progression.[16,17] Considering the cancer type-specific methylation pattern, we analyzed the effects of methylation alterations on gene expressions and patients' survival and found a series of methylation-related oncogenes or tumor suppressor genes. Herein, we found that *LIMD2*, *SYCP2*, and *EPHX3* were associated with better prognosis, while higher expression of *UCLH1*, *STC2*, *PRAME*, *SLC7A4*, *PLOD2*, and *ACADL* implied undesirable prognosis.

Functions of some mrDEGs in the risk model have been identified in previous studies. *PRAME* expression was significantly associated with tumor stage, and positive lymph node metastasis, suggesting *PRAME* is a tumorigenic biomarker.[18] Though studies rarely address *SLC7A4*, one of its family members, *SLC7A5* is a transporter dedicated to essential amino acids and overexpressed in cancer cells to meet the increased demand for nutrients that include glucose and essential amino acids. Recently studies have identified *PLOD2* as a glycolysis-related gene and silencing of *PLOD2* suppresses tumor proliferation and invasion.[19,20] *STC2* was a direct target of miR-381, which suppresses cell proliferation, migration and invasion in HNSCC.[21] In addition, *EPHX3* have been ever addressed in previous survival prediction model of HNSCC or OSCC, and hypermethylation of *EPHX3* will lead to downregulated expression and favorable survival.[22–24] *SYCP2* is involved in the nuclear structure and meiosis, which is found upregulated

