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Identification of *TTC21A* as a Potential Prognostic Marker in Head and Neck Squamous Cell Carcinoma: *In Silico* Analysis

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Abstract. *Background/Aim: Tetratricopeptide repeat domain 21A (TTC21A) plays a crucial role in ciliary function and has been associated with various pathogenic processes, including carcinogenesis. However, its role in head and neck squamous cell carcinoma (HNSCC) has not been elucidated. Materials and Methods: Based on the sequencing and microarray data of HNSCC from publicly available databases, the expression of TTC21A was compared between different subgroups based on clinical and molecular parameters. The survival analysis and regression analysis were conducted using the Kaplan–Meier method and the Cox method, respectively. Functional analysis was performed by the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), and gene set enrichment analysis (GSEA) tools. Immune infiltration analysis was performed based on the expression of TTC21A. Results: TTC21A decreased in tumor tissues and was associated with N stage, histologic grade, HPV infection, and TP53 mutation in HNSCC. TTC21A was an independent indicator of overall survival for patients with HNSCC. A high level of TTC21A expression indicated a favorable prognosis. The TTC21A expression level was involved with immune-related signaling regulation, immune-related gene expression, and immune cell infiltration. TTC21A expression was potent in predicting immunotherapeutic benefits. Conclusion: TTC21A, as a potential predictor of favorable outcomes and immunotherapy response for HNSCC, is related to immune-related signaling regulation, immune-related gene expression, and immune cell infiltration.*

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Head and neck squamous cell carcinoma (HNSCC) is a common malignant tumor worldwide, with more than 54,540 new cases resulting in 11,580 deaths in 2023 in the US alone. Surgery, radiotherapy, and chemotherapy are traditional treatments for HNSCC. Despite standard treatment of HNSCC, the 5-year survival does not improve significantly, and the death rates of the oral cavity and pharynx tumors have increased by 0.4% annually over the last decade (1). It is urgent to explore new effective therapeutic approaches for refractory tumors. In recent years, tumor immunotherapy based on targeting immune checkpoints has been a promising therapy for advanced cancers (2, 3). Immune checkpoint blockade (ICB) treatments have shown significant benefits in survival for patients with cancers, including HNSCC. Pembrolizumab and nivolumab (anti-PD-1 antibodies), were approved by the FDA to treat metastatic and recurrent HNSCC (4, 5). However, >80% of HNSCC patients do not have objective responses to anti-PD-1 treatment, which highlights the urgent need to identify biomarkers to predict the response rate and investigate effective combined therapeutic schedules (4, 6).

Tetratricopeptide repeat domain 21A (*TTC21A*) encodes an intraflagellar transport-associated protein and contains several tetratricopeptide repeat (TPR) domains. *TTC21A* was reported to frequently exist in intraflagellar transport (IFT) proteins and

interact with IFT proteins that seem important for ciliary function (7). IFT is known to be essential for the development and maintenance of flagella, recently, bi-allelic mutations in *TTC21A* were found to induce asthenoteratospermia in humans and mice.

Previous studies have suggested that primary cilia are elaborate antennae and dysfunction of primary cilia affects tumor growth and response to cancer immunotherapies (8). In this study, we sought to investigate the role of *TTC21A* in HNSCC. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and gene set enrichment analysis (GSEA) were used to analyze the potential function of *TTC21A* in HNSCC. Moreover, CIBERSORT and ESTIMATE were used to calculate the relative proportions of immune cells in different tumors to study the relationship of *TTC21A* with the tumor immune microenvironment (TIM). The findings of this research provide insights into the possible effects of *TTC21A* in HNSCC as well as potential mechanism between *TTC21A* and TIM.

Materials and Methods

Data collection. In this study, RNA sequencing data for HNSCC tissues and adjacent non-cancerous tissues were obtained from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/), together with clinicopathological information of HNSCC patients. The expression profiles of GSE30784 and GSE107591 were downloaded from the NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) (9, 10). The R software version v4.0.3 (The R Foundation for Statistical Computing, 2020) was used to process and analyze the raw data.

Kaplan–Meier analysis of the prognostic significance of TTC21A. Clinical information was collected from the TCGA-HNSCC cohort. Samples were divided into two groups based on the median expression of *TTC21A*, namely, *TTC21A*-H and *TTC21A*-L. The survival difference between *TTC21A*-H and *TTC21A*-L groups was compared by performing Kaplan–Meier survival analysis. R packages, "survival" and "survminer" were used to perform the analysis.

Univariate and multivariate Cox regression. To identify the proper terms for creating the nomogram, univariate and multivariate Cox regression analyses were performed. The forest, showing the HR, 95% CI and *p*-value, was created by the 'forestplot' R package. Nomogram was drawn based on the results of multivariate Cox regression, containing terms of age, N stage, M stage and *TTC21A* expression through the 'rms' R package.

Biological function, pathway annotation, and GSEA. Data of RNA sequencing for HNSCC from the TCGA was divided into two groups based on the median expression of *TTC21A*, namely, *TTC21A*-H and *TTC21A*-L. Differential expression genes were identified by performing the limma package (version: 3.40.2) of R software, and "$p<0.05$ and up/down-regulated by at least 1.5-fold" were defined as the thresholds. GO and KEGG analyses were performed by running the Cluster Profiler package (version: 3.18.0) in R software. GSEA was performed using GSEAv4.0.3 software28, and hallmark gene sets from the Molecular Signatures Database (MSigDB) (http://software.broadinstitute.org/gsea/msigdb) were used for pathway analysis.

Immune infiltration analysis. Immune cell infiltration in the TCGA-HNSCC was estimated by the "CIBERSORT" algorithm in R package "immunedeconv", the immune infiltration scores with an abundance of 24 immune cells in different samples were calculated. Spearman correlation analysis of *TTC21A* gene expression and immune infiltration scores was performed. In addition, the "ESTIMATE" and "limma" R packages were employed to measure the immune, and ESTIMATE scores of HNSCC patients based on transcriptomic data.

Validation of gene expression at the protein level. Immunohistochemistry data available at Human Protein Atlas (HPA), a human proteome map (11), is accessed to validate the key gene's protein expression patterns based on the staining intensity levels in HNSCC tissues.

