

Downregulation of CCL22 and Mutation of NOTCH1 in Tongue Squamous Cell Carcinoma Decreases Th2 Cell Recruitment and Expression to Predict Poor Clinical Outcomes

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Abstract

Background: Tongue squamous cell carcinoma (TSCC) exhibits a high rate of local recurrence and cervical lymph node metastasis. The effect of the tumor microenvironment on TSCC remains unclear.

Methods: Transcriptome data and somatic mutation data of TSCC were obtained from the HNSC project of The Cancer Genome Atlas (TCGA). Immune filtration analysis between early stage (clinical stage I-II) and advanced stage (III-IV) was performed using single-sample Gene Set Enrichment Analysis and MCPcounter. Differentially expressed genes were filtered and their related functions were analyzed by Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis. Kaplan-Meier survival analysis and a Cox-regression model were used to evaluate the survival of patients with the CCL22 signature. Maf-tools was used to show an overview of somatic mutations in TSCC.

Results: In patients with TSCC, Th2 cells were significantly increased in patients in the early stage of the disease. The Th2 cell-related chemokine CCL22 was downregulated in patients in the advanced stage. Univariate and multivariate Cox analyses revealed CCL22 as a good prognostic factor for TSCC. A nomogram based on the expression of CCL22 was constructed as a prognostic indicator for TSCC. NOTCH1 mutation was increased in patients in the advanced stage, which inhibited activation of the NOTCH1-Th2 differentiation pathway.

Conclusions: In TSCC, high expression of CCL22 can promote the recruitment of Th2 cells and predict better survival. Mutation of NOTCH1 inhibits the differentiation of Th2 cells. Combined with decreased Th2 cell recruitment and differentiation, tumor progression may occur.

Introduction

Oral squamous cell carcinoma is one of the most common malignant tumors in humans, accounting for 2% of all body tumors [1]. Tongue squamous cell carcinoma (TSCC) is the most common type of oral squamous cell carcinoma. TSCC exhibits a high potential for local recurrence and metastasis to the cervical lymph nodes. Although great progress has been made in treating TSCC, the five-year survival rate of patients has not improved over the last 20 years [2]. Therefore, improving the understanding of the mechanism of TSCC progression may lead to improvements in the prognosis of patients with this disease.

A large amount of evidence indicates that the interaction between tumor cells and tumor microenvironment is of great significance for the occurrence and development of tumors [3-7]. Excessive infiltration of tumor stromal cells, such as regulatory T cells (Tregs) or myeloid suppressor cells, and cytokines may contribute to cancer progression and the acquisition of invasive and immunosuppressive characteristics in patients with TSCC [8]. The correlation between the development of TSCC and other immune microenvironment components remains unclear.

T helper 2 cells differentiate from naïve CD4+ T cells and are thought to mediate humoral immunity. Most studies have indicated that high levels of Th2 cells predict poor survival in colorectal cancer and esophageal cancer [9, 10]. In contrast, in other studies, high Th2 counts and expression levels of IL4, which is secreted by Th2, predicts a good prognosis in B cell non-Hodgkin's lymphoma (NHL) and non-small cell lung cancer (NSCLC). The role of Th2 cells in TSCC has not been studied.

Accordingly, in this study, the immune infiltration between Early stage and Advanced stage of TSCC from the Cancer Genome Atlas (TCGA) was analyzed. According to the results of immune infiltration Th2 cells were found to be significantly enriched in early stage patients. The chemokines of Th2 cells and whole status of gene mutations were investigated to explain the enrichment of Th2 cells.

Materials And Methods

RNA-sequencing data from TCGA

Gene expression data, including the count and fragments per kilobase of transcript per million mapped reads (FPKM) type, and corresponding clinical information of patients with TSCC (178 cases) were downloaded from head and neck squamous cell carcinoma projects of TCGA database (<https://genome-cancer.ucsc.edu/>). Patients diagnosed with TSCC (including tongue and mouth floor) and with complete follow-up data were included. The FPKM data were transformed into transcripts per million reads for further analyses. This study met the publication guidelines described by TCGA. All data used in this study were obtained from TCGA, and thus ethics approval and informed consent were not required.

Acquisition of somatic mutation data

Somatic mutation data were obtained from TCGA database via the GDC data portal (<https://portal.gdc.cancer.gov/>). From the four subtypes of data files, we selected the "mutect2" data. We used maftools [11] package to prepare the somatic mutations in mutation annotation format. The results of mutation analysis were visualized with maftools.

Analysis of immune infiltration

Immune infiltration analysis of TSCC was conducted by MCPcounter and single sample Gene Set Enrichment Analysis (ssGSEA) using GSVA package (<http://www.bioconductor.org/packages/release/bioc/html/GSVA.html>) in R. Based on the signature genes from the literature [12, 13], the relative enrichment score of all immune cells was quantified from the gene expression profile (transcripts per million reads type) for each tumor sample. The *t* test and Spearman correlation were used to evaluate the association between CCL22 expression and immune cells.

Differentially expressed gene (DEG) analysis

Cases were divided into early stage (clinical stage I–II) and advanced stage (clinical stage III–IV) according to the clinical information. The expression data (count type) were compared between the two groups to identify differentially expressed genes (DEGs) using the DESeq2 [14] package in R. A |log2 fold-change| >1.5 and adjusted *P* value < 0.05 were set as the threshold values for DEGs.