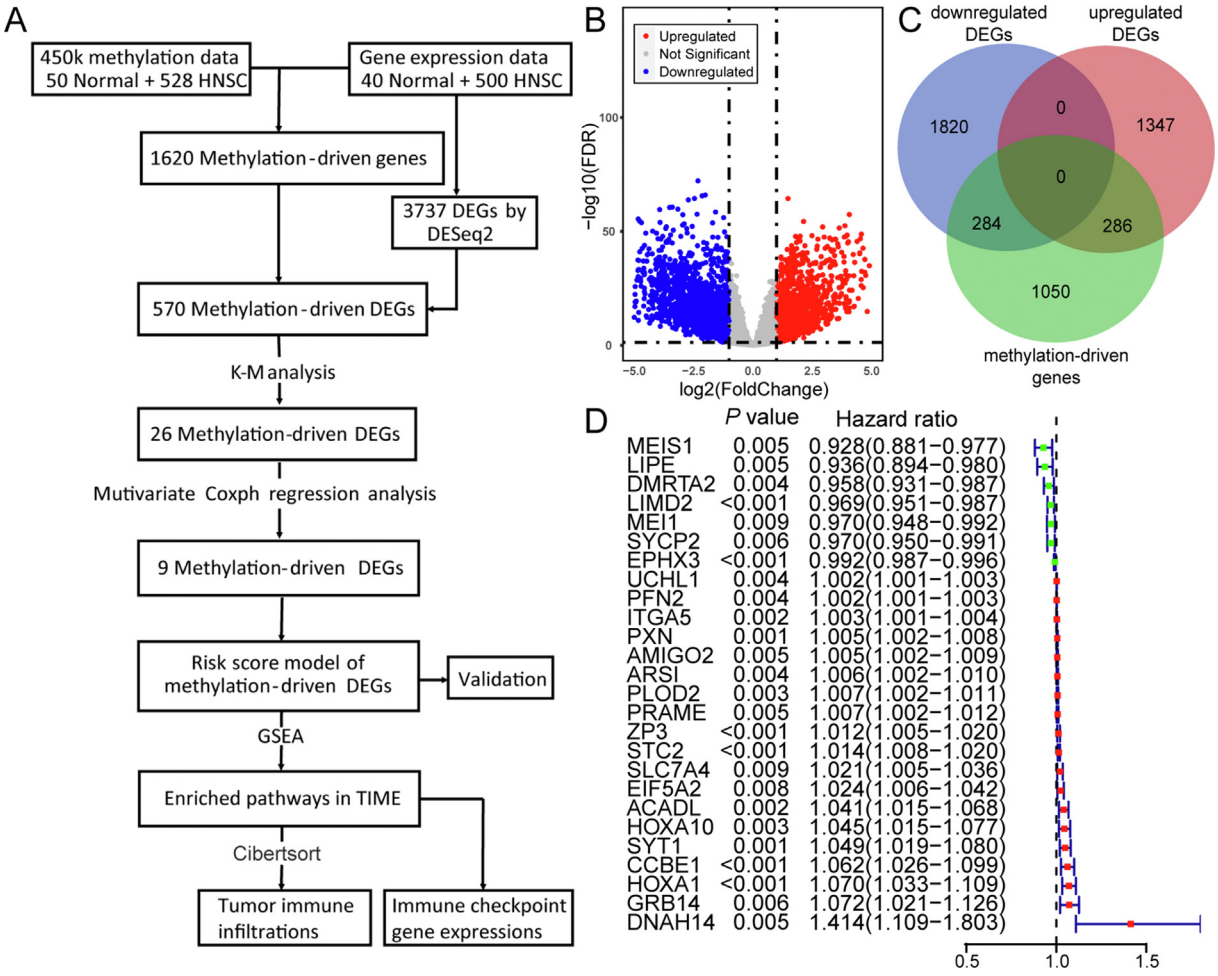

Fig. 1 Selection of methylation-related differentially expressed genes (mrDEGs) in head and neck squamous cell carcinoma (HNSCC) samples. (A) Flow chart of this study. (B) Volcano plot of DEGs using the cutoff of |log-fold change (logFC)| $\geq$ 1 and false discovery rate (FDR) $<$ 0.05. logFC$\leq$1 were downregulated DEGs colored by blue and logFC$\geq$1 were upregulated differentially expressed genes (DEGs) colored by red. (C) Venn plot of upregulated DEGs, downregulated DEGs and methylation-related genes. The overlapping region showed the methylation-related upregulated or downregulated genes. (D) Kaplan-Meier analysis of mrDEGs using the cutoff of $P < 0.01$ in HNSCC patients. Green labeled survival-favorable mrDEGs, red labeled survival-unfavorable mrDEGs. K-M analysis, Kaplan-Meier analysis; GSEA, gene set enrichment analysis; TIME, tumor immune microenvironment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in Human papillomavirus (HPV)-positive HNSCC,[25] when clinical follow-up has shown that HPV positivity was associated with improved survival.

However, the role of other mrDEGs may be controversial among different cancers. *LIMD2* is a component of the signal transduction cascade that links integrin-mediated signaling to cell motility or metastatic behavior in multiple cancers,[26] while our study identified *LIMD2* as a survival-favorable gene. *ACADL* plays a tumor-suppressor role by targeting *Hippo/YAP* signaling in hepatocellular carcinoma, while another report shows that *ACADL* expression is associated with esophageal squamous cell carcinoma progression and poor prognosis.[27,28] Several potential reasons may explain the controversy. First, it may be possible that the same molecule expresses and functions in a cancer-specific pattern. Second, molecules downregulated in cancers may be necessary for the tumor progression and metastasis. For example, it is well-known that E-cadherin is downregulated in cancers, but a recent study found it was required for metastasis of breast cancer.[29] Overall, further studies are needed to elucidate these controversies.

The mrDEGs risk score is highly associated with and enriched in TIME pathways. A previous study found that *PRAME* expression is more frequent in soft tissue sarcomas with low tumor-infiltrating lymphocyte counts.[30] *PLOD2* was positively correlated with the activities of tumor-infiltrating immune cells, including macrophages, neutrophils, *CD4*+ T cells, B cells and dendritic cells.[31] Our study has subclassified the macrophages into M0, M1 and M2 phenotypes, in which M2 phenotype was generally considered as tumor associated one.[32] We found that both M0 and M2 phenotypes increased in the high-risk group while M1 phenotype was higher in the low-risk group. Similarly, the resting or activated *CD4*+ T cells, B cells and dendritic cells distributed differently in groups. Our results suggested the

