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ORIGINAL ARTICLE

Hypoxia signature associated with tumor immune microenvironment in the esophageal squamous cell carcinoma

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Summary

Purpose: *Although the incidence and progression of cancer has remained closely dependent on hypoxia, a critical element in the tumor microenvironment, there is still confusion about the hypoxia's involvement in esophageal squamous cell carcinoma. We tried to investigate the correlation between esophageal squamous cell carcinoma and hypoxia, to establish the hypoxia-related gene signature in this disease.*

Methods: *We retrieved esophageal squamous cell carcinoma cases from Gene Expression Omnibus (GEO) databases. Through conducting univariate and multivariate Cox regression analysis, the genes have been chosen to be contained in the hypoxia-associated signature. After that, we performed a survival analysis and designed to shape ROC curves, so as to confirm the gene signature. And then, the underlying relationship between the gene signature and immune cells has been investigated via the CIBERSORT tool, thus finally figuring out the immune-associated genes regulated by hypoxia.*

Results: *There were four genes in the final signature, including HSPA5, ENO3, GYS1 andPGM2. The results also indicated that patients in the high-risk group displayed worse survival than those in the low-risk group. Additionally, we discovered that there remained great disparity in the infiltration of immune cells between the two groups, that is, activated NK cells, CD8+ T cells, resting NK cells and neutrophils.*

Conclusions: *The hypoxia-related gene signature established and validated in the research was deemed as a latent prognostic factor in esophageal squamous cell carcinoma and may guide the immunotherapy practice.*

Key words: *esophageal squamous cell carcinoma, hypoxia, prognostic factor, immune, GEO*

Introduction

Esophageal cancer is one of the most common malignant tumors of the digestive system, with high morbidity and mortality [1]. Despite advances in diagnosis and treatment techniques, the prognosis of this disease is still very poor, with 5-year survival rates ranging from 15% to 25% [2]. The histologic types of esophageal cancer include two main types: esophageal squamous cell carcinoma and esophageal adenocarcinoma. Esophageal squamous cell carcinoma is the main histological type in China. Compared with other countries, it has the highest morbidity and mortality [3].

Hypoxia is a common feature of malignant tumors, and the increase in expression of related hypoxia genes indicates a poor prognosis for patients [4]. There is an interaction between hypoxia and chemoresistance, radioresistance, invasiveness and angiogenesis. Reduced oxygen supply (hypoxia) is present in most tumors, which is caused by an imbalance between increased oxygen consumption and insufficient oxygen supply. Tumor hypoxia is considered to be an effective target for cancer treatment [5].

It is reported that genes related to hypoxia have prognostic and predictive value. For example,

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hypoxia-related genes can predict the survival rate of head and neck squamous cell carcinoma [6], and hypoxia models also play an important role in the diagnosis, prognosis and immune microenvironment prediction of liver cancer [7]

However, as far as we know, no model has been established based on genes related to hypoxia in esophageal squamous cell carcinoma. Therefore, in this study, we constructed a model based on hypoxia-related genes to explore the potential prognostic value of hypoxia-related genes in patients with esophageal squamous cell carcinoma.

Methods

Data collection and construction of hypoxia model

We secured the RNA-seq data and clinic-associated data of esophageal squamous cell carcinoma from GEO database (GSE53624). Resorting to the STRING database, we have constructed the hypoxia gene PPI network and recognized differentially expressed hypoxia-associated genes pertinent to overall survival time to conducting the research of univariate and multivariate Cox regression analyses. The outcome as $p<0.05$ could be regarded as a kind of standard to indicate a statistically significant difference. Then the risk score of each patient was calculated in accordance with the following formula: risk score= β gene (1) × expressed gene (1) + β gene (2) × expressed gene (2) +···+β gene (n) × expressed gene (N). Using the median risk score as the cut-off point, patients were divided into high-risk and low-risk groups.

Survival analysis and ROC curve analysis

The prognosis of patients with esophageal squamous cell carcinoma could be analyzed through survival and subminer packages in the esophageal squamous cell carcinoma database. We have drawn survival curves using the Kaplan-Meier method and evaluated the 1-year, 3-year, and 5-year survival rates of patients in the esophageal squamous cell carcinoma database. The area under the ROC curve for 1-year, 3-year and 5-year survival rates gradually increased, finally exceeding 0.5,