Functional analysis of DEGs

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted using the enrichGO and enrichKEGG functions of the clusterProfiler R package [15]. An adjusted *P* value (false-discovery rate) less than 0.05 was considered as statistically significant. The GO and KEGG results were visualized using clusterProfiler R and GOpot package [16].

Statistical analysis

Statistical analysis was performed using R (4.0.2). Gene expression in the early and advanced stages was compared by *t* tests. The relationship between clinicopathological features and CCL22 expression was analyzed by *t* test. Clinicopathologic characteristics associated with overall survival were analyzed with the Cox regression and Kaplan-Meier methods. Multivariate Cox analysis was used to determine the influence of CCL22 expression on survival along with other clinical features. The receiver operating characteristic curve was drawn to test the performance of the multiCox model using R package survivalROC. A nomogram was constructed based on the results of multivariate analysis by using rms package in R. Decline analysis curves were drawn to evaluate the performance of the nomogram by using rmda package. All hypothetical tests were two-sided, and *P* < 0.05 was considered as significant in all tests.

Results

Th2 cells were increased in early stage patients with TSCC

To compare the differences in immune cells between the early and advanced stages of TSCC, we first used MCPcounter package to analyze the components of the microenvironment in each tumor sample (Fig. 1A). Among these immune cells, T cells were significantly increased in early stage patients with TSCC (Fig. 1B, *P* = 0.041). To verify which type of T cells changed, ssGSEA was performed by using two kinds of gene signatures from different researches[12, 13]. We defined two signatures as Immunity[13] and 28 Immune Cells[12]. The gene signatures were shown in supplementary data. The immune cell infiltration levels were showed in Fig. 1 C and E. Th2 cells were increased significantly in Early Stage patients in both two gene signatures (Fig. 1 D and F. *P* value was 0.011 and 0.012 for Immunity and 28 Immune Cells, separately).

Screening of differentially expressed genes (DEGs between early stage and advanced stage patients with TSCC and functional cluster analysis

To explore the potential mechanism causing upregulation of Th2 cells, we analyzed DEGs in early and advanced stage samples. A total of 478 DEGs were identified; 348 genes were upregulated and 130 were downregulated. A total of 149 genes were filtered by the threshold of $|log_2 \text{fold-change}| > 1.5$ and adjusted $P < 0.05$ (Fig. 2A). Among these genes, 43 genes were downregulated, and 106 genes were upregulated. The functions of the DEGs in patients with TSCC were predicted by GO and KEGG enrichment analysis. The top 10 GO enrichment items in biological process, molecular function, and cellular component are shown in Fig. S1. The KEGG results showed that chemokine signaling pathway was the most enriched item (Fig. 2B). Combined with the increase in Th2 cells, we predicted that chemokines influence Th2 cell aggregation in patients with TSCC.

CCL22 and CCR4 were upregulated in early stage TSCC and positively correlated with the expression level of Th2 cells

CCL22 and CCL17 are associated with the induction of chemotaxis in T cells and, particularly, Th2 cells by binding to the chemokine receptor CCR4. We compared the expression of CCL22, CCL17, and CCR4 between early and advanced stage samples and found that CCL22 and CCR4 were both upregulated in early stage samples (Fig. 3A and B), whereas there was no significant difference in CCL17 expression (Fig. S2). We next analyzed the correlation between the expression of CCL22 or CCR4 and immune cell infiltration level in MCPcounter for the immunity and 28 immune cells groups by Spearman correlation. The correlations between immune cells and CCL22 or CCR4 are shown in Fig. 3C–H. The results indicate that CCL22 and CCR4 expression is strongly positively correlated with Th2 cells.

Prognostic value of CCL22 in TSCC

To further examine how CCL22 and CCR4 are involved in TSCC development, we analyzed the correlation between CCL22 or CCR4 and clinical parameters. The clinical information of 178 patients with TSCC from TCGA database was analyzed. The clinicopathological features are shown in Table 1. The expression of CCL22 was higher in stages T1 and T2 than in stages T3 and T4 (Fig. 4A), whereas CCR4 was not correlated with the clinicopathological features. The expression level of CCL22 in TSCC tissues was labeled as low or high according to the median value. A Kaplan-Meier curve of overall survival was plotted to analyze the prognosis of patients with TSCC with different expression levels of CCL22. Log-rank test of overall survival revealed that high expression of CCL22 was significantly associated with a better prognosis ($P = 0.037$) (Fig. 4B). Univariate and multivariate Cox regression analyses were performed to identify independent prognostic factors in patients with TSCC (Table 2 and Fig. S3). These results suggest that CCL22 is an independent protective factor in patients with TSCC, whereas CCR4 is not (Fig. 4C). The area under the curve of the multivariate Cox model was 0.7683 (Fig. S4).

Survival prognostic models with CCL22 for TSCC

The results above indicate that CCL22 is an independent prognostic factor in TSCC; thus, we attempted to establish a predicted model for overall survival by fitting CCL22 expression and other characteristics. A nomogram integrating CCL22 expression and other characteristics including age, gender, tumor grade, T

stage, and N stage was constructed (Fig. 5A). A higher point on the nomogram indicated a worse prognosis. The performance of the nomogram with CCL22 was evaluated using a calibration curve, which showed a C-index of 0.7683 (Fig. 5B). We also performed decision curve analysis to evaluate the performance of this prediction model. The prediction nomogram exhibited a higher benefit percentage (Fig. 5C). In summary, this nomogram with CCL22 may be a better model than individual prognostic factors for predicting the survival of patients with TSCC.

