Identification of key genes in head and neck squamous cell carcinoma microenvironment based on gene expression profile

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Primary research

Keywords: head and neck squamous cell carcinoma (HNSCC), tumor microenvironment (TME), ESTIMATE algorithm, The Cancer Genome Atlas (TCGA), prognosis

Posted Date: July 6th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-39972/v1

Abstract

Background: Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent malignancy worldwide with high incidence and mortality. Recently, it has been well established that tumor microenvironment (TME) is intimately associated with cancer progression, in which the infiltration of immune and stromal cells could be exploited as effective biomarkers for cancer diagnosis and prognosis. In this study, we aimed to investigate HNSCC microenvironment to identify significant genes involved in carcinogenesis. Methods: The ESTIMATE algorithm was applied to calculate immune scores and stromal scores based on the gene expression profiles downloaded from The Cancer Genome Atlas (TCGA) database and the correlation of these scores with clinical parameters and prognosis was further analyzed. Differential expression analysis and functional enrichment analysis were performed by R software. Results: Immune scores and stromal scores were calculated by ESTIMATE algorithm and their correlations with clinicopathological characteristics of HNSCC patients were identified. Differentially expressed genes (DEGs) based on two scores were identified and subjected to functional analysis, demonstrating their close associations with tumor immunity. Hub genes were selected from which CCR2, CCR4, CCR8 and P2RY14 were found to be significantly associated with the survival rate of HNSCC patients. Conclusions: In summary, we made a comprehensive analysis of HNSCC microenvironment and illustrated several key immune-related genes, which may provide evidences for the development of precision immunotherapy.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a highly aggressive and lethal malignancy all over the world with the 5-year survival rate remaining at around 50%[1]. In recent years, the development and application of immune checkpoint inhibitors have proved to be promising and of great value for HNSCC treatment, such as anti-PD-L1 and anti-CTLA4 immune-related therapeutic methods[2]. Therefore, a more comprehensive and profound understanding of HNSCC microenvironment is crucial for the early diagnosis, effective treatment, and precise prognosis evaluation for HNSCC patients.

Tumor microenvironment (TME) is a dynamic and complicated system, including immune cells, stromal cells, extracellular matrix, and a variety of inflammatory cytokines and soluble factors[3]. It was illustrated that alterations in TME components occur in almost every step of the multi-stage process of tumor carcinogenesis[4]. Among all the components, immune and stromal cells are the most major non-tumor elements that play indispensable roles in cancer development and progression[5]. It was demonstrated that immune cells have the ability of mediating tumor cells phenotypes through multiple mechanisms. Previous studies suggested that macrophages actively participated in the whole process of tumorigenesis, influencing the proliferation, migration and invasion of malignant cells[6]. Meanwhile, stromal cells in the TME, such as fibroblasts, could regulate the growth and metastasis of solid tumor[7]. More importantly, a variety of innovative strategies focused on targeting TME components have underwent clinical trials and turned out to be promising[4]. Hence, investigation of TME may facilitate the development and improvement of therapy methods for some tricky diseases.

Conventional methods for evaluating immune and stromal cells in TME include immunohistochemistry assay and flow cytometry, both of which are based on the detection of specific marker proteins for different cell types[8]. However, these methods could not provide an in-depth and well-rounded understanding of diverse immune composition in TME due to various limitations. As an alternative, Estimation of STromal and Immune cells in MAlignant Tumours using Expression data (ESTIMATE) algorithm was designed to predict the infiltration of immune and stromal cells in tumors through analyzing the transcriptome data of TME-related genes[9]. Also, with the advent of the big data era in which abundant transcriptome profiles and detailed clinical information of cancer patients could be obtained from public database such as The Cancer Genome Atlas (TCGA), bioinformatics-based analysis has become more and more widespread. Until now, ESTIMATE algorithm has been applied to an array of tumors including thyroid cancer[10], bladder cancer[11], hepatocellular carcinoma[12], and cervical squamous cell carcinoma[13] with high efficacy, providing valuable information for the diagnostic and prognostic assessment of cancer patients.

In the current study, to our knowledge, it is the first time that immune and stromal scores in HNSCC microenvironment have been elaborated using ESTIMATE algorithm. A series of TME-related genes that predict the initiation and prognosis of HNSCC were identified and put into further analysis. Overall, this study presented a comprehensive landscape of HNSCC microenvironment which could be utilized for inventing more effective and personalized immunotherapies.

Materials And Methods

Database

Gene expression profiles and corresponding clinical information of HNSCC patients were obtained from TCGA (https://gdc.nci.nih.gov/). Detailed clinical parameters of HNSCC samples were concluded in Table 1.

Table 1
Baseline patient and primary tumor characteristics (n = 515)

Variable	No. of samples	%
Gender		
Male	375	72.8
Female	140	27.2
Age at diagnosis		
≤ 65	337	65.4
> 65	178	34.6
Grade		
I-II	365	70.9
III-IV	146	28.3
Missing	4	0.8
T stage		
I-II	189	36.7
III-IV	265	51.5
Missing	61	11.8
TNM stage		
I-II	100	19.4
III-IV	342	66.4
Missing	73	14.2
Lymph node involvement		
True	245	47.6
False	173	33.6
Missing	97	18.8

Estimate Algorithm

The immune scores and stromal scores of HNSCC microenvironment were calculated through ESTIMATE package in R language. Relationship of stromal/immune scores between different clinical parameters were evaluated by the wilcox test. The Kaplan-Meier plots were performed to show survival difference

between HNSCC patients with relative low or high stromal/immune scores. P value < 0.05 was considered statistically significantly different.