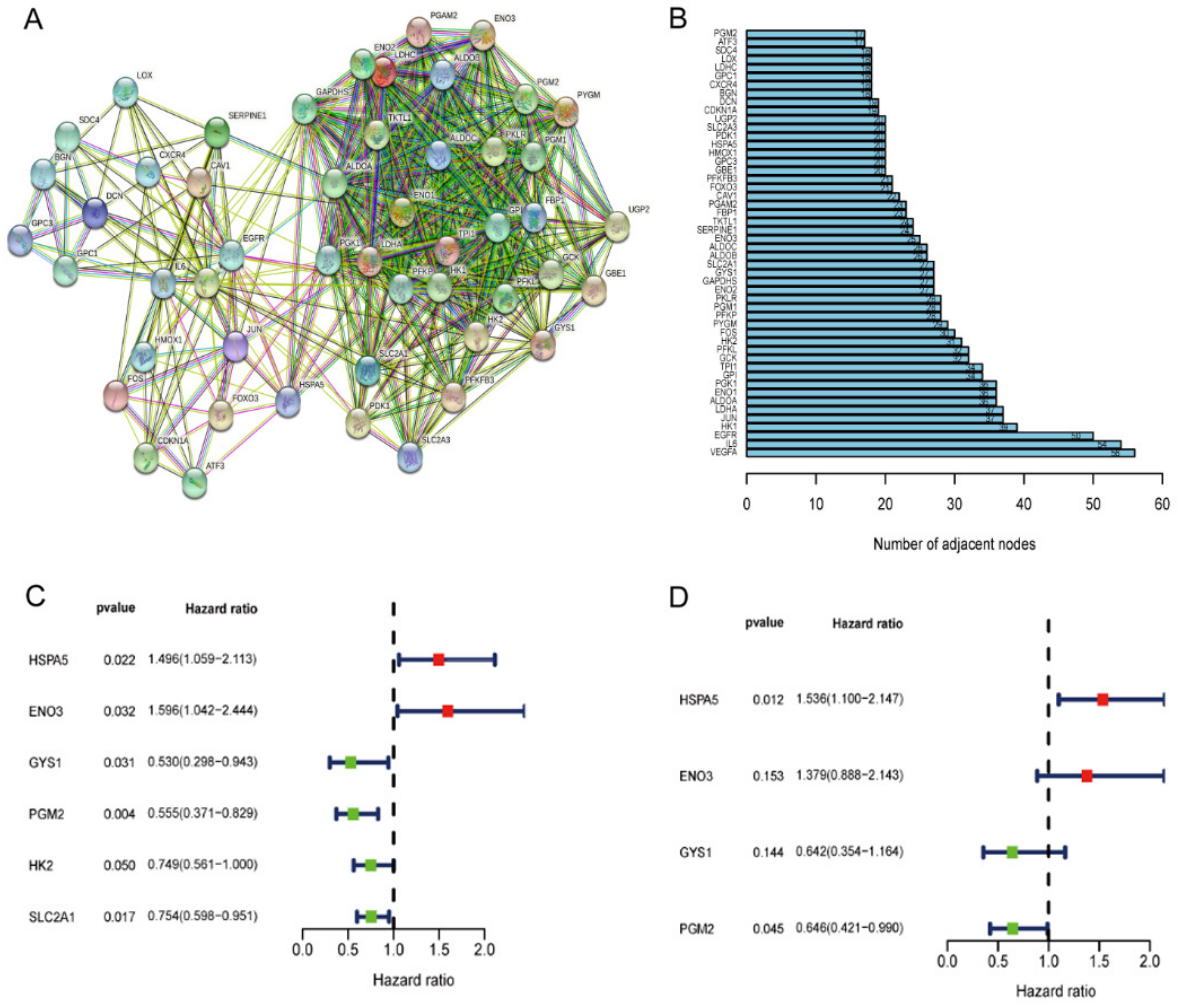

Figure 1. Screening of hypoxia-related genes. **A:** Protein interactions among hypoxia-related genes. **B:** Extracted 50 genes with the most connected nodes. **C:** univariate Cox regression analysis showed that 6 hypoxia related genes were associated with the prognosis of patients **D:** 4 hypoxia related genes were selected and multivariate Cox regression was used to establish the hypoxia risk model.

which was defined as the threshold for the model to accurately predict survival.

Hypoxia-related gene set and immune cells

Standardization of the mRNA expression matrix could be of supreme significance for the exploration of the underlying relationship between gene markers and immune cells in the context of lung adenocarcinoma. After that, we employed the CIBERSORT tool to estimate the content of 22 human immune cells, divided the cases into two groups grounded on the median risk, and utilized the "vioplot" software package in R to visualize the data.