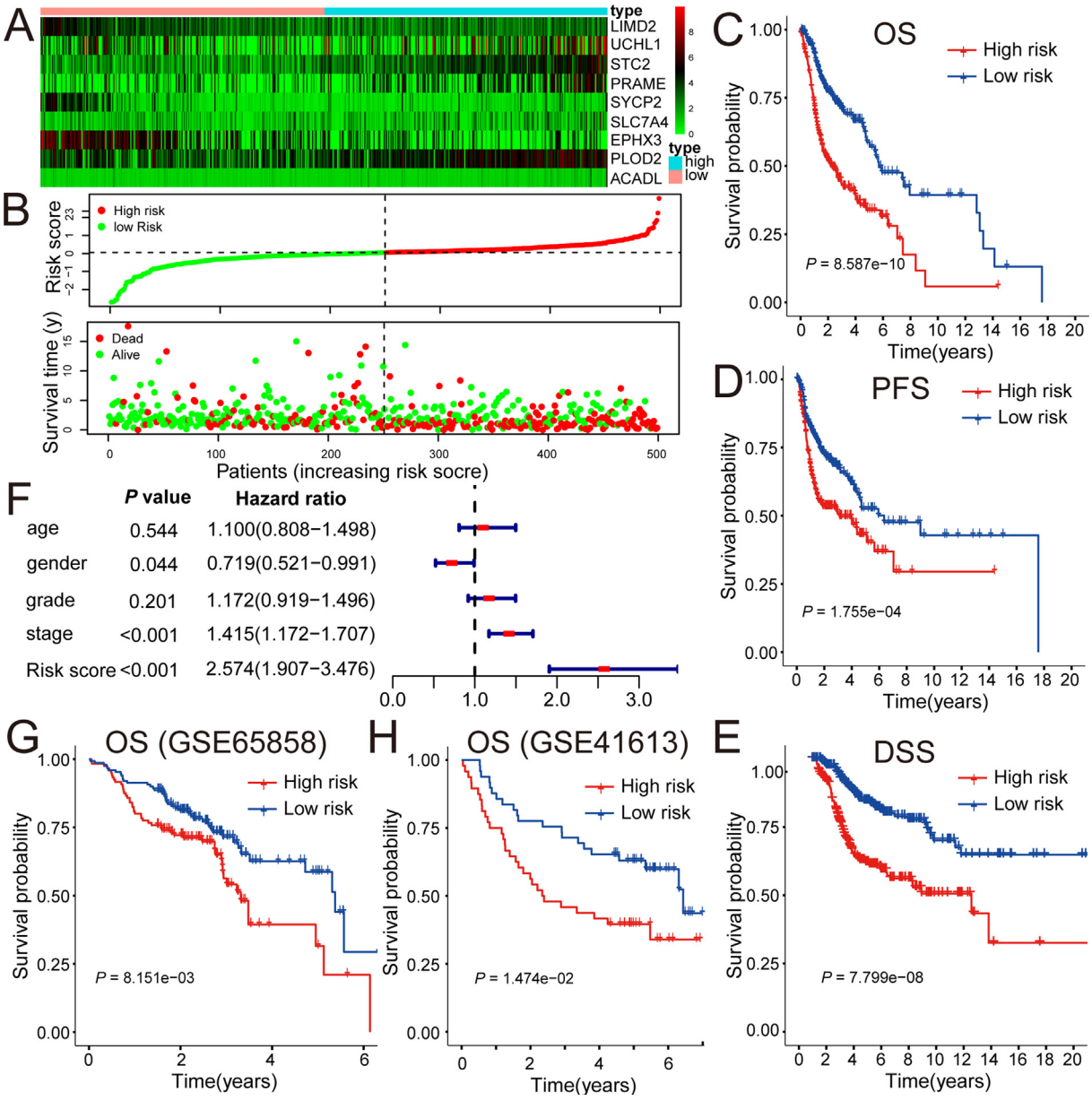

Fig. 2 Survival prediction by risk factor model of methylation-related differentially expressed genes (mrDEGs) in head and neck squamous cell carcinoma (HNSCC) samples. (A) expressions of mrDEGs in HNSCC patients divided by median mrDEGs risk scores. (B) Survival time in HNSCC patients sequenced with increasing risk score. (C) Kaplan-Meier analysis of overall survival (OS) in the training set. (D) Kaplan-Meier analysis of progression-free survival (PFS) in the training set. (E) Kaplan-Meier analysis of disease-specific survival (DSS) in the training set. (F) Multivariate Cox regression of risk score and clinical factors in HNSCC patients in the training set. (G) Kaplan-Meier analysis of OS in the validation set (GSE65858). (H) Kaplan-Meier analysis of OS in the validation set (GSE41613).

potential of immunomodulation functions of other mrDEGs, which might need more experimental confirmation.

T-cell Receptor Signaling is the essential basis of arising immunotherapy and may participate in the resistance to chemotherapy.[33,34] The current immunotherapy was mainly achieved by antibodies blocking *CTLA-4* or *PD-1* pathway.[35] Evidence is cumulating that *LAG3*, *LGALS9*, *HAVCR2* and *TIGHT* could be the next-generation immunotherapy checkpoints.[36–39] Previous studies found a significant correlation among *PD-L1* and *PD-1* expression in immune cells and *PRAME* expression in tumor cells of salivary duct carcinoma.[40] No published studies have addressed the association between other mrDEGs and immunotherapy checkpoints. Our study firstly revealed that methylation might also drive the alterations in TIME and could be a potential target of immunotherapy.