Identification Of Differentially Expressed Genes (degs)

Utilizing R software, we screened out 953 and 1019 DEGs based on immune scores or stromal scores, respectively. The cut-off criteria were set as FDR < 0.05 and $|Log_2FC| > 1.0$. Volcanic plot and heatmap were depicted to illustrate the distribution of DEGs in the low vs. high immune score or stromal score groups (Fig. 3a, b). Through the intersection of overlapping genes, a total of 235 commonly upregulated genes and 9 commonly downregulated genes were finally identified and manifested in the venn diagram (Fig. 4a). These commonly DEGs were selected for further analysis.

Functional Enrichment Analyses

Functional enrichment analysis of DEGs was conducted by the clusterProfiler package in R (version 3.6.1). Through identifying the enriched biological processes, molecular functions, cellular components, and functional pathways of these DEGs, Gene Ontology (GO) analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis help to better understand the potential functions of DEGs.

Protein-protein Interaction Network (ppi) Construction And Hub Gene Identification

To give a comprehensive landscape of DEGs, a PPI network was constructed by the STRING database. Based on the interactive connections between DEGs, the top 30 genes with the most connectivity were screened out as hub gene by R software and subjected to further corresponding analyses.

Survival Analysis

Survival analysis was conducted using the survival R package. The Kaplan-Meier survival curve was drawn to demonstrate the relationship between hub genes expression and HNSCC patient prognosis. The patients were divided into two groups (high vs. low) based on the expression level of selected genes with median determined as the cut-off. The log-rank test was performed to assess differences between survival curves. P < 0.05 was considered to be significant.

Statistics Analysis

Associations of stromal/immune/ESTIMATE scores with clinicopathological characteristics of HNSCC patients were analyzed by the wilcox test in R. The correlation between selected genes and the overall

survival of HNSCC patients was evaluated by the survival package in R. All the statistical analyses were performed by R (version 3.6.1) and P value < 0.05 was considered as statistically significant throughout this study.

Results

Correlation between immune conditions and clinical characteristics

A total of 515 HNSCC patients with genome data were obtained from TCGA database and the detailed information of patient clinical parameters was summarized in Table 1. Based on the ESTIMATE algorithm, immune scores of the samples used in this study were distributed between -928.73 and 2809.55, while stromal scores ranged from -1974.36 to 1960.05. Subsequently, we investigated the correlations between stromal/immune/ESTIMATE scores with clinicopathological characteristics of HNSCC patients. As shown in Figure 1, a higher immune or ESTIMATE score was significantly associated with female when compared to male. Higher immune/ESTIMATE score could be observed in patients with advanced tumor grade, indicating the importance of microenvironment in tumorigenesis. Moreover, advanced T stage was correlated with fewer immune infiltration.

With the aim of detecting the potential prognostic value of immune scores or stromal scores for HNSCC patient survival, we divided the patients with available follow-up information into relative high and low group. It was suggested that the overall survival of clinical cases with the high-score group of immune scores is significantly longer than those in the low immune score group (Figure 2).

Identification of differentially expressed genes (DEGs)

Utilizing R software, we screened out 953 and 1019 DEGs based on immune scores or stromal scores, respectively. The cut-off criteria were set as FDR < 0.05 and $|Log_2FC| > 1.0$. Volcanic plot and heatmap were depicted to illustrate the distribution of DEGs in the low vs. high immune score or stromal score groups (Figure 3a, b). Through the intersection of overlapping genes, a total of 235 commonly upregulated genes and 9 commonly downregulated genes were finally identified and manifested in the venn diagram (Figure 4a). These commonly DEGs were selected for further analysis.

Functional enrichment analysis

To gain an in-depth understanding of the biological functions of selected DEGs, we performed functional enrichment analysis. The top 10 BP terms, CC terms and MF terms were presented in Figure 4b. As was shown, DEGs were primarily enriched in neutrophil activation, neutrophil degranulation, neutrophil mediated immunity, regulation of inflammatory response, leukocyte differentiation and so on. Regarding cellular component, various kinds of membrane, extracellular matrix, and specific granule were the main enriched terms. Furthermore, a variety of binding including peptide binding, heparin binding, and immunoglobulin binding dominated the molecular function category. For KEGG pathway enrichment

analysis, the results suggested the main enriched pathways included several immune-related and cancer-related pathways, emphasizing the important roles of DEGs in tumor microenvironment (Figure 4c).

PPI network construction and hub gene identification

To illuminate the interactive relationships among DEGs, a PPI network was constructed by the STRING database. The confidence level was set as 0.900 and the connected network was plotted in Figure 5a. The top 30 genes with the most connectivity were screened out and determined as hub gene by R software (Figure 5b). Afterwards, hub gene network was constructed (Figure 6a) and underwent GO/KEGG enrichment analysis. The enriched results were concluded in Figure 6b and c. Specifically, selected genes mainly participate in functions involving neutrophils and leukocytes. Moreover, these genes were primarily enriched in natural killer cell mediated cytoxicity, cytokine-cytokine receptor interaction, and chemokine signaling pathway, which were all closely associated with tumor immunity.

Survival analysis of hub gene

To access the prognostic value of hub gene in HNSCC patients, survival analysis was conducted through R. It was shown in Figure 7 that a total of four genes (CCR2, CCR4, CCR8, and P2RY14) were significantly associated with patient survival rate. To be specific, patients with relative higher expression of CCR2, CCR4, CCR8, or P2RY14 had better overall survival. Our results implied the potential of utilizing these genes as prognostic indicator for HNSCC patients.

Discussion

HNSCC is ranked as the sixth most common malignancy around the world[14]. In spite of constant improvement of traditional therapeutic methods and development of innovative treatment methods, the prognosis of HNSCC patients remains desperately poor. Therefore, a comprehensive and in-depth understanding of the underlying mechanisms involved in HNSCC progression may contribute to the discovery of effective cancer therapy.