The HALLMARK gene set and gene symbols have been successively downloaded from the GSEA website (https://www.gsea-msigdb.org/gsea/index.jsp) to extract genes pertaining to hypoxia. Among all tumor samples in the entire transcriptome for GSEA, only genomes with p values <0.05 and FDR q value <0.06 were being of critical importance.

Results

Extraction and screening of hypoxia-related genes

At the beginning, the HALLMARK gene set has been downloaded. Besides, the Gene Set Enrichment Analysis (GSEA) website (https://www.gsea-msigdb.org/gsea/index.jsp) was available to the

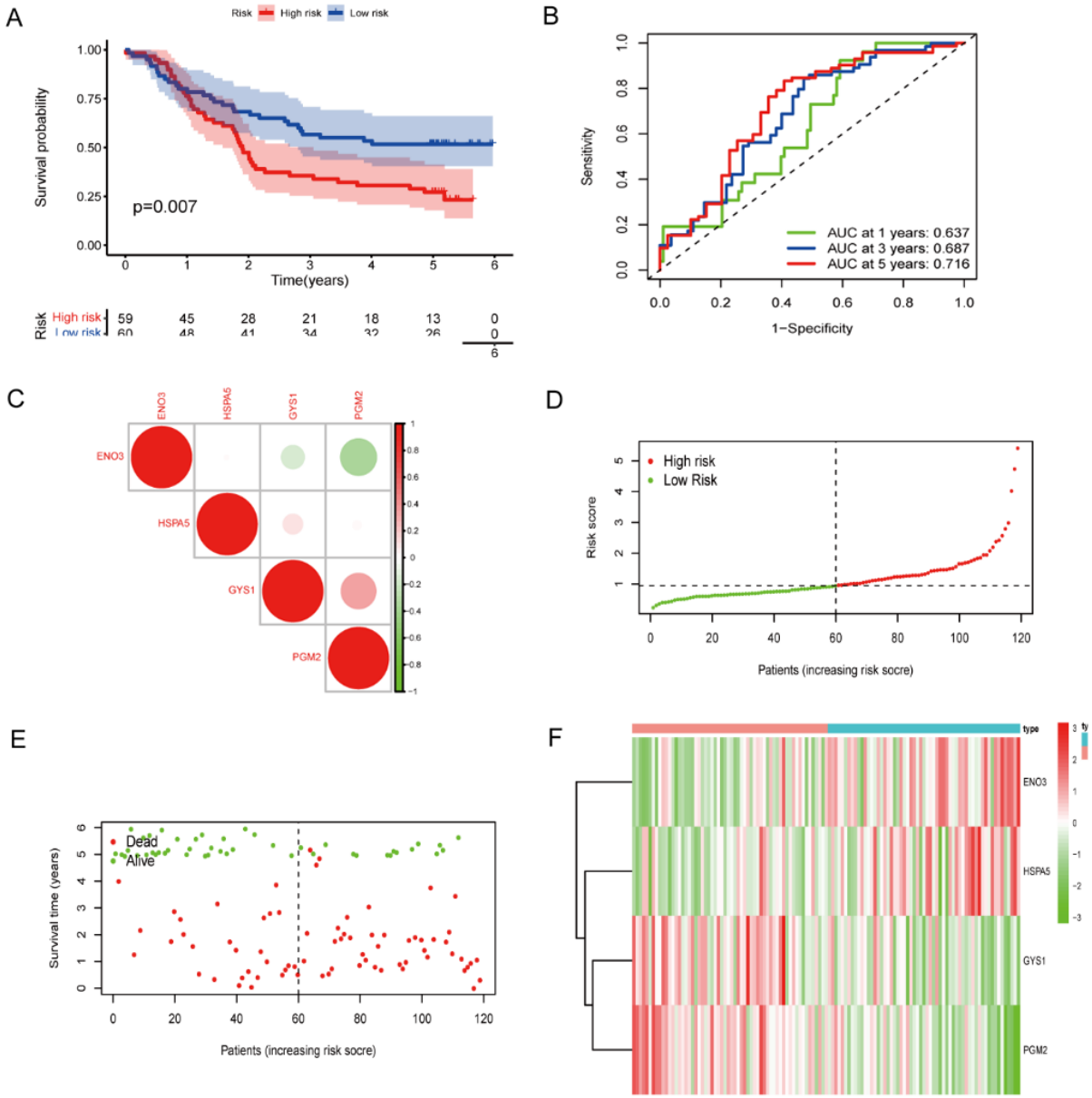

Figure 2. The predictive value of hypoxia risk score for the prognosis of esophageal squamous cell carcinoma. **A:** Kaplan-Meier overall survival analysis of esophageal squamous cell carcinoma hypoxia high and low risk groups. **B:** ROC curve shows the prognostic value of the patient's hypoxia risk score. **C:** Spearman correlation analysis of 4 hypoxia genes. **D:** The relationship between the hypoxia risk score and the patient's risk level. **E:** Distribution of patients' survival status. **F:** Expression of four hypoxia-related genes in high and low hypoxia risk groups.