However, this study has some limitations. First, the risk score model's validation could not be performed in multiple populations due to the limited data source. We validated its efficacy in an independent GEO dataset, and we expected

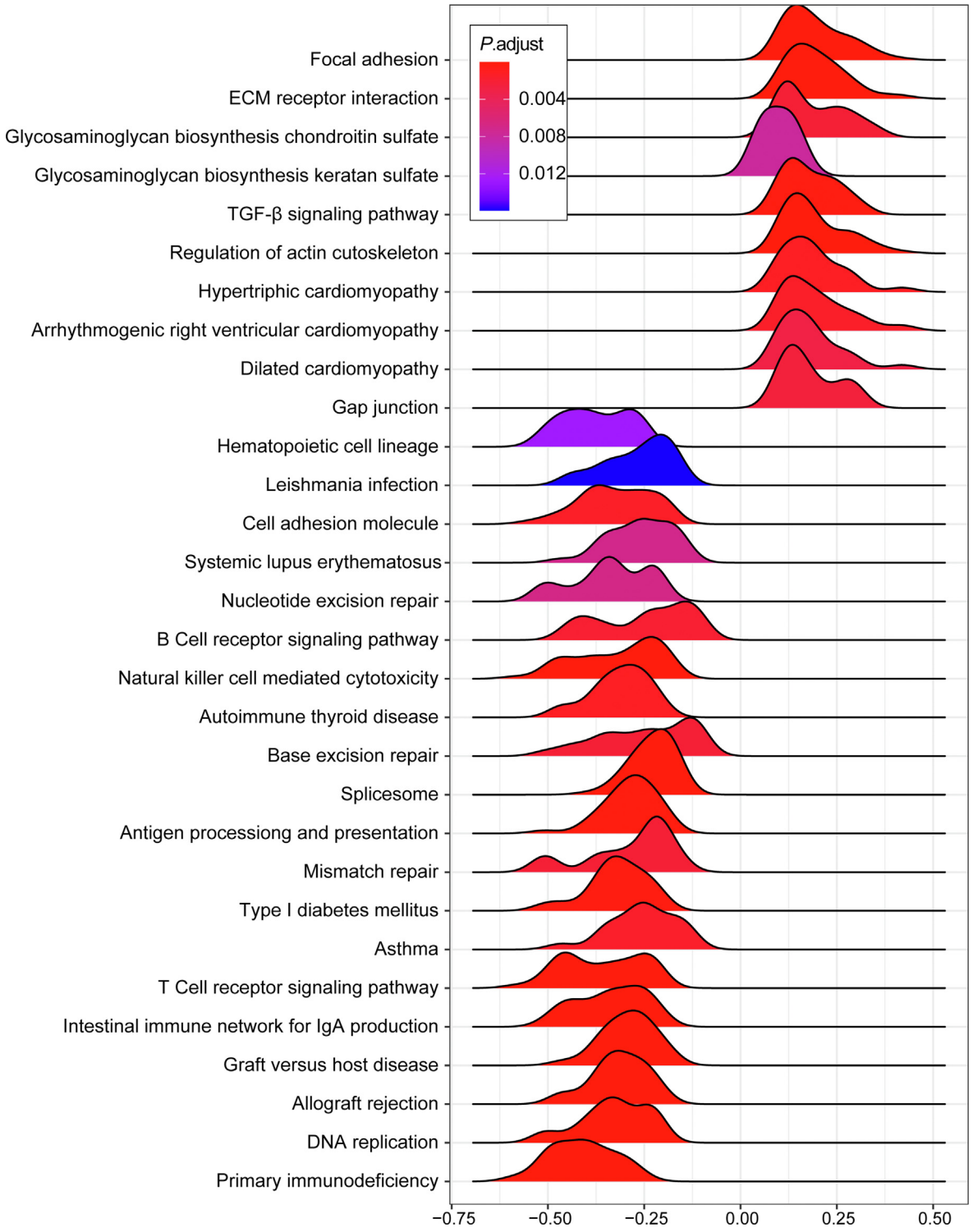

Fig. 3 Pathway enrichment of methylation-related differentially expressed genes (mrDEGs) in head and neck squamous cell carcinoma (HNSCC) samples. A false discovery rate (FDR) less than 0.05 and an absolute value of the enrichment score (ES) greater than 0.5 were defined as the cutoff criteria. *P*.adjust, adjusted *P* value.

that genomic databank with more HNSCC samples could be established. Second, we did not test our risk score's prediction efficacy on the immunotherapy in late-stage HNSCC patients exclusively. Early-stage HNSCC patients are not indicated to the immunotherapy due to the efficiency of surgery. Further validation should be conducted when late-stage samples are cumulative.

