Comprehensive co-expression analysis reveals TMC8 as a prognostic immune-associated gene in head and neck squamous cancer

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Abstract. The occurrence and prognosis of head and neck squamous cell cancer (HNSC) is closely associated with human papillomavirus (HPV) infection. Transmembrane channel-like 8 (TMC8) is a key gene affecting the susceptibility of HPV and that plays an important role in T cell regulation. However, the mechanism by which TMC8 affects T cells and whether it further affects the prognosis of patients with HNSC remains unclear. In the present study, oral cancer cell lines and independent tumor specimens were used to detect TMC8 expression in HNSC. Differential expression of TMC8, methylation status, function and associated signaling pathways were further analyzed. Then, multiple databases were cross-analyzed for the relationship of TMC8 with immune cell infiltration and its impact on the prognosis of numerous types of cancer. The results showed that TMC8 was upregulated in HNSC and high expression was predictive of an improved prognosis. Furthermore, TMC8 was concentrated in multiple immune-associated signaling pathways and the expression of TMC8 was associated with the infiltration of $CD4^+$ T cells and their subsets, including $CD8^+$ T cells, B cells and macrophages, suggesting that TMC8 may play an anti-HPV role by regulating $CD4^+$ T cells. Thus, TMC8 plays an anti-HPV role by regulating the infiltration level of $CD4^+$ T cells, and could therefore be used as a potential prognostic marker for patients with HNSC.

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Introduction

Head and neck squamous cell carcinoma (HNSC) is the sixth most common malignancy in the world (1), which predominantly develops from squamous cell epithelia according to Chaturvedi *et al* in 2013 (2). The main risk-factors of HNSC are cigarette smoking, excessive alcohol use and the presence of human papillomavirus (HPV). Although the overall survival (OS) time and quality of life have been enhanced by improved standard treatment and supportive care (3), HNSC prognosis remains poor with a 5-year OS rate of ~50% worldwide by 2011 (4).

In recent years, some biomarkers have been identified and used in the diagnosis of HNSC (5). For example, matrix metalloproteinases (MMPs), which promote tumor invasion and metastasis, have been found to be significantly increased in the serum of patients with head and neck cancer and are promising biomarkers for the diagnosis of HNSC (6). In addition, DNA methylation is a major epigenetic change that often precedes the malignant proliferation of cells. The identification of DNA methylation changes is of great significance for the early detection of tumors (7). At present, the methylation status of genes such as p16, cyclin-dependent kinase and stratifin have been associated with HNSC (8,9). However, the sensitivity and specificity of these biomarkers reported in literatures are quite different.

Since immune-associated mechanisms have a critical role in HNSC, immunotherapies represent a promising strategy for treatment (10,11). Immune checkpoints can respond to pathogens by regulating the balance of immune stimuli and inhibitory signals, or as regulators of mutant/overexpressing T cell immune responses (12,13). Previous research has demonstrated that the interaction between programmed cell death protein-1 (PD-1) and PD ligand-1 is a critical immune checkpoint. Inhibiting PD-1 has been found to exhibit high treatment efficacy for melanoma and is now approved for the treatment of HNSC (14,15). However, current anti-PD-1 immunotherapies do not generate good responses from patients with advanced HNSC. According to reports, the median OS time was 7.5 months, and some patients show resistance (16,17). Additionally,

several studies have reported that patients with a greater number of tumor-infiltrating lymphocytes display increased survival in HPV-positive and -negative oropharyngeal disease (18-21). Therefore, there is a need to elucidate the specific immune phenotypes of tumor-immune relationships and identify novel immunological targets for the treatment of HNSC.

As a protein-coding gene, transmembrane channel-like 8 (TMC8) is not yet fully understood. TMC6 and TMC8 null mutations have been shown to result in severe susceptibility to cutaneous (β-type) HPV infections, causing a rare syndrome termed epidermodysplasia verruciformis (EV) (22,23). Moreover, an association between TMC8 variants and susceptibility to skin and cervical cancer has been observed (23,24). Notably, patients with HNSC are often accompanied by HPV infection, but the expression level of TMC8 and its relationship with patient prognosis and clinical stage has not yet been reported. Previous studies have shown that T lymphocytes exhibit high levels of TMC8 gene expression, indicating that TMC8 plays a multifunctional role in the mechanisms associated with tumor infiltration (25,26). However, the impact of this gene on the OS time of patients with HNSC and the underlying function of TMC8 in tumor-immune interactions remains unclear.