gene symbol set and could provide the names of all hypoxia-associated genes. Then, the expression data and clinic-associated information of esophageal squamous cell carcinoma was assessed from the GEO database. We extracted the gene expression data pertinent to hypoxia, and then we searched for interacting genes/proteins (STRING) and PPI values between hypoxia-related genes (Figure 1A) via the search tool of the protein-protein interaction (PPI) network database (http://string-db. org/cgi/input.pl). Furthermore, the top 50 core genes have been recognized by counting the number of adjacent nodes for each protein (Figure 1B) and strictly screened the prognostic-related genes through univariate Cox regression analysis (Figure 1C). Among the identified genes, GYS1, PGM2, HK2, and SLC2A1 were closely related to low risk, while HSPA5 and ENO3 were connected with high risk of potential malignant tumors. Multivariate Cox regression analysis of genes pertaining to patient prognosis unveiled four genes (Figure 1D), which could be utilized to construct a prognostic

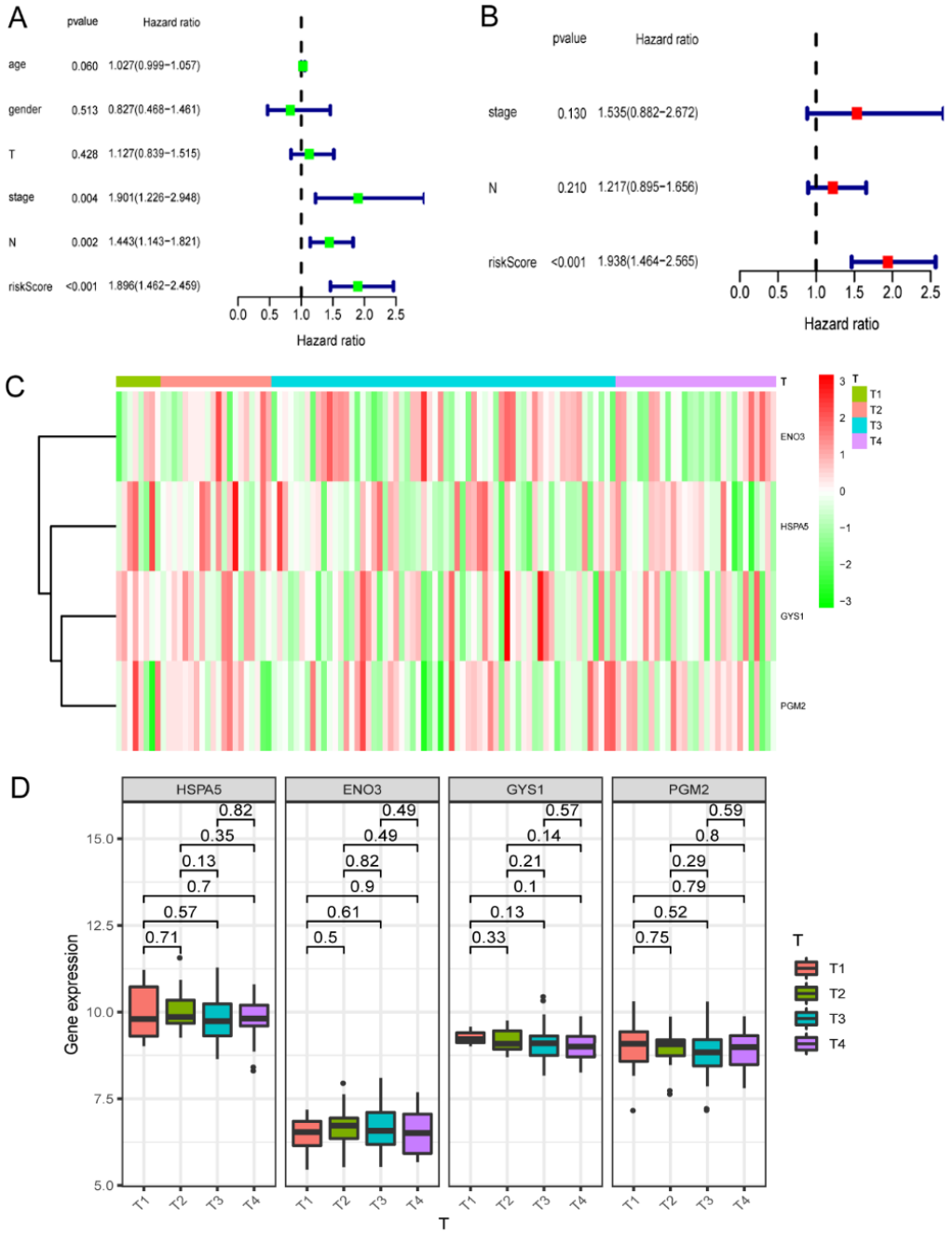

Figure 3. The impact of different clinical features on the prognosis of esophageal squamous cell carcinoma. **A** and **B:** Multivariate and univariate analyses of independent prognostic analysis. **C** and **D:** the expression levels of four hypoxia related genes in different T stages.

model. The risk coefficients of HSPA5, ENO3, GYS1, and PGM2 were 1.536, 1.379, 0.642 and 0.646, respectively.

The influence of hypoxia-associated genes on prognosis

By conducting multivariate Cox regression analysis, four hypoxia-associated genes pertinent to esophageal cancer prognosis have been detected. The expression levels of these genes were multiplied by the corresponding coefficients, so as to secure the risk score for each patient. Subsequently, we resorted to the median risk score value to classify the patients in the GEO database into high-risk and low-risk groups, respectively. A tremendous difference between the high-risk group and the low-risk group ($p<0.05$; Figure 2A) could be mirrored from the following survival analysis. Additionally, the precision of estimating the accuracy of the survival rate from this model could be effectively verified through ROC curve analysis. It illustrated that the accuracy of predicting the 1-year, 3-year, and 5-year survival rates of patients in the GEO database has gained