NOTCH1 mutation in advanced stage samples reduced Th2 cell differentiation

To identify somatic mutations in patients with different stages of TSCC, mutation data were downloaded and visualized using the maftools [11] package. Enrichment analysis of all mutated genes in early and advanced stage patients indicated that the RTK-RAS pathway and NOTCH1 pathway were the major pathways affecting the development of TSCC (Fig. 6A and B). The waterfall plot revealed the top six mutated genes in patients with TSCC. According to this plot, the rate of NOTCH1 mutation was significantly reduced in early stage patients (Fig. 6C). We then performed ssGSEA analysis to evaluate the activation level of the NOTCH1 signaling pathway. The gene signatures are shown in Table S1. However, NOTCH1 signaling pathway activation did not differ between early and advanced stage patients (Fig. 6D). Previous studies have suggested that NOTCH1 can induce Th2 cell differentiation [17, 18]. Gene signatures were derived from KEGG and are shown in Table S1. We repeated ssGSEA to evaluate activation of the NOTCH1-Th2 cell differentiation pathway and found that in advanced stage patients, this pathway was downregulated (Fig. 6E). These results suggest that the reduction of Th2 cells in advanced stage samples is caused by decreased CCL22 and Th2 cell differentiation which is induced by mutation of NOTCH1.

Discussion

The progression of TSCC is affected by several factors, including gene mutation, dysregulation of long non-coding RNA, and changes in the tumor microenvironment [19-21].

With the development of single-cell sequencing technology, increasing numbers of immune cells signatures have been identified and immune cells in the tumor microenvironment have become characterized in more detail [13, 22, 23]. The composition of various components in the microenvironment can be analyzed using the expression profile obtained by RNA sequencing.

Various mechanisms leading to immune cell dysfunction in the tumor microenvironment have been successively identified. The immune microenvironment can directly or indirectly affect the occurrence and development of tumors. Its mechanisms include promoting tumor angiogenesis, changing the biological characteristics of tumors cells, or establishing an appropriate tumor microenvironment to promote tumor progression [4]. Liu et al. [24] suggested that alterations in the Treg/Th17 balance in TSCC promote disease progression. Macrophages in the tumor microenvironment of TSCC can promote the invasion and migration abilities of tumor cells [25]. In our study, Th2 cells were downregulated in the advanced

stage samples. Th2 cells are differentiated from naïve CD4⁺ T cells and mainly mediate humoral immunity but the role of Th2 cells in tumors is unclear. Studies have indicated that increased Th2 cells or cytokines secreted by Th2 cells indicates a poor prognosis in colorectal cancer, esophageal cancer, and prostate cancer [9, 10, 26]. However, a study of B cell non-Hodgkin's lymphoma suggested that high expression of IL4, which is secreted by Th2 cells, is strongly correlated with reduced levels of proliferation and a greater survival duration [27]. Another study of non-small cell lung cancer suggested that high Th2 and low Th17 levels indicate good prognosis. [28]. Our results suggest that high Th2 levels play a protective role in TSCC.

Changes in the Th2 cell expression level are related to the recruitment and differentiation of these cells. CCL22 and CCL17 are chemokines that regulate Treg and Th2 cell recruitment via binding to their receptor CCR4. In our study, CCL17 expression did not differ between the early and advanced stage, whereas CCL22 was significantly decreased in the advanced stage. Overexpression of CCL22 in human tumors was reported to be associated with high infiltration of Treg cells, along with augmented tumor growth and poor prognosis in breast, gastric, and liver cancer [29-33]. Studies of CCL22 and Th2 cells are limited but indicate that CCL22 is a prognostic factor in breast and colorectal cancer [34, 35]. A study of TSCC suggested that high expression of CCL22 in TSCC influences the balance of M1- and M2-like macrophages and leads to worse prognosis [36]. However, in our study, CCL22 was a protective prognostic factor, contrasting the results of previous studies. The expression of Treg cells between early and advanced stage patients did not significantly differ (Fig. S5). We hypothesized that CCL22 only affects the recruitment of Th2 cells in patients with TSCC, rather than Treg cells, suggesting that CCL22 affects the prognosis of patients and progression of TSCC by regulating Th2 cells. Our results suggest a new idea that CCL22 can be used as a treatment target in TSCC.

Gene mutation plays a very important role in the development and progression of cancer. Recent large-scale genome sequencing efforts have validated TP53 as the most common mutation in head and neck squamous cell carcinoma [37]. A study of the mutations landscape of TSCC revealed that *TP53*, *FAT1*, *CDKN2A*, *NOTCH1*, and *PIK3CA* are the most frequently mutated genes [38]. In our study, *NOTCH1* mutation was significantly reduced in early stage patients. NOTCH signaling is involved in different types of malignant tumors [39] and has mostly been found to be altered in head and neck squamous cell carcinoma [40]; NOTCH can act as an oncogene or tumor suppressor depending on the cellular context [41]. The role of NOTCH as a tumor suppressor pathway in oral squamous cell carcinoma has been suggested in several studies [37, 42, 43]. Activated NOTCH1 exerts an anti-proliferative effect in tongue tumor cells by downregulating Wnt/β-catenin signaling and inducing apoptosis and cell cycle arrest [44].