Gene expression and clinical datasets for immune checkpoint blockade. To explore the forecast efficacy of *TTC21A* in patients after immunotherapy, immunophenoscores (IPS) of HNSCC patients from the TCIA database (https://tcia.at/) were downloaded and the relationship between IPS and *TTC21A* expression was analyzed. Meanwhile, we collected data from GSE35640 and GSE91061 from the GEO dataset. Samples were divided into two groups based on the response to immunotherapy, and *TTC21A* mRNA expression was compared between the two groups.

Results

Aberrant low expression of TTC21A in HNSCC. We evaluated the distribution of *TTC21A* based on the TCGA-HNSCC dataset and found that *TTC21A* expression was significantly down-regulated in tumor tissues (Figure 1A). Then the expression of *TTC21A* between paired tumors and adjacent tissues was compared and showed that *TTC21A* was attenuated in the majority of tumor tissues (Figure 1B). In addition, we employed two independent datasets from GEO, the result confirmed that tumor tissues held a decreased level of *TTC21A* in HNSCC (Figure 1C and D).

High TTC21A expression was associated with a favorable prognosis in HNSCC. HNSCC samples were classified as *TTC21A*-Low or *TTC21A*-High group according to the median expression. As shown in Table I, *TTC21A* expression was significantly associated with N stage, histologic grade, overall survival (OS), disease-specific survival (DSS), and progress-free interval (PFI). K-M curves showed that the *TTC21A*-High group had a considerably longer OS, DSS, and PFI compared with the *TTC21A*-Low group (Figure 1E-G).

Univariate analysis was performed to analyze the importance of prognosis factors of OS in HNSCC, as shown in Figure 1H, *TTC21A* expression ($p<0.001$), N stage ($p=0.026$), and M stage ($p=0.002$), was significantly associated with OS in HNSCC.

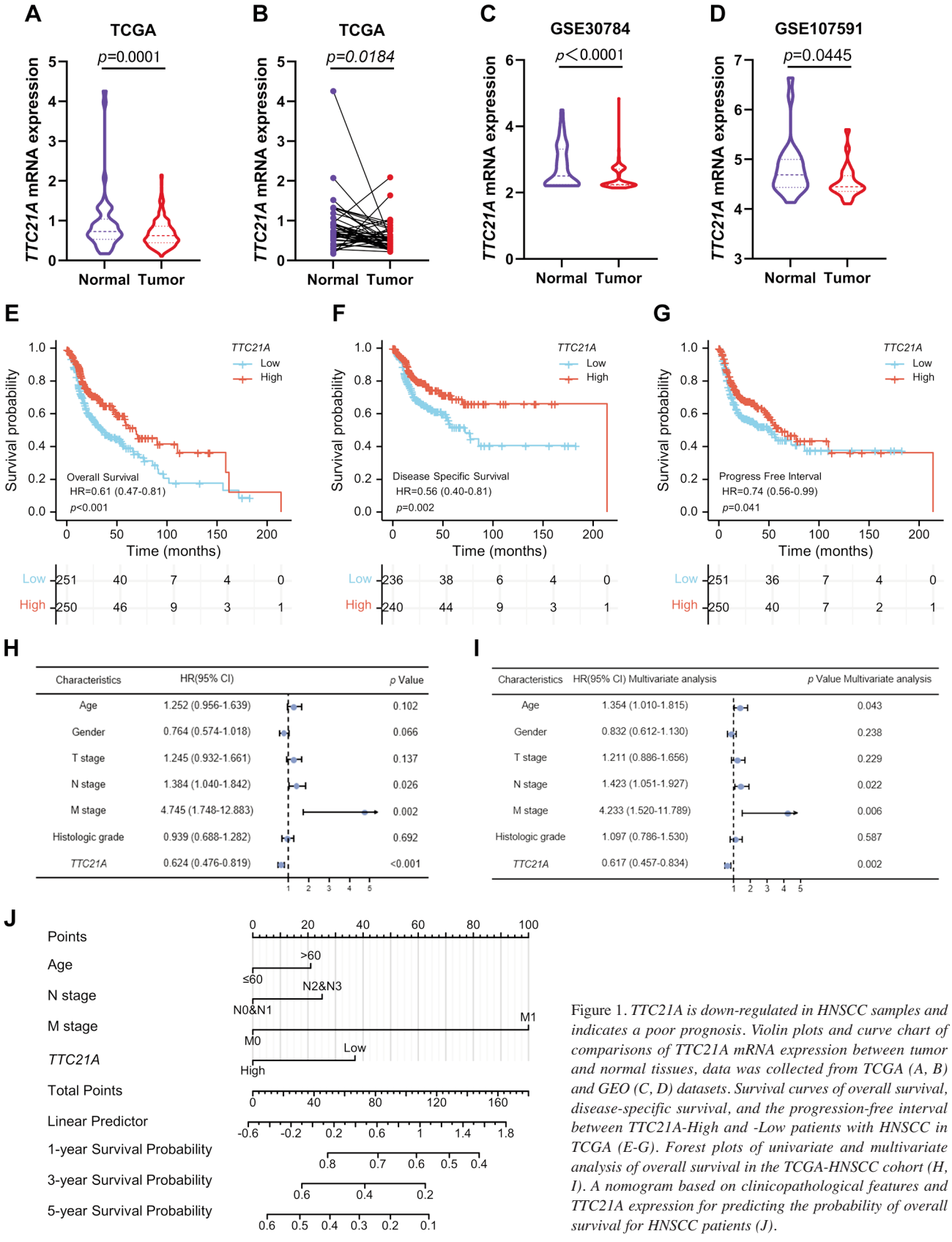

Figure 1. *TTC21A is down-regulated in HNSCC samples and indicates a poor prognosis. Violin plots and curve chart of comparisons of TTC21A mRNA expression between tumor and normal tissues, data was collected from TCGA (A, B) and GEO (C, D) datasets. Survival curves of overall survival, disease-specific survival, and the progression-free interval between TTC21A-High and -Low patients with HNSCC in TCGA (E-G). Forest plots of univariate and multivariate analysis of overall survival in the TCGA-HNSCC cohort (H, I). A nomogram based on clinicopathological features and TTC21A expression for predicting the probability of overall survival for HNSCC patients (J).*

Table I. *Correlation between TTC21A and clinical characteristics in the TCGA-HNSCC cohort.*