In recent years, tumor microenvironment (TME) is receiving increasing attention because of its essential roles during tumor progression. TME is the place where tumor cells could interact with the immune system, so as to influence tumor evolution, progression, recurrence and drug resistance. For example, a study found comprehensive interactions between tumor cells and the surrounding stroma, which could be exploited as a potential therapy target for lung cancer[15]. Another study illustrated the intimate participation of TME components in multiple oncogenic process[16]. Also, TME composition alterations may affect the malignant transformation of solid tumor, providing a promising target for immunotherapy. There already have been several studies applying ESTIMATE algorithm to calculate the immune and stromal status. Trough deciphering immune-related genes by applying ESTIMATE, a study explored significant genes which have predictive values on clear cell renal cell cancer patient survival[17]. To further understand the TME of cutaneous melanoma, the ESTIMATE algorithm was utilized to access immune and stromal status and found out effective prognostic immune-associated biomarkers[18]. In

addition, another study suggested that high immune scores were closely associated with the better overall survival rate of cancer patients, implying the importance of TME in patient prognosis[13].

In the current study, we made a relative well-rounded understanding of HNSCC TME composition to investigate potential significant mechanisms underlying cancer progression. Immune scores, stromal scores, and ESTIMATE scores in individual HNSCC samples have been evaluated by ESTIMATE algorithm. Analysis results showed that immune or ESTIMATE score was significantly associated with gender, tumor grade, and T stage, implying the significance of immune infiltration in cancer progression. Through bioinformatics-based differential analysis, 235 commonly upregulated genes and 9 commonly downregulated genes were finally identified. GO analysis suggested that the candidate DEGs primarily participate in neutrophil activation, neutrophil degranulation, and neutrophil mediated immunity, all are indispensable biological processes involved in tumor immunity[19, 20]. Neutrophils, known as key effector cells in innate immunity, were demonstrated to play critical roles in carcinogenesis. A published study summarized that neutrophils may present protumor or antitumor functions according to different phenotypes in the tumor microenvironment[19]. Another study suggested that neutrophils participate in various aspects of cancer initiation and progression, especially in tumor angiogenesis and metastasis[21]. Therefore, investigation of significant genes with the ability of regulating neutrophil activity may help to discover efficient strategies to mediate the tumor-supporting functions of neutrophils during tumorigenesis. Regarding cellular component, a noticeable enriched term of DEGs was extracellular matrix. Intimate and complex communications between tumor cells and the ECM have been established and this result emphasized the roles of DEGs in HNSCC progression[22–24]. What is more,

KEGG analysis results suggested these genes showed enrichment in cytokine-cytokine receptor interaction, cell adhesion molecules (CAMs), B cell receptor signaling pathway, and Toll-like receptor signaling pathway, all were functional processes involved in HNSCC pathogenesis. Due to the relative straightforward representation of intricate relations between genes presented by interactive network, a PPI network based on DEGs was constructed in this study. Analysis results showed that FPR2 was the most significant gene with the highest connectivity degree, followed by C3AR1. Formyl peptide receptor 2 (FPR2) was originally found to be critical in mediating mucosal homeostasis and inflammatory responses. Several studies have uncovered its roles in altering neutrophils' functions and influencing leukocytes recruitment[25, 26]. More importantly, FPR2 was demonstrated to regulate the malignant transformation of human cancer cells through affecting multiple biological processes including cancer cell proliferation, migration, and invasion[27–29]. Moreover, a study found FPR2 could promote antitumor host defense through protecting macrophages from M2 polarization[30]. Complement C3a receptor 1 (C3AR1) was verified to induce CD8 + T proliferation by amplifying antigen presenting cell costimulatory molecule expression and innate cytokine production[31]. Also, in vitro experiments implemented by a research team demonstrated the regulatory functions of C3AR1 in tumor cell migration and invasion[32].

Subsequently, the top 30 key genes were subjected to functional enrichment analysis and the results were similar to those of DEGs, implying their significant influence. Then, survival analysis was conducted and four genes (CCR2, CCR4, CCR8, and P2RY14) were screened out to be promising prognostic markers for

HNSCC. CCR2, CCR4, and CCR8, as different types of C-C motif chemokine receptor, were demonstrated to mediate the cross-talk between immune cells and cancer cells. To be specific, a study found that mice treated with CCR2 antagonist presented reduced tumor growth and metastasis and it suggested a bidirectional cross-talk between tumor associated macrophages and cancer cells via CCR2 signaling[33]. In hepatocellular carcinoma, blockade of CCL2/CCR2 signaling also led to reduced tumor progression and improved survival rate. To be details, CCL2/CCR2 axis blocking could effectively inhibit the recruitment of inflammatory monocytes, infiltration and M2-polarization of tumor associated macrophages, so as to result in the reversal of the immunosuppression status of the tumor microenvironment[34]. A study provided evidences about the involvement of CCR4 in Th1/Th2 regulation and suggested the combination of anti-CCR4 antibody with other immune modulators could be a potential treatment approach for human solid tumors[35]. In another animal modal, anti-CCR4 treatment to dogs with spontaneous bladder cancer resulted in improved prognosis and lower therapy-related toxicities[36]. For CCR8-based mAb therapy, the antitumor activity was associated with increased tumor-specific T cells and enhanced infiltration of $CD4^+$ and $CD8^+$ T cells, implying the active involvement of CCR8 in cancer-related immune responses[37]. The blockade of CCR8 signals was implicated to be an attractive potential strategy for therapeutic intervention in multiple cancers[38]. Collectively, these findings identified CCR2, CCR4, and CCR8 could be exploited as novel theraputic target for tumor immunotherapy and further antibody treatment should be tested in clinical trials before putting into practice. Purinergic receptor P2Y14 (P2RY14) was regarded to be a key front line player able to modulate innate mucosal immunity[39]. However, researches focused on its relationship with tumorigenesis have not been performed. More in-depth investigation of these key genes may contribute to the development of more innovative and efficient treatment strategies.