a sound momentum (Figure 2B), clearly stating that the model can exactly forecast the survival rate. We have also analyzed the interactions between hypoxia-related genes (Figure 2C) with an aim to evaluate the survival rate of patients more intuitively in the high-risk and low-risk groups. The risk curve was employed to further demonstrate the relationship between patient risk and survival, in which the risk scores of the two groups of patients were plotted (Figure 2D). We also found that the survival time of the low-risk group was longer than that of the high-risk group. Besides, the number of deaths in the low-risk group decreased over time (Figure 2E). Finally, we compared the expression level of each gene in the model between high-risk and low-risk populations (Figure 2F).

The influence of different clinical characteristics on the prognosis of intestinal tumors

Considering that clinical characteristics have certain effects on the prognosis of patients, we analyzed the effect of clinical features in the GEO database on the prognosis of patients. Firstly, univariate Cox regression analysis was performed to appraise the influence of two clinical features on

Table 1. GESA enrichment pathway in high-risk group

Enrichment pathway name	*SIZE*	*ES*	*NES*	*NOM p value*	*FDR q value*	*FWER p value*
Epithelia lmesenchymal transition	194	0.50	1.31	0.24	1.00	0.65
Hedgehog signaling	36	0.44	1.29	0.17	0.69	0.67
myogenesis	192	0.36	1.23	0.23	0.58	0.73
Pancreas beta cells	40	0.42	1.08	0.32	0.71	0.82
Apical junction	195	0.27	1.03	0.43	0.68	0.86
WNT beta catenin signaling	41	0.32	1.01	0.46	0.60	0.87
Angiogenesis	35	0.38	0.98	0.50	0.55	0.89
Notch signaling	32	0.31	0.97	0.49	0.50	0.90
UV response dn	139	0.27	0.87	0.62	0.57	0.93
TGF beta signaling	52	0.25	0.81	0.64	0.61	0.95

Table 2. GESA enrichment pathway in low-risk group

Enrichment pathway name	*SIZE*	*ES*	*NES*	*NOM p value*	*FDR q value*	*FWER p value*
P53 pathway	192	-0.48	-1.91	0.00	0.06	0.03
Fatty acid metabolism	148	-0.45	-1.79	0.00	0.06	0.10
Heme metabolism	189	-0.42	-1.74	0.00	0.06	0.14
Reactive oxygen species pathway	47	-0.53	-1.89	0.00	0.04	0.04
Glycolysis	188	-0.39	-1.64	0.02	0.10	0.27
Xenobiotic metabolism	187	-0.44	-1.50	0.02	0.20	0.46
Oxidative phosphorylation	187	-0.44	-1.75	0.03	0.07	0.13
KRAS signaling dn	190	-0.41	-1.33	0.03	0.31	0.63
MTORC1 signaling	183	-0.42	-1.71	0.03	0.07	0.18
Cholesterol homeostasis	66	-0.44	-1.54	0.04	0.17	0.41