Clinical features		TTC21A-low	TTC21A-high	*p*-Value
Age, n (%)	≤60	128 (25.5%)	117 (23.4%)	0.395
	>60	123 (24.6%)	133 (26.5%)	
Sex, n (%)	Female	77 (15.3%)	57 (11.4%)	0.055
	Male	174 (34.7%)	194 (38.6%)	
Race, n (%)	Asian	5 (1%)	5 (1%)	0.592
	Black or African-American	27 (5.6%)	20 (4.1%)	
	White	212 (43.7%)	216 (44.5%)	
T stage, n (%)	T1	15 (3.1%)	18 (3.7%)	0.153
	T2	62 (12.7%)	82 (16.8%)	
	T3	70 (14.4%)	61 (12.5%)	
	T4	98 (20.1%)	81 (16.6%)	
N stage, n (%)	N0	113 (23.5%)	126 (26.2%)	0.006
	N1	54 (11.2%)	26 (5.4%)	
	N2	70 (14.6%)	84 (17.5%)	
	N3	3 (0.6%)	4 (0.8%)	
M stage, n (%)	M0	239 (50.1%)	233 (48.8%)	1.000
	M1	3 (0.6%)	2 (0.4%)	
Histologic grade, n (%)	G1	43 (8.9%)	19 (3.9%)	<0.001
	G2	164 (34%)	136 (28.2%)	
	G3	39 (8.1%)	80 (16.6%)	
	G4	0 (0%)	2 (0.4%)	
	Yes	213 (42.7%)	196 (39.3%)	
OS event, n (%)	Alive	119 (23.7%)	165 (32.9%)	<0.001
	Dead	132 (26.3%)	86 (17.1%)	
DSS event, n (%)	Alive	156 (32.7%)	191 (40%)	0.002
	Dead	80 (16.8%)	50 (10.5%)	
PFI event, n (%)	Alive	143 (28.5%)	165 (32.9%)	0.041
	Dead	108 (21.5%)	86 (17.1%)	

OS, Overall survival; DSS, disease-specific survival; PFI, progression-free interval.

Interestingly, multivariate analysis revealed *TTC21A* as an independent prognostic factor of OS for HNSCC (HR=0.617, 95%CI=0.457-0.834, *p*=0.002) (Figure 1I). To further verify the prognostic value of *TTC21A* in HNSCC, a nomogram was then conducted to predict *TTC21A* and other prognostic factors of OS at 1, 3, and 5 years, as shown in Figure 1J, a higher score on the model suggests a worse prognosis.

Correlation between TTC21A and molecular classifications of HNSCC. Molecular classifications of HNSCC have identified distinct subtypes of HNSCC with heterogeneous clinical features and outcomes. Then, we compared the expression of *TTC21A* mRNA based on the classifications of these molecular signatures. The NAT classification includes subtypes of atypical, basal, classical, and mesenchymal (12-14). Interestingly, the atypical subtype, which predicts the longest recurrence-free survival time, here showed the highest level of *TTC21A* mRNA compared to the other three subtypes (Figure 2A). The *TTC21A* mRNA was also comparable among the Locati *et al*. defined subtypes: Cl1, Cl2, and Cl3 (15). The Cl1, which refers to the "immune strong" subtype, held the highest expression of *TTC21A* (Figure 2B), suggesting a potential association between *TTC21A* and TIM in HNSCC.

HPV status is an important factor that is involved in the progression and prognosis of HNSCC, Figure 2C shows that HPV positive group had enhanced expression of *TTC21A*. TP53 mutations are the most frequent somatic genomic alterations in HNSCC and serve as an independent prognostic factor (16). Here, we found that HNSCC tumors with TP53 mutation had a decreased level of *TTC21A* mRNA expression (Figure 2D), suggesting potential links between *TTC21A* and TP53 mutation. In addition, the level of *TTC21A* was also observed to be associated with the expression of genes related to ferroptosis, and N6-Methyladenosine (m6A) (Figure 3).

Predicted functions and pathways of the changes in TTC21A expression. To identify *TTC21A*-related signaling pathways comprehensively, enrichment analysis was performed by KEGG and GO tools. We found that *TTC21A* expression was positively correlated with immune terms, including allograft rejection, antigen processing and presentation, T cell activation, T cell differentiation, and T cell proliferation (Figure 4A). In