The present study explored HNSCC microenvironment using TCGA database, which included a large number of clinical samples and available updated clinical information. However, there still exist some limitations which need to be elaborated. Firstly, since we retrieved transcription data and clinical information from TCGA database, the potential of selection bias may lead to biased conclusion. Moreover, biological algorithm-based findings in this study need further validation before putting into practice, such as some confirmatory experiments including in vitro and in vivo biological research.

In summary, our study utilized TCGA database and illustrated TME-related DEGs based on ESTIMATE algorithm. Analysis of the correlation between gene expression with patient prognosis was performed, discovering several immune-related prognostic markers for HNSCC patients. The relative comprehensive understanding of HNSCC microenvironment may guide the development of more effective immune-based therapies.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

No parts of this manuscript are being considered for publication elsewhere.

Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by National Key R&D Program of China (2017YFC 0840100, 2017YFC 0840110).

Authors' contributions

Y and QX designed the study protocol. XL analyzed the data and JY performed statistical analysis. XL and JY completed the figures. XL wrote the manuscript and QX made revisions. All authors approved the final version of the manuscript.

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Figures

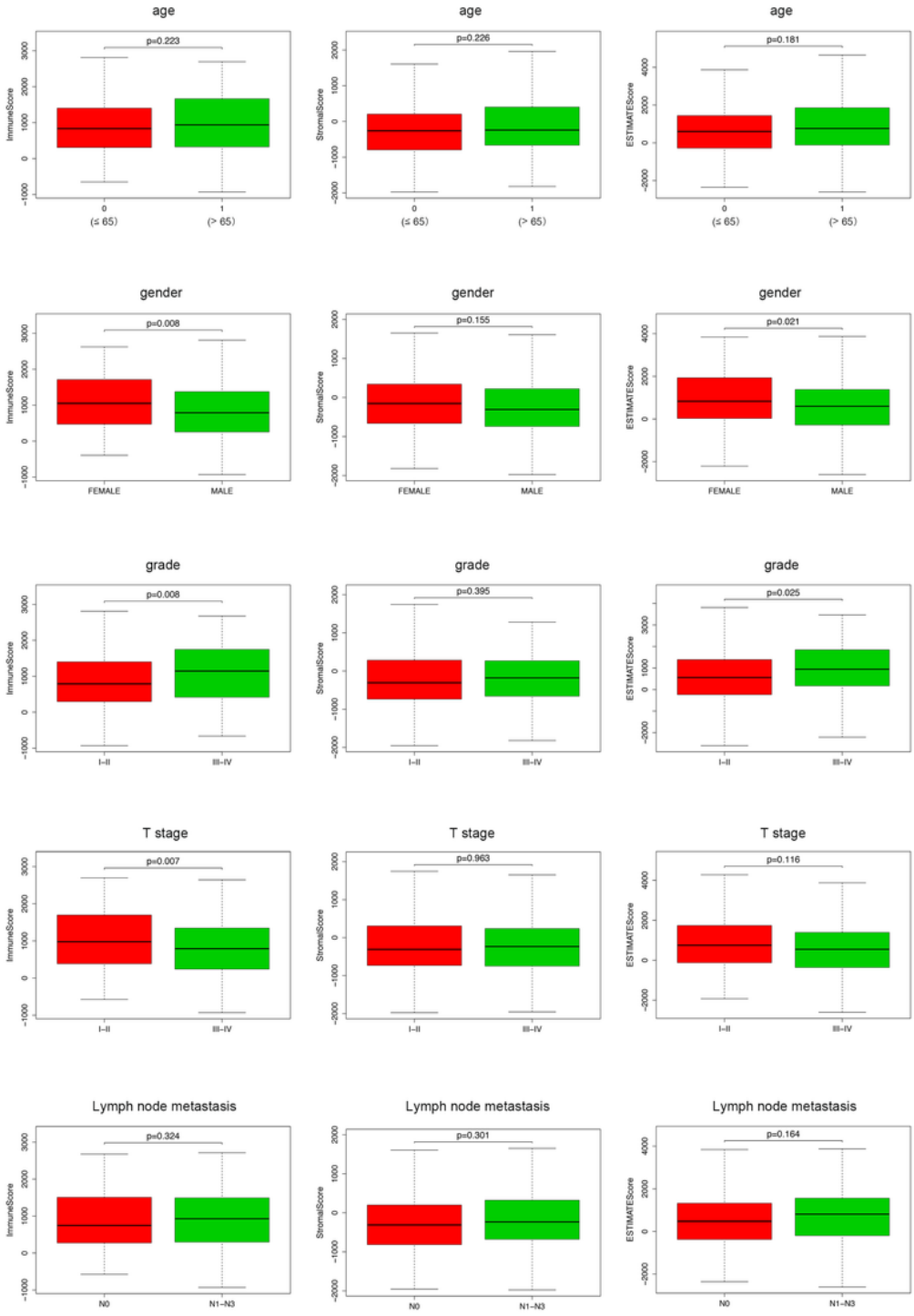

Figure 1

The correlation of immune, stromal, or ESTIMATE scores with clinical characteristics of HNSCC patients.