patient survival time and prognosis (Figure 3A). We have discovered that survival and prognosis can be deeply affected not by the patient's gender but by stage and risk score. Multivariate analysis of these factors reflected that the p values of risk score were both <0.05, demonstrating that this variable constituted an independent prognostic factor (Figure 3B). We have also discovered differences in the expression levels of hypoxia-associated genes in different T-stage patients in esophageal cancer (Figure 3C), but the disparity in the expression levels of these four genes in different T-stages (T1, T2, T3, and T4) and patients was not conspicuous (p>0.05).

GSEA analysis

We have noticed gaps in the enrichment levels and relevant pathways of hypoxia-related genes between high-risk and low-risk groups. Also, we have compared pathways between different risk groups through software to understand the level of pathway enrichment. As displayed in Table 1, in contrast to low-risk groups, the high-risk groups in the esophageal squamous cell carcinoma database were mainly enriched in angiogenesis, TGFβ signaling, β-catenin signaling, etc., while in the high-risk group, the enriched pathway was not significant (p >0.05). By contrast, the pathways enriched in the low-risk group were mainly concentrated in the p53 signaling pathway and various metabolic pathways, as shown in Table 2.

Immune cell infiltration and differential immunity related genes

Resorting to the online platform "track tumor immune phenotype" (http://biocc.hrbmu.edu.cn/TIP/index.jsp), we screened immune-associated genes and sought for those that were critical to regulate this immune cell type. We drew a heat map

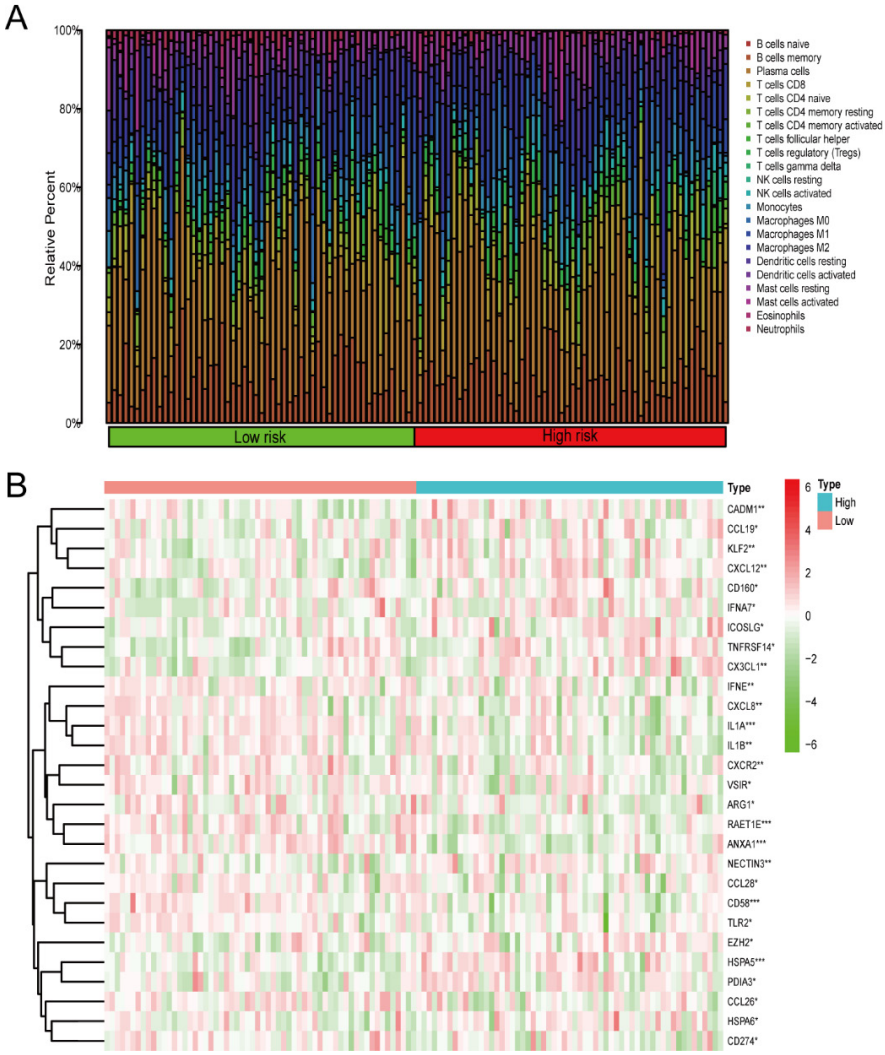

Figure 4. Analysis of immune cells and immune genes in the high and low hypoxia risk groups of patients with esophageal squamous cell carcinoma. **A:** The relative proportion of immune infiltration. **B:** The expression difference of immune-related cells. ***p<0.001, **p<0.01, *p<0.05.