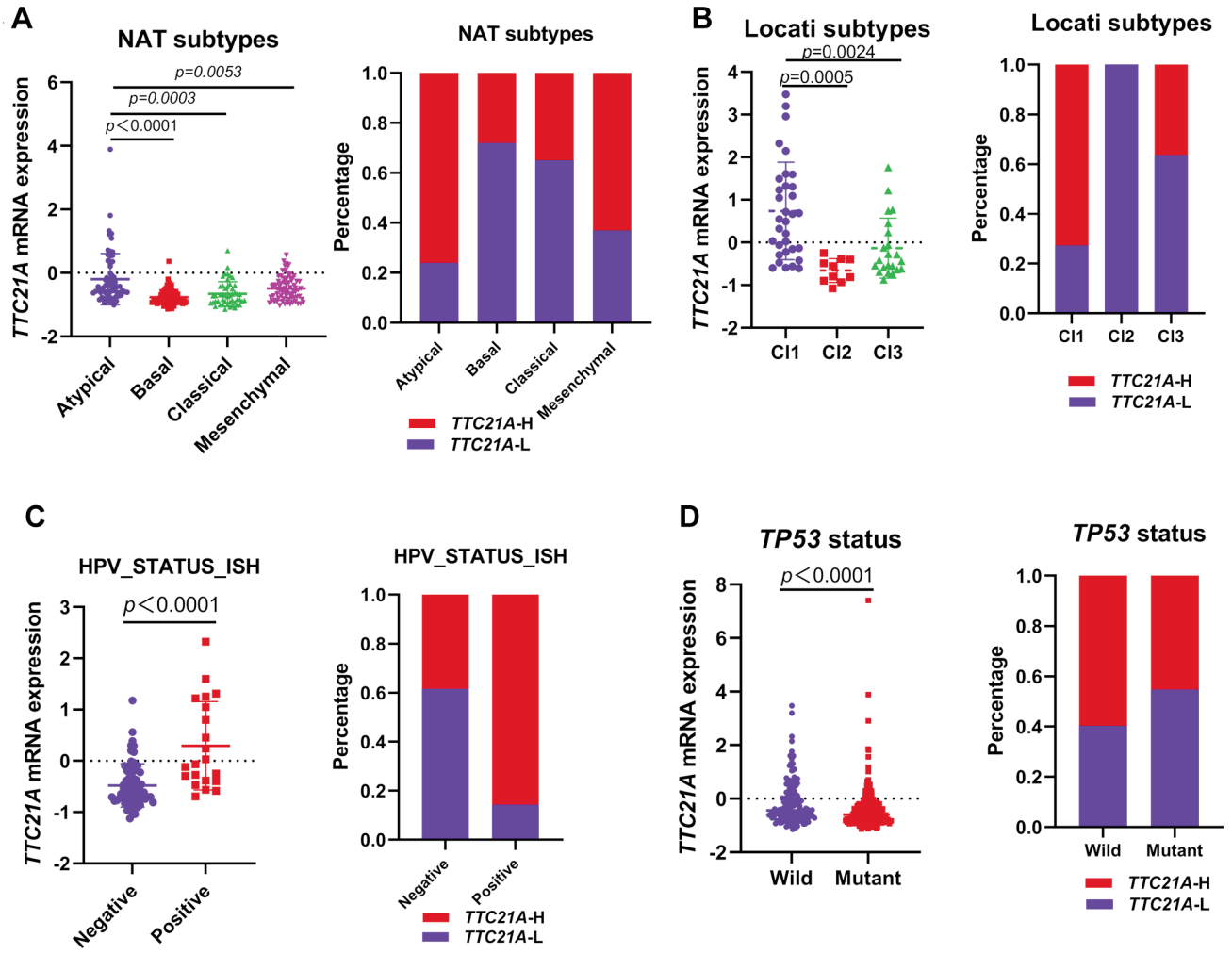

Figure 2. *Correlation between TTC21A expression and molecular classifications of HNSCC. Comparison of TTC21A expression in molecular subtypes defined by Chung (A), and Locati (B). The proportion of TTC21A expression in different HPV infection (C) and TP53 mutation (D) statuses.*

addition, GSEA was performed to reveal the global transcriptomic variations associated with *TTC21A* expression. The most significantly enriched signaling pathways were immune-related terms, including interferon-gamma response, allograft rejection, primary immunodeficiency, and intestinal immune network for IG-A production (Figure 4B). These data suggest a potential link between *TTC21A* and immune-related pathways.

Association of TTC21A with immune cell infiltration and immune-related gene expression. To investigate the underlying relationship between *TTC21A* expression and the abundance of tumor-infiltrating immune cells in HNSCC, the Spearman correlation coefficients of *TTC21A* expression and distribution of 24 immune cells were calculated based on CIBERSORT. The enrichment of most immune cells (14 of 24) was significantly positively correlated with *TTC21A* expression ($p<0.05$) (Figure 5A), among them, CD8 T cells, Tem, NK cells, T cells, pDC, and Cytotoxic cells were the top 6 most correlated ones. Figure 5B showed that the *TTC21A*-High group held enhanced immune cell infiltration compared with the *TTC21A*-Low group. Given that most of these positively correlated immune cells play important roles in the anti-tumor immune response, we speculated a promoting role of *TTC21A* in anti-tumor immunity in HNSCC.

To further elucidate the correlation between *TTC21A* expression and activation of anti-tumor immunity, the expression of 24 HLA (human leukocyte antigen) genes was compared between *TTC21A*-Low and *TTC21A*-High groups. We found that 18 HLA genes had significantly higher expression levels in the *TTC21A*-High group (Figure 6A). In addition, levels of 46 immune checkpoint genes (17) were

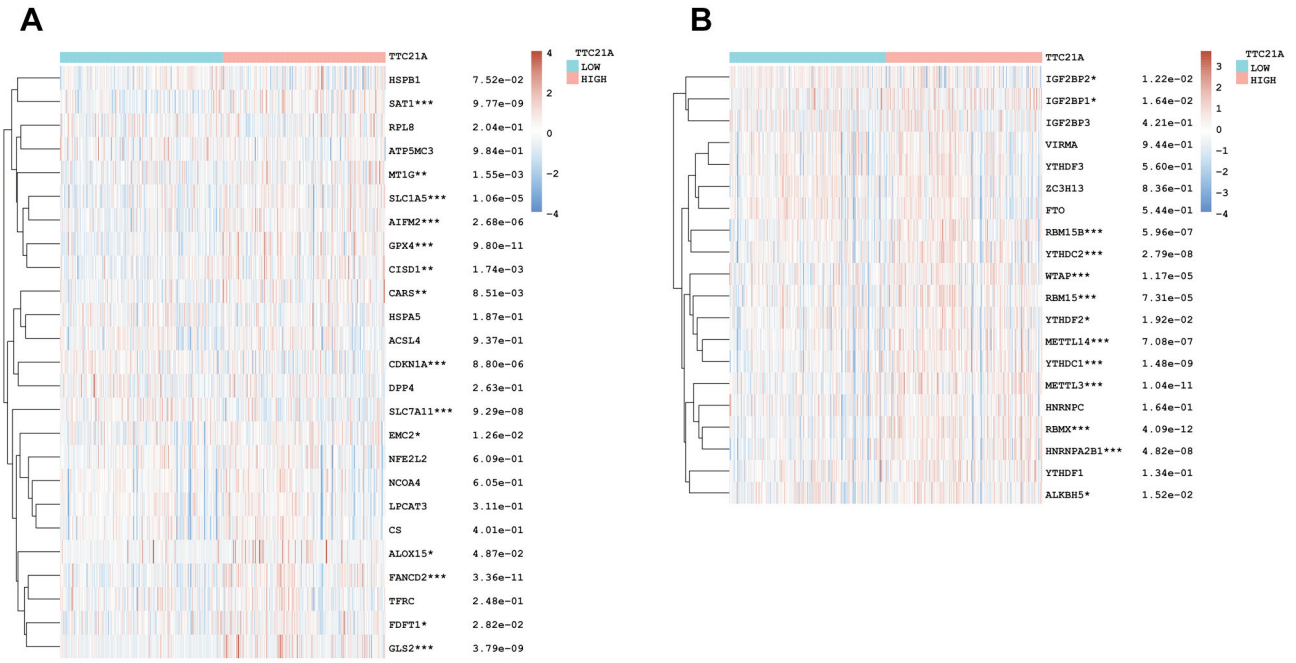

Figure 3. *Correlation between TTC21A expression and ferroptosis and N6-methyladenosine. Heatmaps of comparison of ferroptosis (A), and N6-methyladenosine (B) related genes expression in TTC21A-High and TTC21A-Low subgroups. *p<0.05, **p<0.01, ***p<0.001.*