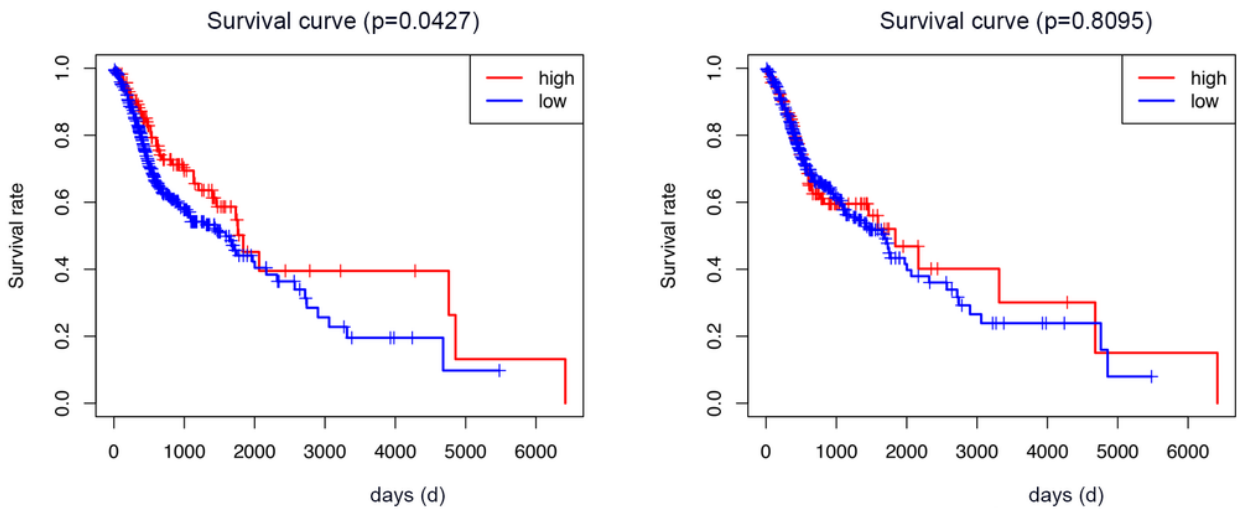

Figure 2

Association of immune or stromal scores with the overall survival rate of HNSCC patients.

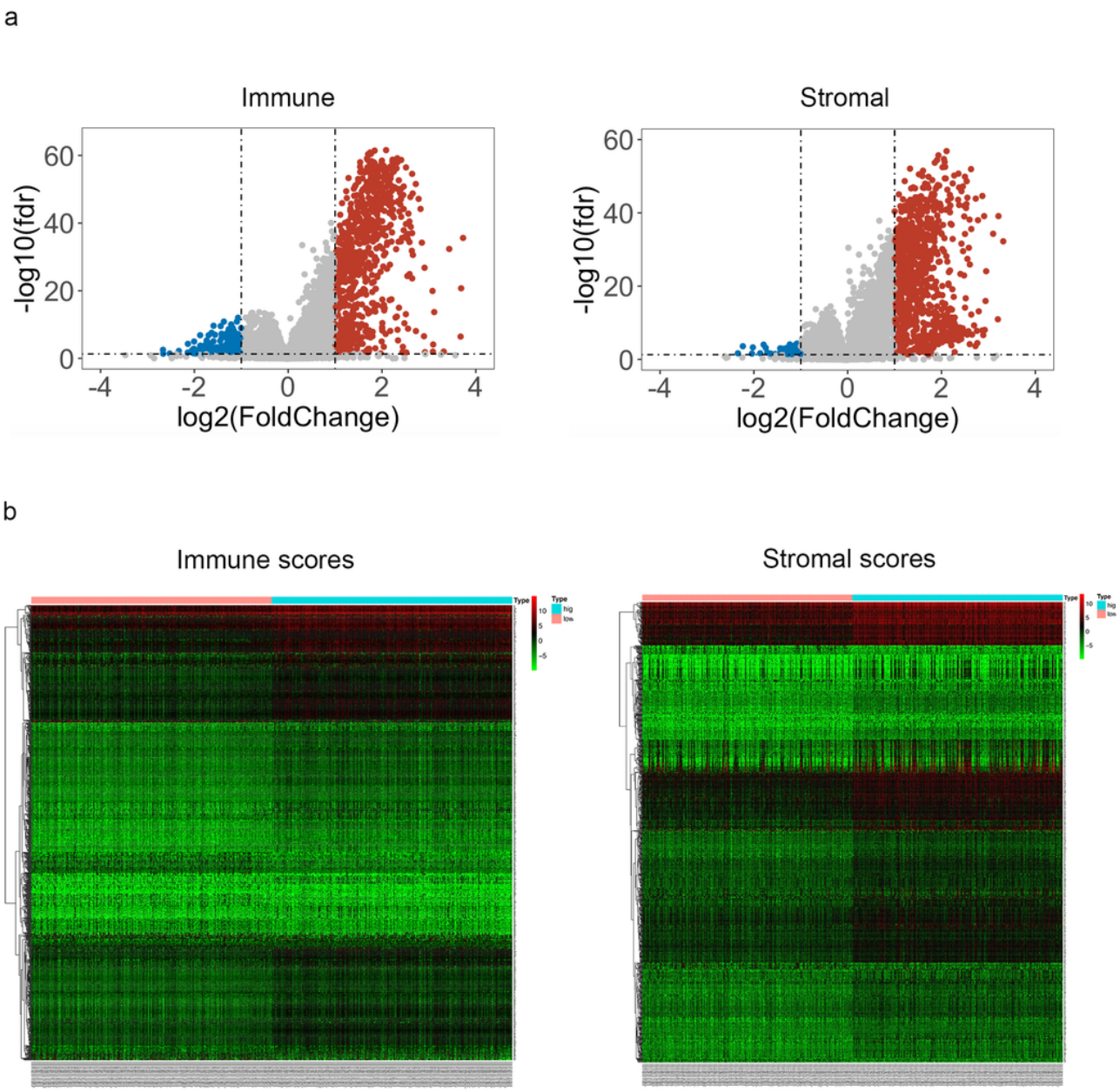

Figure 3

Differentially expressed genes (DEGs) identified by immune score or stromal score. a Volcanic plot showing the distribution of DEGs in the low vs. high immune score or stromal score groups. The cut-off criteria were set as FDR < 0.05 and |Log2FC| > 1.0. Blue indicates downregulation and red indicates upregulation. b The heatmaps were drawn to show the DEGs profiles in the low vs. high immune score or stromal score groups. Green means downregulated genes and red means upregulated genes.