However, in our study, the activation level of the NOTCH1 signaling pathway did not differ between early and advanced stage patients. Previous studies suggested that NOTCH1 can induce Th2 cell differentiation [17, 18]. The NOTCH-Th2 differentiation pathway was upregulated in early stage patients. These results indicate that NOTCH1 mutation affects activation of the NOTCH-Th2 differentiation pathway, thus reducing the number of Th2 cells.

According to our results, increased expression of CCL22 in TSCC may activate the recruitment of Th2 cells, whereas mutations of NOTCH1 may inhibit Th2 cell differentiation. These two mechanisms influence the expression level of Th2 cells, leading to the progression of TSCC. However, there were several limitations to this study. First, the data were obtained from a public database. Fresh tumor tissue should be analyzed to verify our results. Second, our findings were mainly based on bioinformatics and algorithms, and validation using biological experiments was not performed.

In conclusion, in TSCC, high expression of CCL22 can promote the recruitment of Th2 cells and predict better survival. Mutation of NOTCH1 inhibits the differentiation of Th2 cells. Combined with decreased Th2 cell recruitment and differentiation, these effects can lead to tumor progression.

Declarations

Availability of data and materials

All data used in this study were obtained from TCGA, and hence ethics approval and informed consent were not required. (<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>)

Authors' contributions

WC and XFL conceived the project and designed the research. XJL and ZQL carried out the data download and wrote the manuscript. WKZ and ZQL performed the statistical analysis. WC aided in drafting the manuscript. The study supervisors are WC and XFL. All authors read and approved the final manuscript

Conflict of Interest Statement

The authors declare that they have no competing interests.

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Tables

Table 1 Clinicopathological parameters of patients with tongue squamous cell carcinoma based on TCGA

Characteristics	n=178
Survival time (days, mean (SD))	798.92 (760.64)
Age (mean (SD))	59.19 (13.39)
Gender (%)	
Female	58 (32.6)
Male	120 (67.4)
Tumor_grade (%)	
G1	21 (11.8)
G2	122 (68.5)
G3	35 (19.7)
Tstage (%)	
T1	24 (14.0)
T2	56 (32.7)
T3	41 (24.0)
T4	50 (29.2)
Nstage (%)	
N0	70 (42.7)
N1	24 (14.6)
N2	68 (41.5)
N3	2 (1.2)
Clinical stage (%)	
Stage I-II	54 (30.3)
Stage III-IV	124 (69.7)

Table 2. Univariate and multivariate Cox regression analyses of clinicopathological parameters and overall survival

Characteristics	Hazard ratio (95% CI) in univariate analysis	P value in univariate analysis	Hazard ratio (95% CI) in multivariate analysis	P value in multivariate analysis
CCL22	0.774 (0.641–0.934)	0.008	0.799(0.643–0.992)	0.042
GATA3	0.878 (0.701–1.100)	0.257		
CCR4	0.846 (0.674–1.063)	0.152		
Age	1.017 (0.998–1.036)	0.087	1.016 (0.993–1.040)	0.175
Gender	1.108 (0.693–1.771)	0.669	1.062 (0.624–1.806)	0.826
Tumor_grade	1.415 (0.954–2.099)	0.084	1.469 (0.942–2.291)	0.090
Tstage	1.528 (1.209–1.932)	0.000	1.415 (1.077–1.859)	0.013
Nstage	1.518 (1.167–1.974)	0.002	1.417 (1.091–1.840)	0.009