compared between *TTC21A*-Low and *TTC21A*-High groups. The heatmap showed that most immune checkpoint genes (35 of 46) held enhanced levels in samples belonging to the *TTC21A*-High group (Figure 6A), including immunotherapeutic target genes, like CD274 (p=0.038), CTLA4 (p=7.53E-11), and LAG3 (p=8.11E-08). Immunohistochemical staining of *TTC21A*, CD274, and CD8A was obtained from two HNSCC patients in the HPA database. Interestingly, the tissues from patient 4117 had positive staining of TTC21A (weak), CD274 (strong), and CD8A, while the tissues from patient 3470 had no staining of TTC21A, CD274, or CD8A (Figure 7A). Data at the transcription level also indicated that the *TTC21A*-High group held a higher level of *CD274* and *CD8A* (Figure 6B). This result suggests that patients belonging to the *TTC21A*-High group may have a higher likelihood of response to immune checkpoint blockade.

TTC21A expression could predict the clinical benefit of ICB. Based on the above results, we further investigated the potential regulatory role of *TTC21A* on the TIM of HNSCC, by assessing the ESTIMATE score and immunoscore (18). As expected, the *TTC21A*-High group held a significantly higher level of immune abundance (Figure 7B). Saloura (19) reported that a higher T-cell-inflamed phenotype (TCIP) score predicted improved antitumor immune responses in patients with HNSCC. We evaluated scores of TCIP based on the Messina 12-chemokine gene signature (20), and the result revealed that the TCIP-High group expressed a significantly higher level of *TTC21A* mRNA (Figure 7C). In addition, IFN-γ–related signatures have been reported to predict response to immune checkpoint blockade in human solid tumors, including HNSCC (21-23). Expression scores were calculated based on "IFN-γ (6-gene)" and "expanded immune (18-gene)" signatures (21) in HNSCC samples from TCGA, we found that the *TTC21A*-High group got significantly higher IFN scores compared with the *TTC21A*-Low group (Figure 6C). A higher immunoscore, TCIP score, or IFN-γ–related score represents a higher potential for anti-tumor immunity, which suggests that patients with high *TTC21A* expression would be more likely to benefit from ICB therapy.

Two methods were used to verify the ability of *TTC21A* expression in the prediction of the immunotherapy response. Recent studies have reported the role of IPS in predicting the efficacy of immunotherapy (24), we analyzed the relationship of IPS between *TTC21A*-Low and *TTC21A*-High groups to evaluate the potential of *TTC21A* application in immunotherapeutic benefits. As shown, the IPS-CTLA4-PD1 was significantly different between the two groups (p<0.05) (Figure 7D). Melanoma was used as a model for the development of immunotherapy (25). Based on the GSE35640 and GSE91061 datasets, we evaluated the expression level of *TTC21A* between the different statuses of ICB response, as

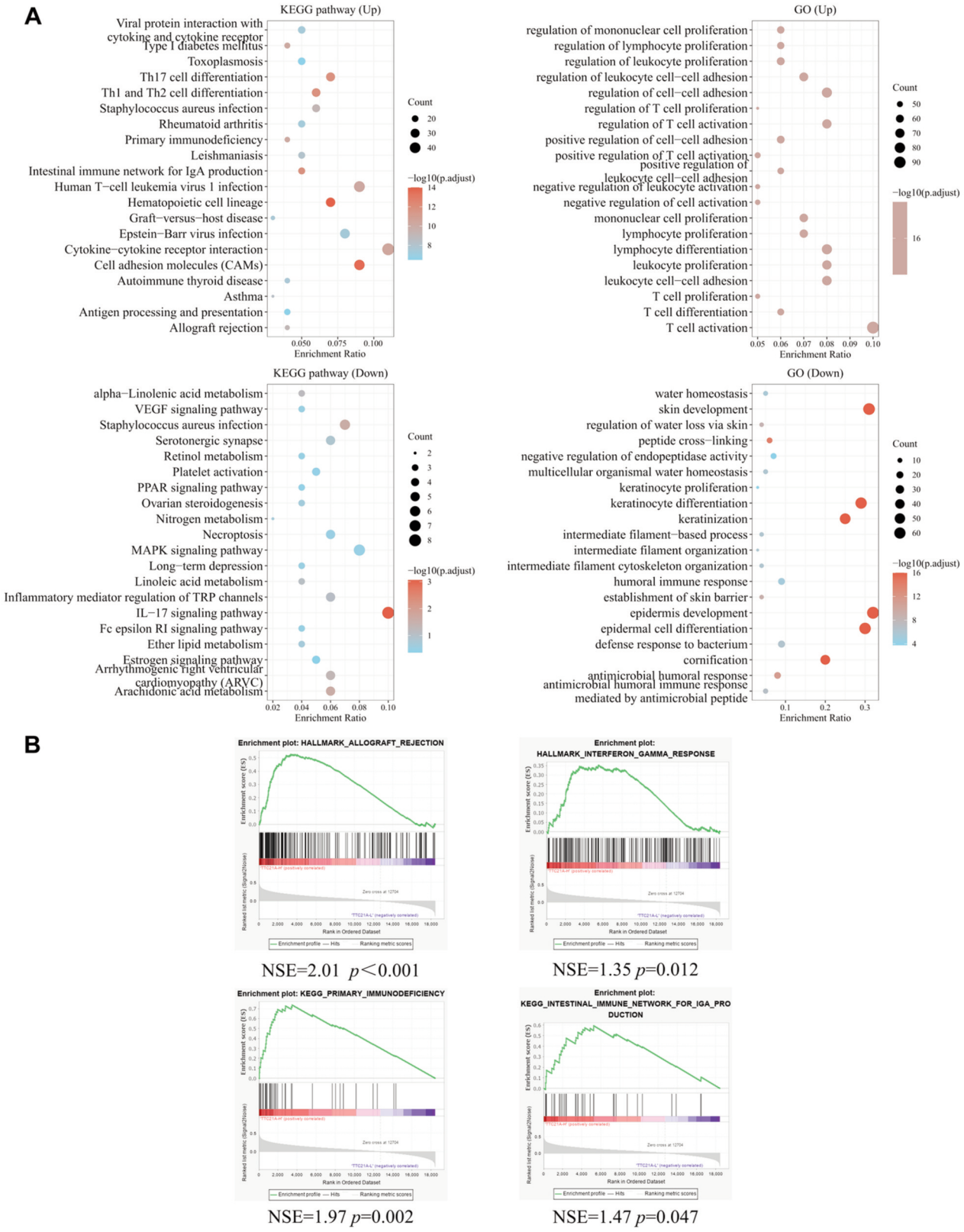

Figure 4. *Predicted functions and pathways associated with TTC21A expression in HNSCC. Bubble diagrams of KEGG and GO analysis (A). Enrichment plots of GSEA (B).*