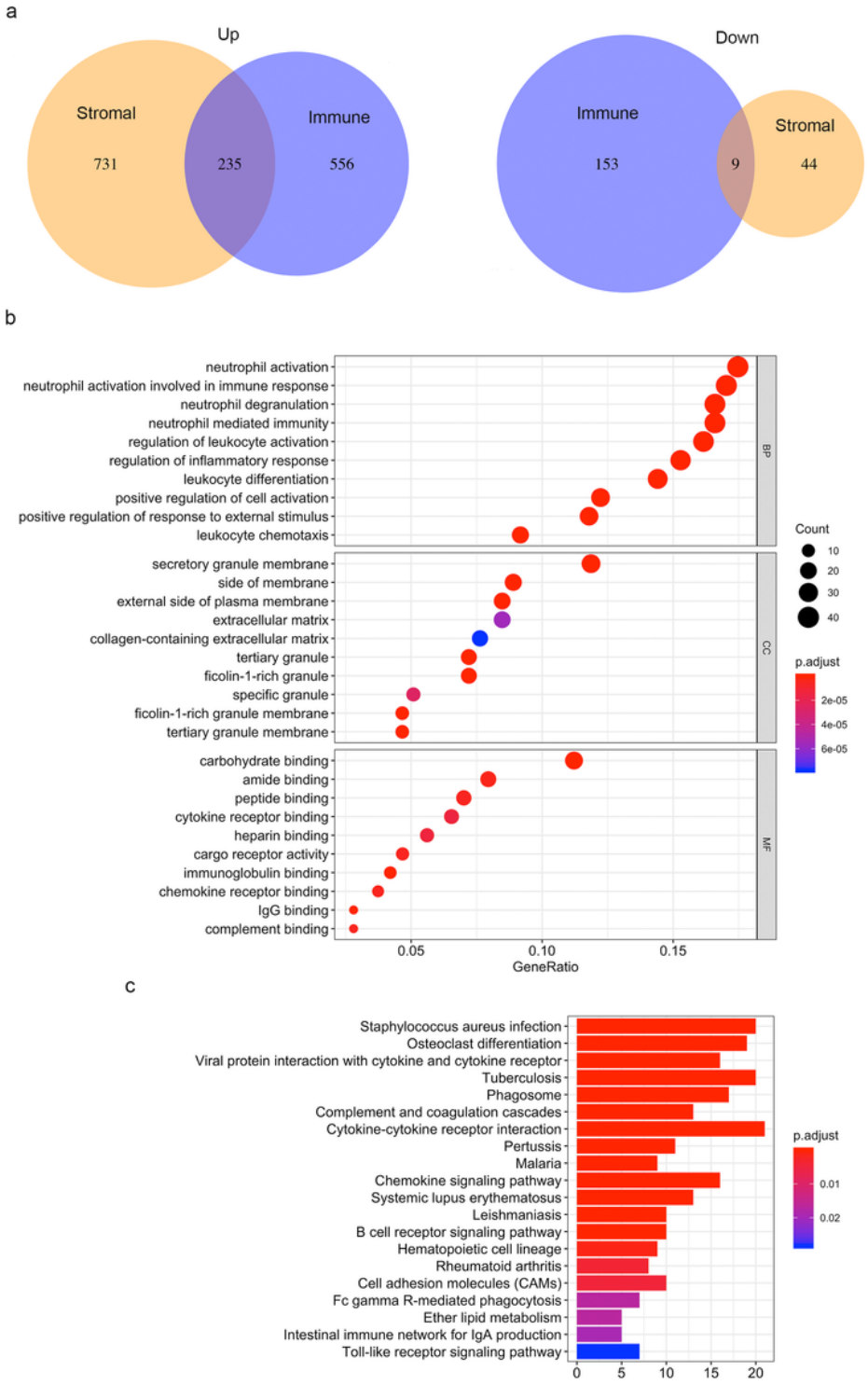

Figure 4

Functional enrichment analysis of DEGs. a Venn plot of commonly upregulated or downregulated genes with immune score and stromal score. b Top 10 biological process (BP) terms, cellular components (CC) terms, molecular functions (MF) terms enriched by DEGs. c Top 30 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched by DEGs.