Figures

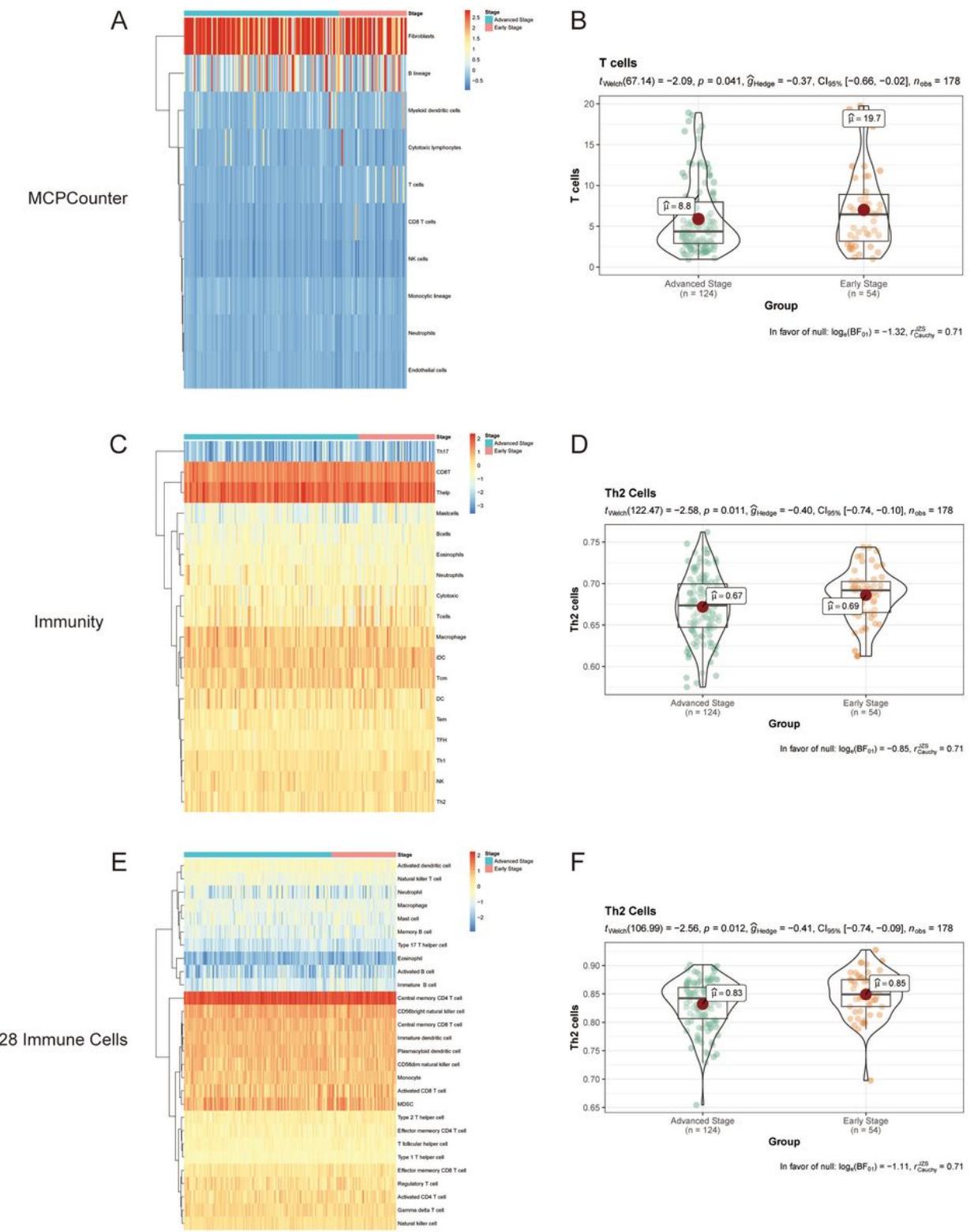


Figure 1

Immune infiltrating cells in TCGA datasets. Th2 cells were increased in early stage samples. (A) Distribution of tumor immune-infiltrating cells in TCGA samples calculated by MCPcounter. (B) Relative expression level of T cells in early and advanced stage samples calculated by MCPcounter. (C) Distribution of tumor immune-infiltrating cells in TCGA samples calculated by ssGSEA using gene signature from the literature [13]. We named this signature as Immunity (D) Relative expression level of

Th2 cells in early and advanced stage samples according to Immunity signature. (E) Distribution of tumor immune-infiltrating cells in TCGA samples calculated by ssGSEA using gene signature from the literature [12]. We named this signature as 28 Immune cells. (F) Relative expression level of T cells in early and advanced stage samples according to 28 Immune cells signature. In heatmap, red represents a higher distribution, whereas blue represents a lower distribution.