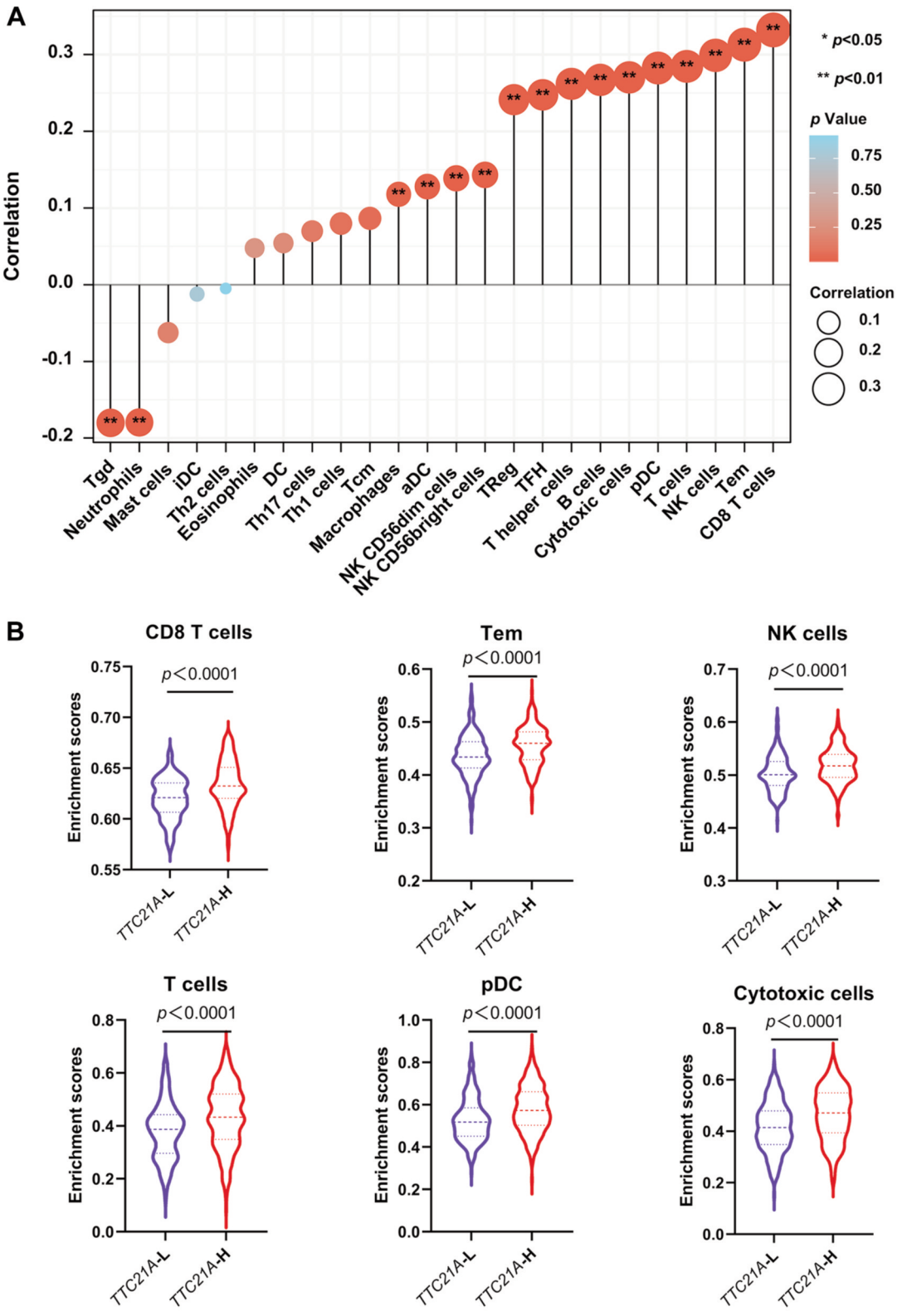

Figure 5. *High expression of TTC21A correlates with high immune cell infiltration. Lollipop charts of the correlation between TTC21A expression and the abundance of 24 immune cells (A). Violin diagrams of the top 6 enrichment immune cells between the TTC21A-High (TTC21A-H) and TTC21A-Low (TTC21A-L) groups (B).*