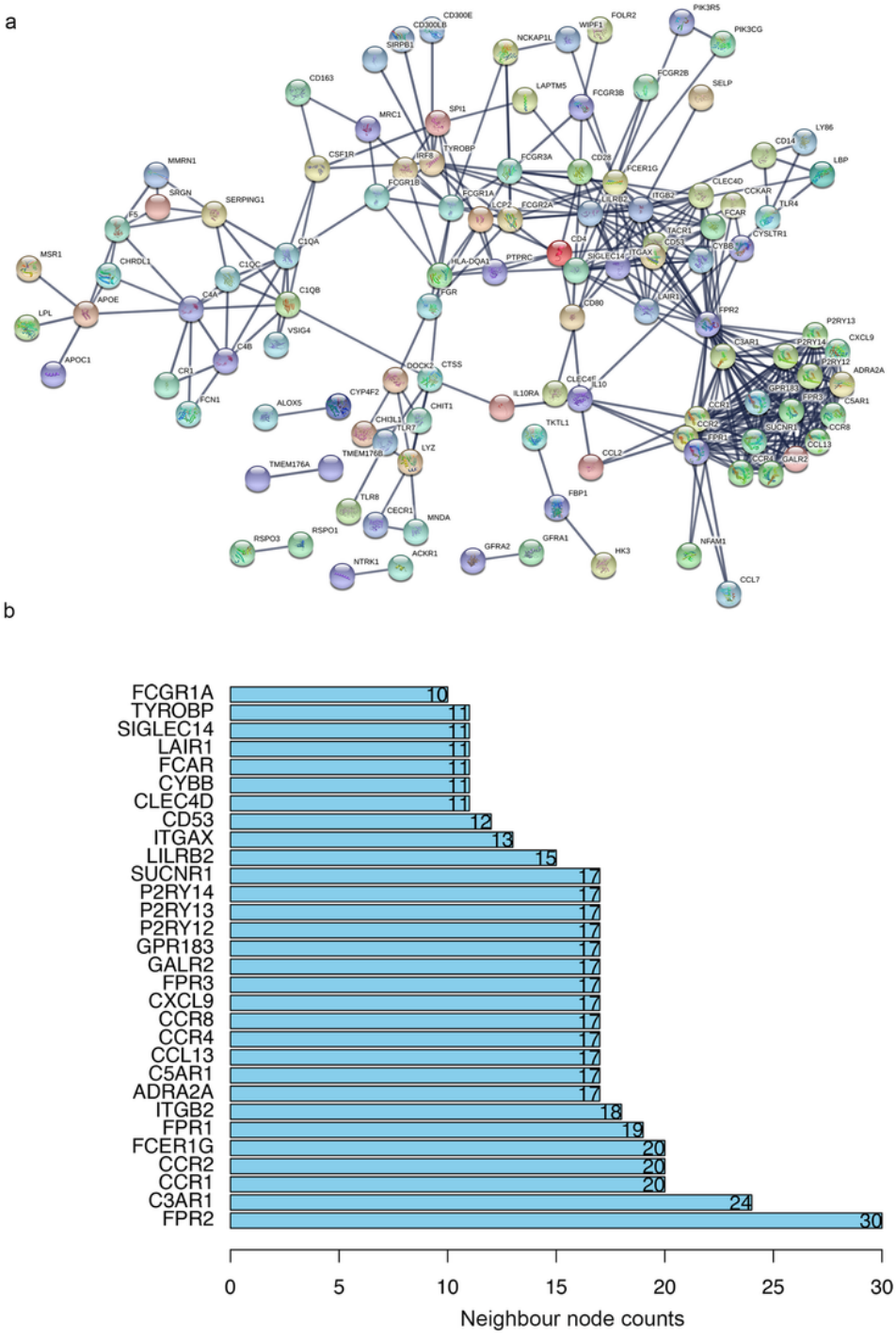

Figure 5

PPI network construction and hub gene identification. a A comprehensive PPI network of DEGs was constructed by STRING database. b The top 30 significant genes with the most connectivity inside PPI network were screened out.