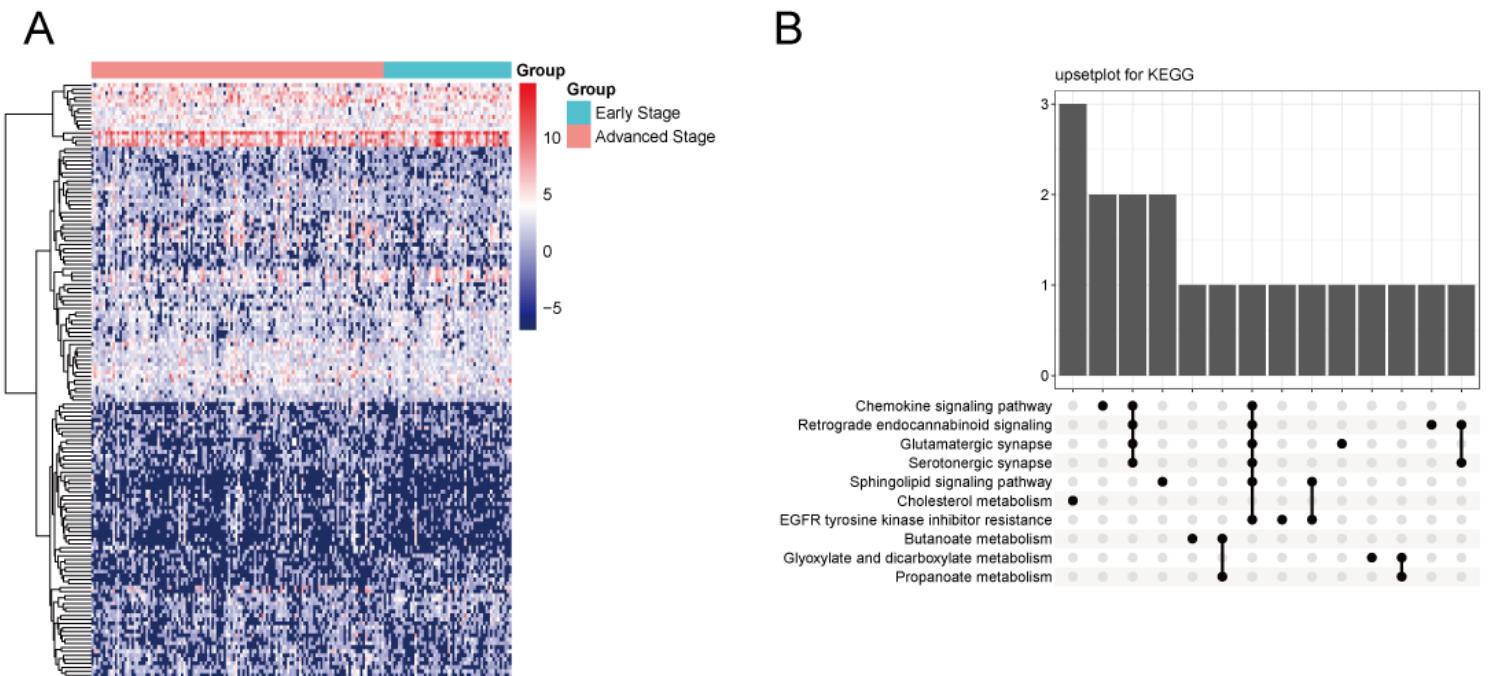
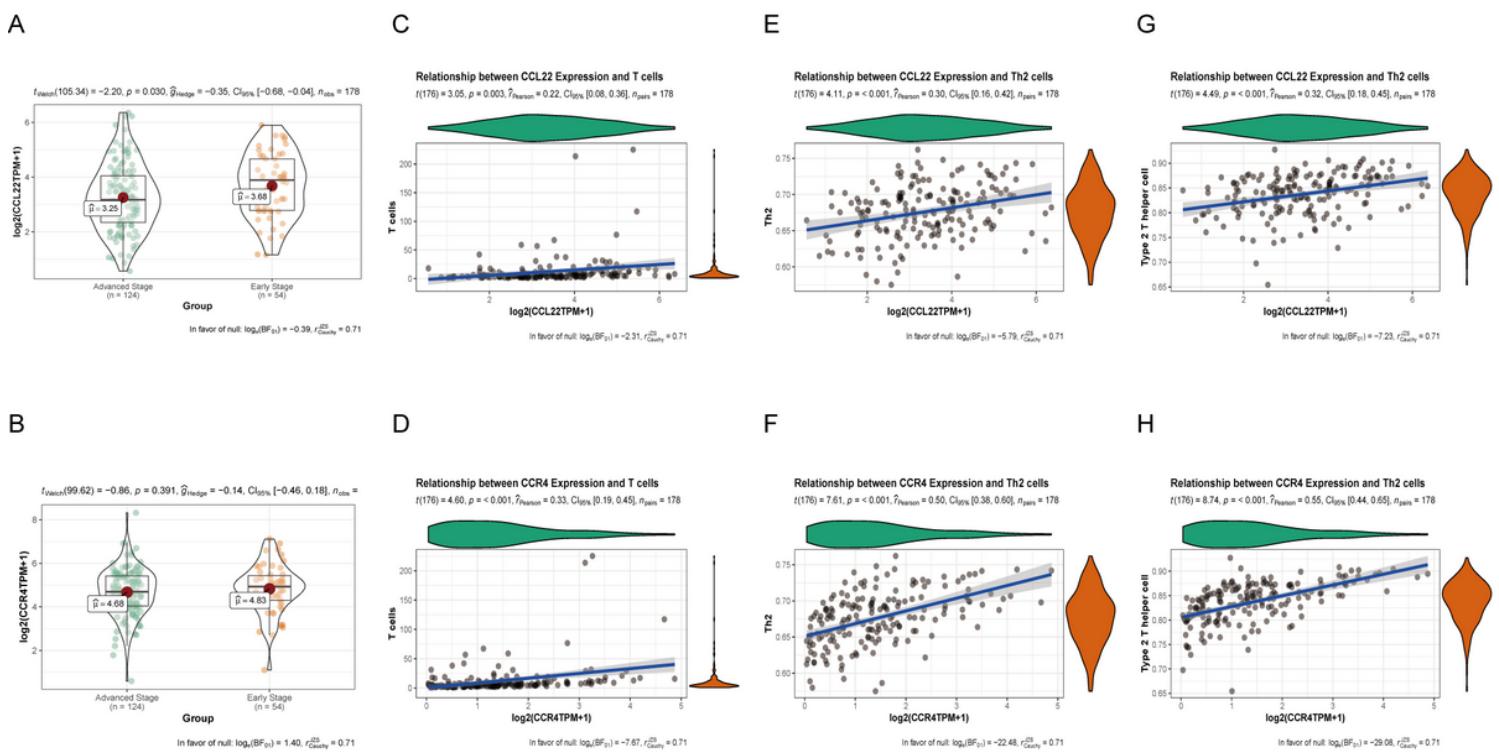
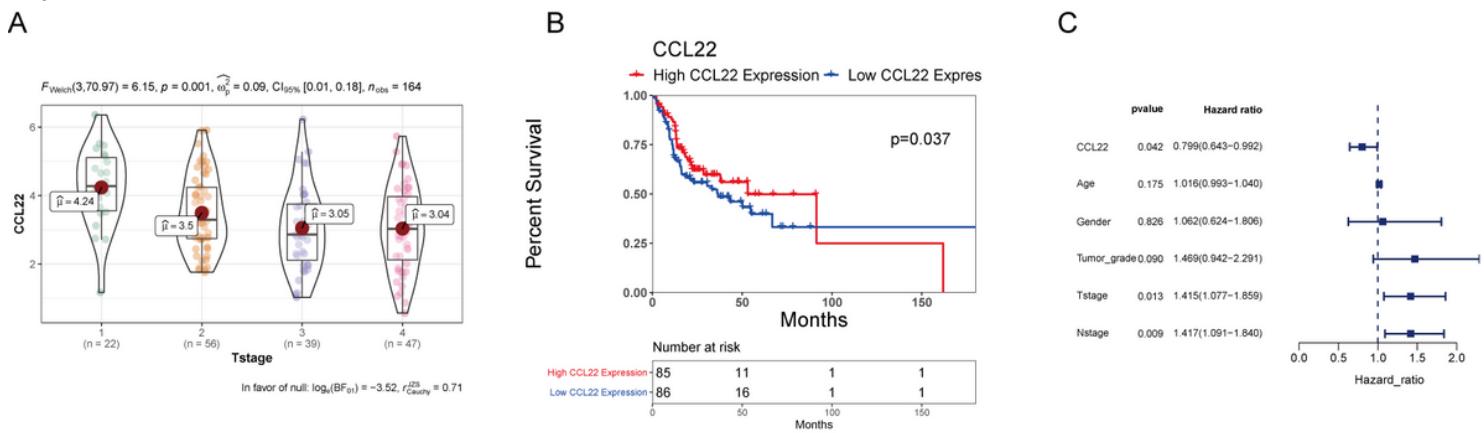