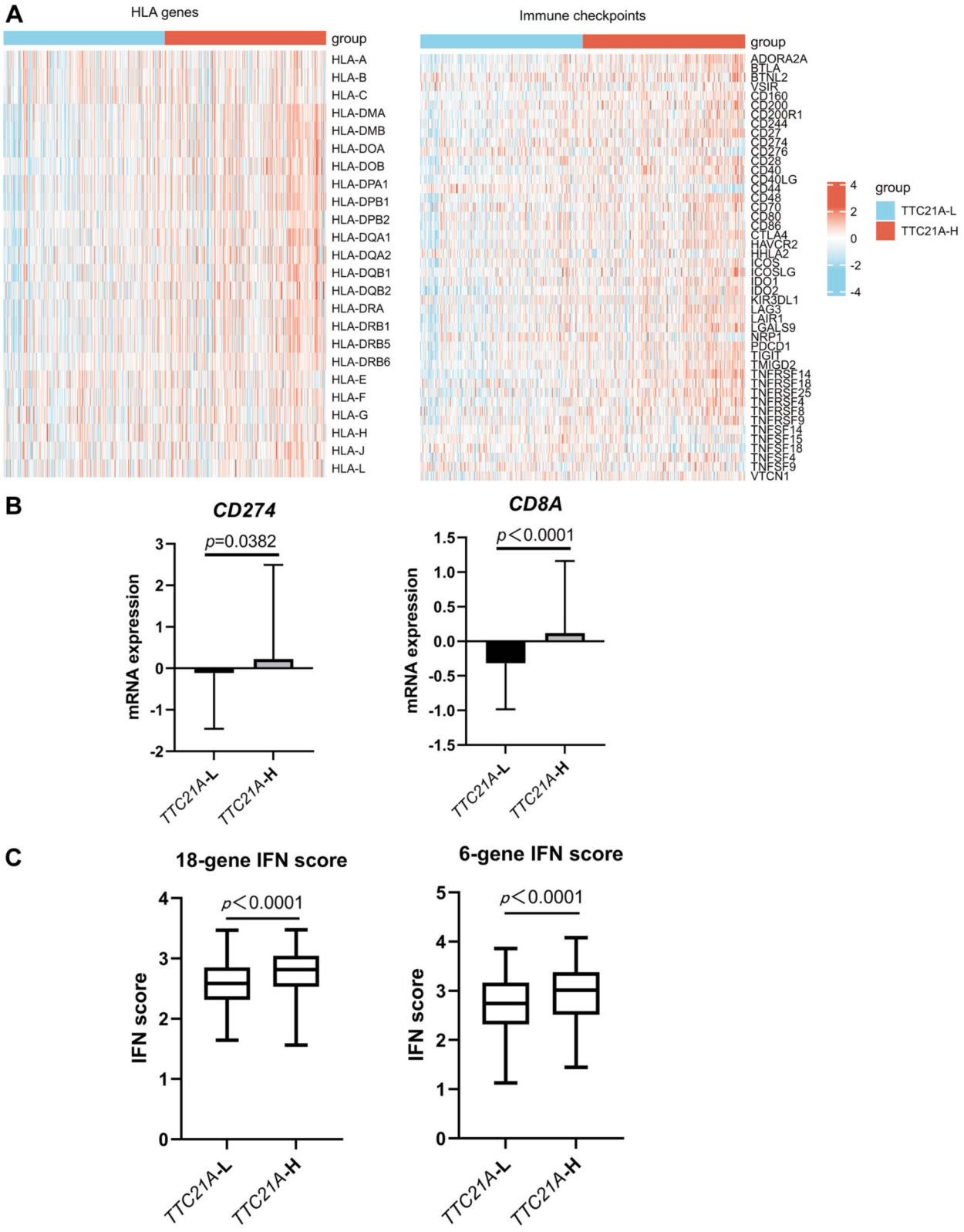

Figure 6. *TTC21A correlates with a high level of immune-related gene expression. Heatmaps of the expression of HLA genes and immune checkpoints between TTC21A-High (TTC21A-H) and TTC21A-Low (TTC21A-L) groups (A). Comparison of the expression of CD274 and CD8A between TTC21A-H and TTC21A-L (B). The proportion of IFN scores in TTC21A-H and TTC21A-L groups (C).*

expected, patients with higher *TTC21A* expression were more likely to respond to ICB treatment (Figure 7C).

Discussion

HNSCC is the sixth most common malignant carcinoma worldwide (26). Immunotherapy based on targeting immune checkpoints is a promising treatment for recurrent or metastatic patients with HNSCC, however, the response rate is inadequate (4, 5, 27), and studies focusing on biomarkers for predicting immunotherapy response are meaningful. In recent years, advances in sequencing technologies have promoted the development of high-throughput analysis, allowing investigation of potential drivers of malignant carcinoma, including HNSCC (28-31). *TTC21A* is a protein-coding gene and contains several TPR domains, which was found to play roles in spermatogenesis and male fertility (7, 32). Reports on the role and mechanism of *TTC21A* in human tumors are limited. In addition, two recent studies on the relationship of *TTC21A* with tumors suggest that whether *TTC21A* infers a favorable prognosis is tumor-specific (33, 34). In this study, we analyzed datasets collected from the TCGA and GEO and found that the expression of *TTC21A* was down-regulated in HNSCC tissues. The *TTC21A*-High group had a better prognosis than the *TTC21A*-Low group. *TTC21A* was associated with clinical parameters and molecular features and could serve as an independent prognostic indicator in HNSCC. Functional analysis suggested that *TTC21A* levels were associated with immune-related pathways, genes, and cells in HNSCC. Further, we uncovered that ICB treatment responders showed a higher level of *TTC21A*, suggesting the predictive role of *TTC21A* in ICB response.

HNSCCs are molecularly heterogeneous tumors and can be classified into different subgroups based on the molecular and biological characteristics of tumors. Our data showed that HNSCC patients in different subgroups had different levels of *TTC21A* expression, suggesting potential relationships between *TTC21A* and molecular pathways in HNSCC. Sequencing-based KEGG and GO analysis revealed that *TTC21A* expression was associated with immune-related functions, including immune cell differentiation, immune cell proliferation, and antigen processing and presentation. Further, GSEA results suggested that *TTC21A* may link to the activity of immune-related signaling, like allograft rejection, interferon-gamma response, primary immunodeficiency, and intestinal immune network for IgA production. These results raise the possibility that *TTC21A* plays a role in regulating the tumor microenvironment.

The tumor microenvironment is the constitutive element in cancer immunity, multiple cytokines, cytokine receptors, and immune cells are included. Herein, we revealed that *TTC21A* level was positively correlated with infiltration of most immunocytes (19/24) based on CIBERSORT analysis, implying an immune-hot character of *TTC21A*-High tumors. Further, we noted that *TTC21A* expression was strongly correlated with CD8 T cells, Tem, NK cells, T cells, and Cytotoxic cells. Among them, CD8 T cells, NK cells, and cytotoxic T-cells are known as the main cytotoxic effector cells of the immune system and play a critical role in ICB response (35), this result implies a possible function of *TTC21A* in the prediction of ICB response. The expression of immune checkpoints and HLA genes are considered to influence cancer response to checkpoint blockade immunotherapy (36). Our data showed that *TTC21A*-High tumors had higher levels of most HLA and immune checkpoints. Indeed, we compared the expression of *TTC21A* between the different statuses of ICB response and found that ICB responders showed higher expression of *TTC21A*. The above results suggest *TTC21A* may be related to the tumor microenvironment and the ICB response in HNSCC.