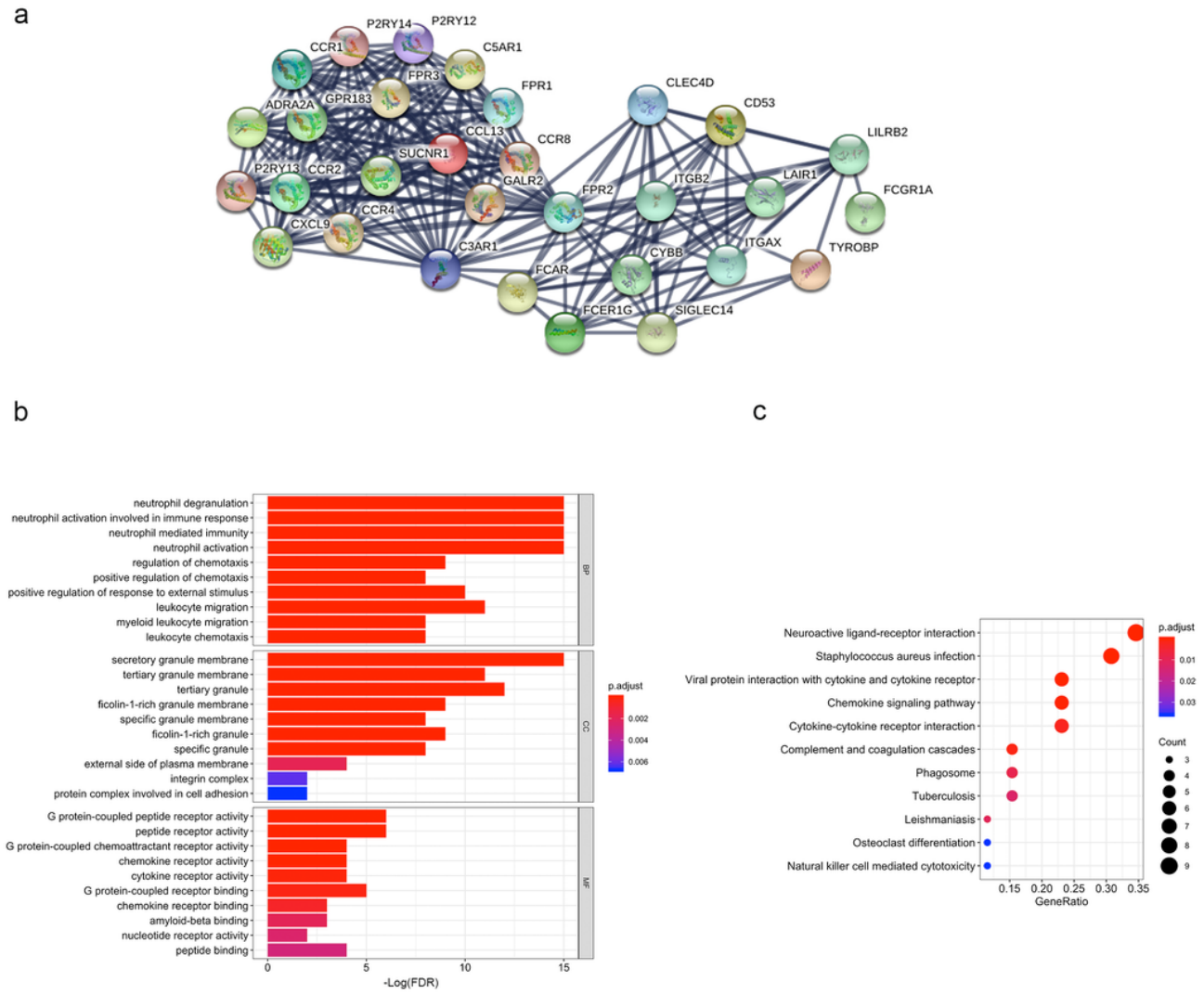

Figure 6

Bioinformatics analysis of hub gene. a A comprehensive PPI network of hub gene was constructed by STRING database. b Gene Ontology (GO) analysis of the selected genes. c Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the selected genes.

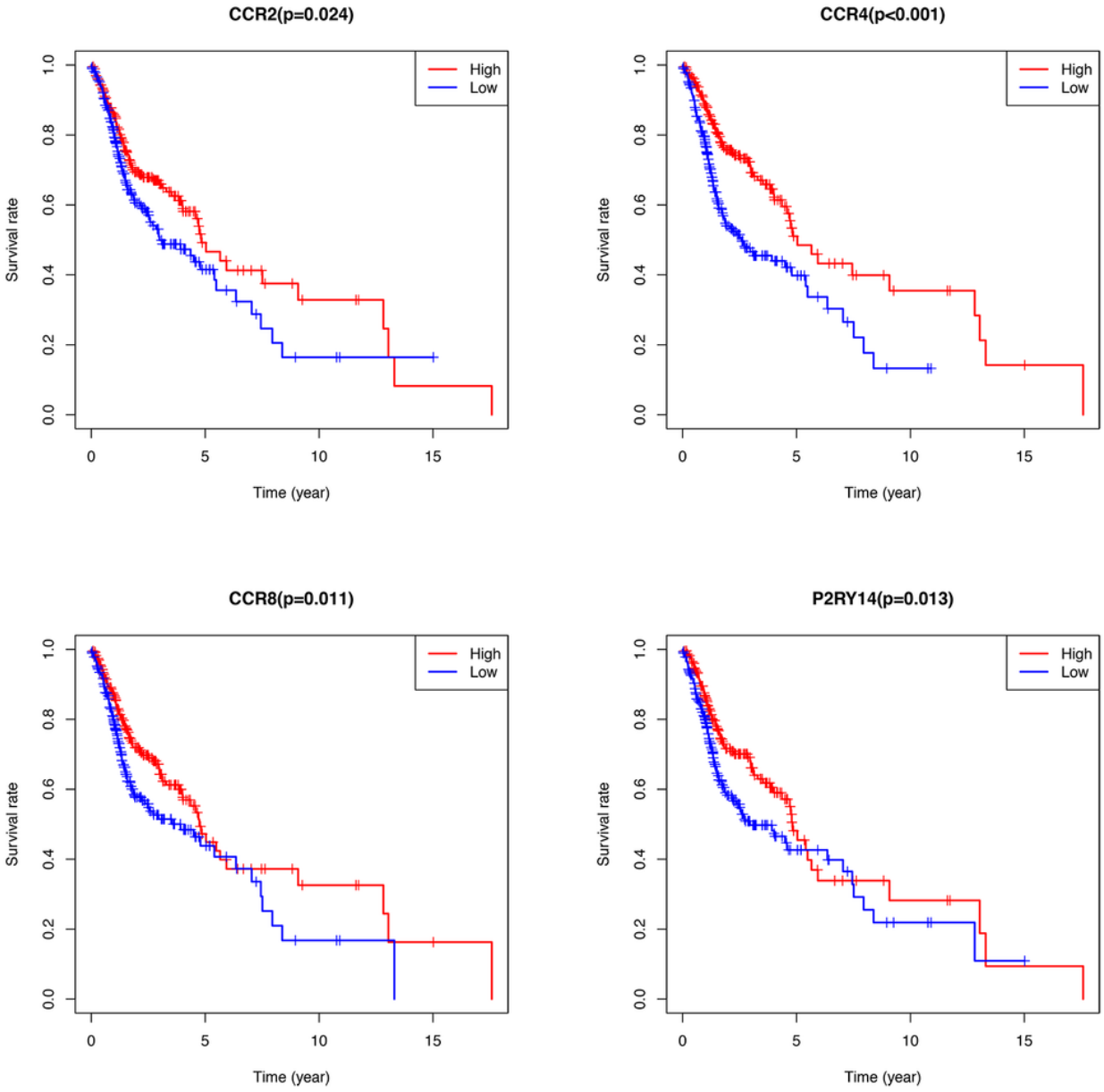

Figure 7

Kaplan-Meier survival curves showing the prognostic value of CCR2, CCR4, CCR8, and P2RY14 in HNSCC patients.