to visualize the expression of these genes pertaining to hypoxia-associated genes in the high-risk and low-risk patient groups in the GEO database (Figure 4A). There remained significant differences in the expression levels of multitudinous immune-related genes ($p < 0.05$).

Additionally, the infiltration rate of immune cells in each risk group has been evaluated grounded on the hypoxia-associated gene model. As shown in Figure 4B, the penetration rate of immune cells in the high-risk and low-risk groups in the GEO database could demonstrate that there remained significant differences in the infiltration rate of the four types of immune cells in the high-risk and low-risk groups, namely activated NK cells, resting NK cells, neutrophils and CD8+T cells (Figure 5, $p<0.05$).

Discussion

Due to the rapid and uncontrolled proliferation of tumors, the supply of oxygen is restricted. Insufficient blood supply or hypoxia is a typical microenvironmental feature of almost all solid tumors [8]. Oxygen is essential for energy metabolism to drive cell bioenergy. The rapid proliferation of tumors extends beyond the surrounding vasculature, causing normal oxygen levels to drop by 2-9%, while hypoxia levels drop by less than 2%. Areas with low oxygen content are often called hypoxic areas. Extensive reviews have reported the clinical significance of hypoxia in tumor treatment [9,10].

Most studies on hypoxic phenotypes focus on a single gene. Such studies used high-throughput biological technology to systematically and comprehensively detect the expression of hypoxia-related genes to predict the prognosis of patients with esophageal squamous cell carcinoma [8-10].

In our research, we screened genes related to hypoxia in the intestine and found that core genes (HSPA5, ENO3, GYS1, PGM2) are closely related to the prognosis of patients. Studies have shown that HSPA5 contributes to the survival of head and neck cancer by maintaining lysosomal activity [11]. In hepatocellular carcinoma, HSPA5 (GRP78) promotes liver cancer cell proliferation and anti-apoptotic response by combining with up-regulated CD5L [12]. As oxygen becomes limited, cells will mitigate mitochondrial respiration and augment ATP production through anaerobic fermentation of glucose [13]. Hypoxia inducible factor (HIF) functions as a power-