Figure 2

Screening of differentially expressed genes (DEGs) and functional analysis. (A) Heatmaps of DEGs between early and advanced stage samples. (B) Upset plot of KEGG pathway. The bar chart above represents the number of genes in each group. The dotted line at the bottom represents genes involved in each pathway.

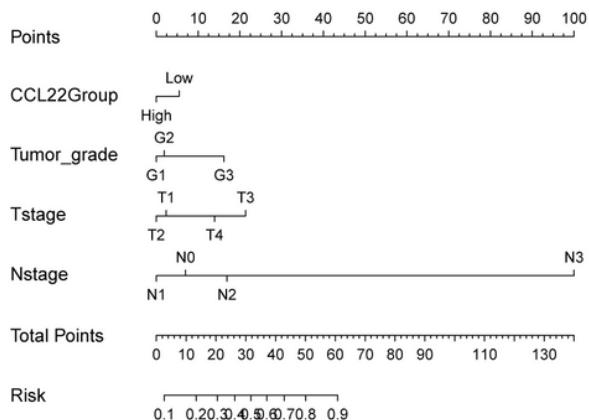
**Figure 3**

Correlation between CCL22/CCR4 and T/Th2 cell levels. Expression level of CCL22 (A) and CCR4 (B) between early and advanced stage sample. The correlation between CCL22 (C)/CCR4 (D) and T cells, using MCPcounter. The correlation between CCL22 (E)/ CCR4 (F) and Th2 cells, using Immunity signature. The correlation between CCL22 (G)/CCR4 (H) and Th2 cells, using 28 Immune cells signature. Violin plots in the margin of the dotplot represent the distribution of CCL22/CCR4 and T/Th2 cell expression.

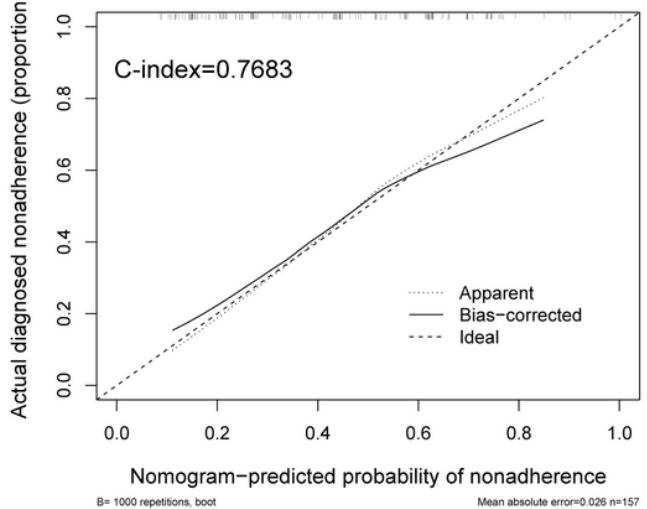
**Figure 4**

Clinical relevance and prognostic value of CCL22. (A) CCL22 expression level in different T stage samples. (B) Kaplan-Meier curve of overall survival with high and low expression of CCL22. (C) Forest plot of independent prognostic factors.