Interestingly, the substantial association between *TTC21A* and HPV status, *TP53* status, ferroptosis-related genes, and m6A regulators in HNSCC was also observed in this study. HPV infection is a favorable prognostic indicator in HNSCC and may enhance anti-tumor immunity (37). *TP53* mutation is the most frequent somatic genomic alteration and is associated with a poor prognosis in HNSCC (38). In addition, *TP53* mutations also have a potential impact on the immune response (39). Ferroptosis is an iron-dependent form of cell death, activation of ferroptosis induces tumor suppression and promotes anti-tumor immune response in HNSCC (40). Yi recently reported that m6A regulators were associated with prognosis, PD-L1 expression, immunoscore, and immune cell infiltration in HNSCC (41). Given the functional role of HPV infection, *TP53* mutation, ferroptosis-related genes, and m6A regulators in the tumor microenvironment and anti-tumor immune response, associations between *TTC21A* and these above 4 features may provide potential mechanisms for *TTC21A* in regulating immune infiltration in HNSCC.

Conclusion

In summary, we reported that *TTC21A* mRNA levels decreased in HNSCC, and were associated with a favorable prognosis. *TTC21A* expression was linked with the expression of immune checkpoints and HLA genes, and the abundance of infiltrating immune cells, indicating a potential regulatory role of *TTC21A* in the tumor microenvironment. In addition, ICB responders showed enhanced *TTC21A* expression than non-responders. Although more in-depth studies are needed, our study suggests that *TTC21A* may regulate the tumor microenvironment by linking to HPV status, *TP53* status, ferroptosis-related genes and m6A regulators, we propose some ideas about the predictive role of *TTC21A* on the anti-tumor immune response in HNSCC.

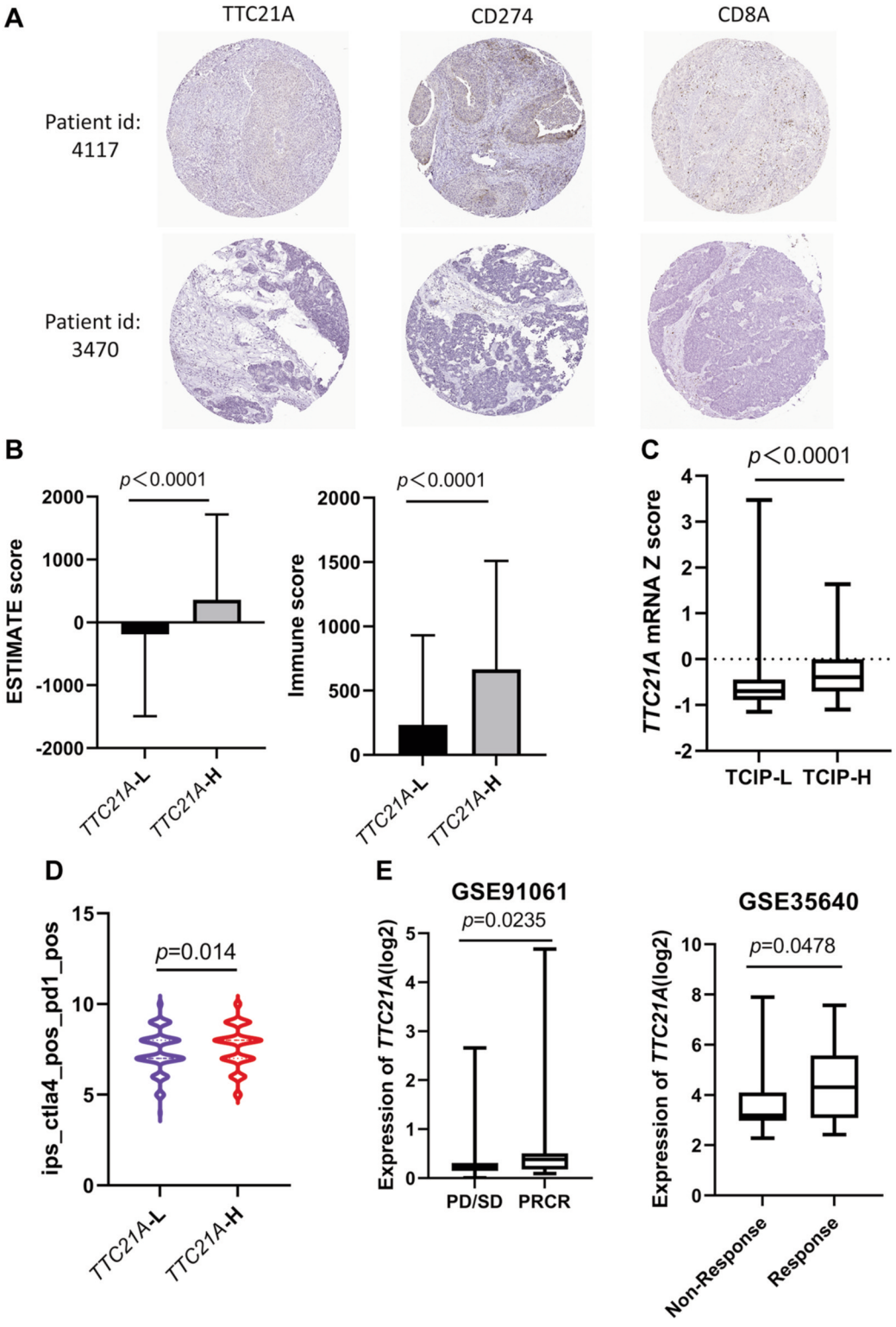

Figure 7. *TTC21A expression predicted the clinical benefit of ICB. Immunohistochemical validation of TTC21A, CD274, and CD8A in patients with HNSCC from the HPA (A) (11). Distribution of ESTIMATE score, and Immune score in TTC21A-High (TTC21A-H) and TTC21A-Low (TTC21A-L) groups (B). The proportion of TTC21A expression between TCIP-High (TCIP-H) and TCIP-Low (TCIP-L) groups (C). Violin chart of immunotherapy efficacy based on the TCIA database (D). Comparison of TTC21A in patients with different responses to ICB treatment (E). PD/SD, progressive disease/stable disease. PRCR, part response, or complete response.*

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

LW and HC designed the study. All authors contributed to data analysis, drafting, and revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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