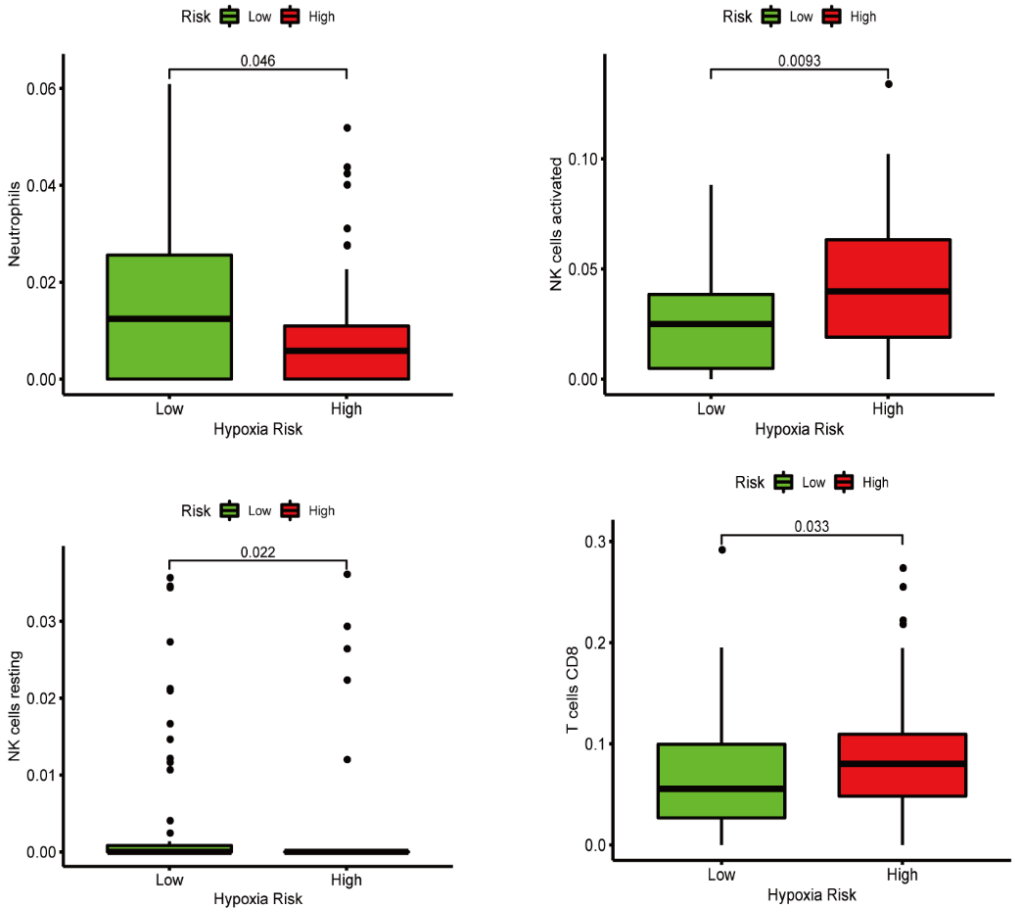

Figure 5. Differences in the level of immune cell infiltration between high and low risk groups.

ful engine for glucose metabolism by modulating the transcription of key enzymes [14]. As reflected in recent studies, GYS1 is definitively a brand-new hypoxia inducible gene. Hypoxia-induced GYS1 necessitates HIF activity and HR sequence in GYS1 promoter to play a series of roles [15].

We also found that the expression levels of these four hypoxia-associated genes were closely entwined with the occurrence, evolvement and metabolism of esophageal squamous cell carcinoma.

The patients have been classified into high-risk group and low-risk group, so as to further figure out the relationship between the expression of these four genes and the prognosis of patients. Subsequent survival analysis showed that the survival rate of low-risk group was significantly higher than that of high-risk group. We have also verified the reliability of the model resorting to the results of ROC curve, and analyzed the impact of other factors (gender, TNM stage, and our recommended risk score) on the prognosis of patients. It could be concluded that our recommended risk score is closely linked to the prognosis of patients. In the meantime, multivariate analysis showed that risk score can be deemed as an independent prognostic factor.

After determining the enrichment pathways of high-risk and low-risk groups in GEO database via GSEA, we have discovered that these pathways mainly pertinent to metabolism have been extracted. Previous studies have also demonstrated that hypoxia in tumors enhances the activity of hypoxia inducible factor-1 (HIF-1) accompanied by changes in mitochondrial metabolism, which increases the transport of glucose into cells, thus being the main reprogramming of cancer cell metabolism [16].

The established researches have reflected that the rising in glycolysis rate is significantly relevant to the activation of multiple immune cell types, including macrophages, DC, T cells and B cells [17].

Hypoxia has strong capacity to absorb immune cells into the tumor microenvironment and bring about the immune escape through the action of immune cells [18]. Numerous studies have illustrated that hypoxia places an irreplaceable position in tumor immunotherapy. Nonetheless, its underlying mechanism remains to be studied.

In our study, we observed an increase both in activated NK cells and CD8 + T cells in the high-risk group and in resting NK cells and neutrophils in the low-risk group between the high-risk and low-risk groups of ESCC database construction. During the process of screening immune regulation-related genes, we have observed that there is a substantial amount of gene expression difference between high-risk and low-risk groups, which further indicates the correlation between hypoxia and immunity in esophageal adenocarcinoma and squamous cell carcinoma. As for tumor immunotherapy, it has not considered the hypoxic microenvironment and its influence on the therapeutic effect in clinical practice, and many anti-cancer drugs have been displayed to prevent HIFs [19,20]. Apparently, considering the essential role of hypoxia in regulating tumor progression and immunosuppression, it is possible that its target can be considered in new cancer combination therapies.

Nevertheless, the current research has certain limitations without sufficient experimental evidence. Therefore, the research experts urgently necessitate further research on cell lines and animal experiments to verify the current findings and investigate the molecular mechanism.

Conclusions

Hypoxia plays an important role in the tumor microenvironment and screening genes probably affect the infiltration of immune cells. Besides, we have discovered that there remains a correlation between these genes and the hypoxia risk score. Meanwhile, the findings clearly demonstrate that hypoxia-associated genes can well forecast the prognosis of esophageal squamous cell carcinoma and may be constructive for the immune infiltration of esophageal squamous cell carcinoma. Hence, the analysis of the correlation between hypoxia and immune cells may boost immunotherapy against tumors.

Conflict of interests

The authors declare no conflict of interests.

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