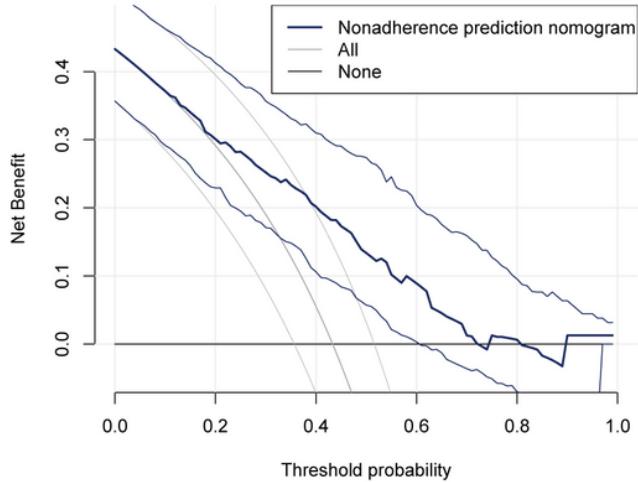
A



B



C

**Figure 5**

Establishment and evaluation of survival prognostic models for TSCC. (A) Nomogram for predicting the probability of 1-, 3-, and 5-year survival of patients with TSCC. (B) Calibration curve for evaluating the accuracy of nomogram for patients with TSCC. (C) Decision curve analysis for evaluating the performance of the nomogram. The lines on both sides represent confidence intervals

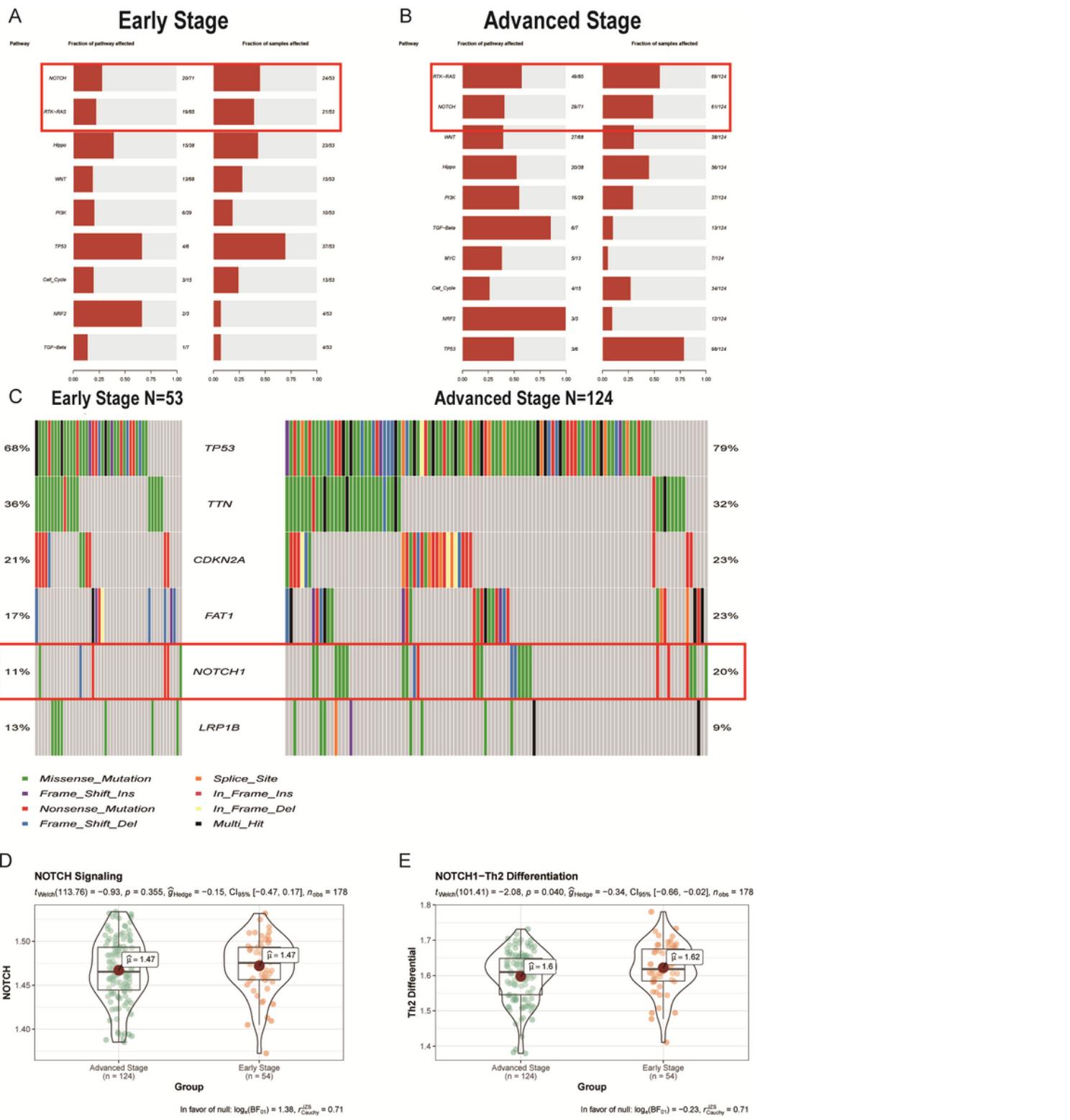


Figure 6

NOTCH1 mutation in TSCC samples reduced Th2 cell differentiation. Pathways enriched by mutation genes in early (A) and advanced (B) stage samples. RTK-RAS and NOTCH signaling were the top 2 pathways in TSCC. (C) Comparison of mutated genes between early and advanced stage samples. NOTCH1 mutation was significantly reduced in early stage (11% vs 20%). (D) NOTCH signaling activation score calculated by ssGSEA between early and advanced stage. There was no significant difference

between the 2 groups. (E) NOTCH1-Th2 differentiation pathway activation score calculated by ssGSEA between early and advanced stage. NOTCH1-Th2 differentiation pathway showed greater activation in early stage samples ($P = 0.040$).

Supplementary Files

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