In conclusion, risk score constructed by mrDEGs could predict HNSCC patients' prognosis, which may correlate with TIME and immune checkpoint expressions.

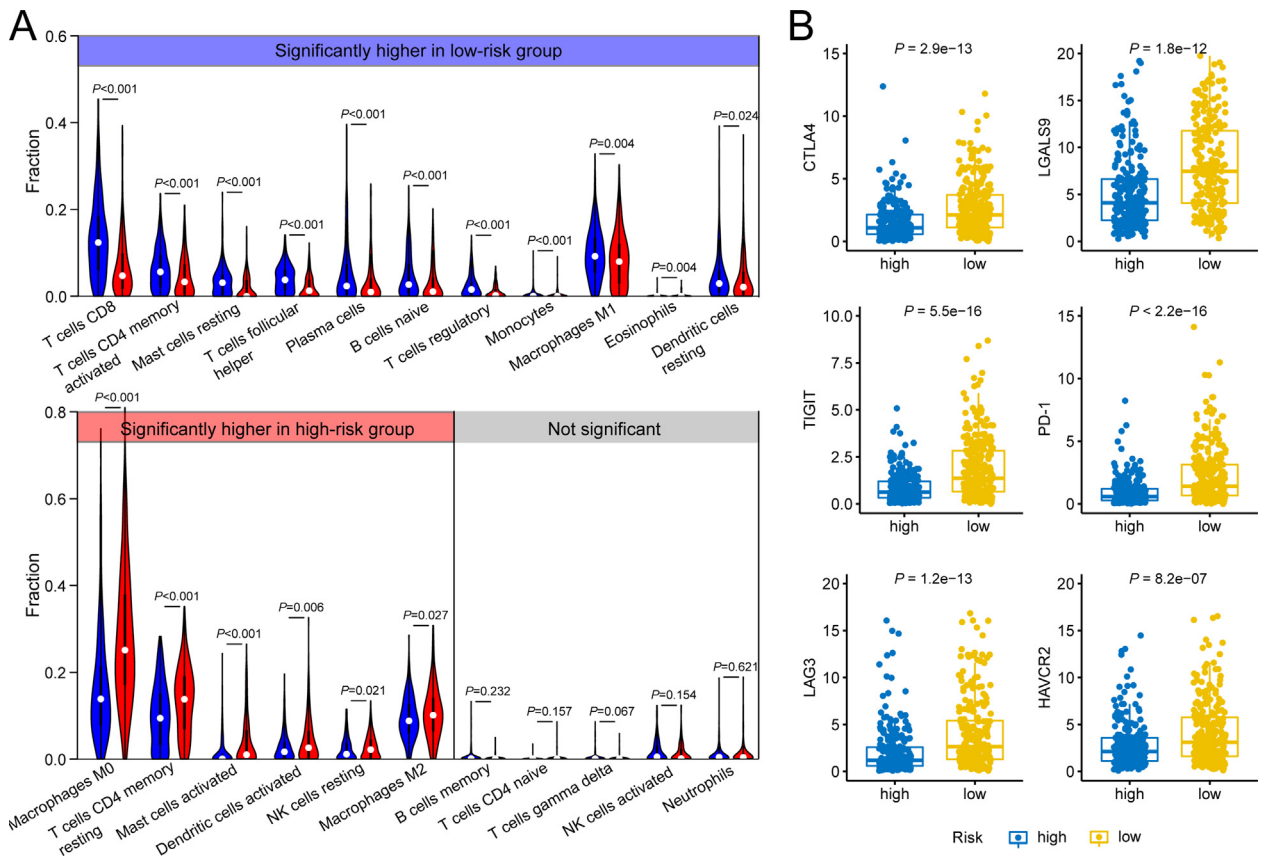

Fig. 4 Relationship between methylation-related differentially expressed genes (mrDEGs) and immunomodulation. (A) Fractions of immune cell infiltrations in low-risk and high-risk head and neck squamous cell carcinoma (HNSCC) groups. (B) gene expressions of immune checkpoints in low-risk and high-risk HNSCC groups.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

We would like to appreciate Dr. Xia Guo (Ph.D., College of Foreign Languages and Cultures, Sichuan University) for her kind assistance in the English writing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2022.07.010.

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