Oral squamous cell cancer is a typical representative of HNSC, accounting for the majority of HNSC (1,4). As shown in the workflow of Fig. 1, the expression of TMC8 was examined in oral cancer cell lines, clinical specimens and The Cancer Genome Atlas (TCGA) database and its relationship with the OS time of patients with HNSC was also evaluated. Moreover, its function and role in the immune cell network, as well as its impact on prognosis in multiple cancers (pan-cancers) were analyzed. The purpose of the present study was to explore the role of TMC8 in the immune microenvironment and to further clarify its impact on the incidence and outcome of HNSC.

Materials and methods

Acquisition of clinical specimens. Oral squamous cell carcinoma (OSCC) and adjacent normal tissues were collected from patients with HNSC at the Peking University Shenzhen Hospital (Shenzhen, China) between January 2017 and December 2019. HNSC was diagnosed and classified by two independent pathologists based on the World Health Organization classification system (27). The specimens were all taken from the tongue cancer resection process, and the distance between tumor tissue and adjacent normal tissue was greater than 2 cm. Specimens from patients with a history of preoperative chemotherapy were excluded. The study was approved by The Ethics Committee of Peking University Health Science Center (Guangdong, China). Informed consent was obtained from patients before the study began.

Immunochemical staining. The above mentioned, obtained from the operating room without pre-embedded OSCC samples were fixed in 10% neutral buffered formalin for 24 h at room temperature, dehydrated in gradient alcohol solution (50, 70, 80, 95, 95 and 100 alcohol, each for 1 h) and paraffin-embedded. Paraffin-embedded tumor sections with a thickness of 5-μm were deparaffinized with xylene I for 15 min, xylene II for 10 min, and rehydrated with 100% ethanol and 100, 95, 95 and 80% ethanol for 5 min each. Sections were then washed twice for 5 min each with dH2O. Slides were heated in a microwave in 1X citrate antigen retrieval Solution (cat. no. MVS-0066; MBX Bioscience) until boiling, followed by incubation at the boiling state for 20 min. After cooling, sections were washed in PBS three times for 5 min each. Antigen retrieval was achieved by blocking with 3% H_2O_2 for 30 min at room temperature. The aforementioned washing steps were repeated and then blocking was performed using goat serum (cat. no. ZLI-9022; OriGene Technologies, Inc.) at room temperature for 20 min. Then the sections were incubated with antibodies against TMC8 (cat. no. ab69859; 1:75; Abcam) overnight at 4°C. The slides were then incubated with a biotin-labeled secondary antibody UltraSensitive™ SP (Mouse/Rabbit) IHC kit (cat. no. KIT-9710; MBX Biotechnologies, Inc.) to TMC8 for 30 min at room temperature. The aforementioned washing steps were repeated and the slices were stained with DAB (cat. no. DAB-0031; MBX Biotechnologies, Inc.) at room temperature for ~1 min. All images shown are wide-field light microscopy images acquired at sufficient resolution.

Cell culture. Human oral squamous cell carcinoma cell lines SCC9, SCC15, SCC25 and CAL27 were obtained from the American Type Culture Collection. Human oral keratinocyte (HOK) cells were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. SCC15, SCC25, CAL27 and HOK cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin sulfate (all Gibco; Thermo Fisher Scientific, Inc.). SCC9 cells were cultured in 1:1 DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS, 1% penicillin and streptomycin sulfate, 1% sodium pyruvate (Gibco; Thermo Fisher Scientific, Inc.) and 400 ng/ml hydrocortisone (MedChemExpress). All cells were cultured at 37°C and 5% CO_2.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR). Total RNA from frozen tissues or cultured cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (cat. no. RR047A; Takara Bio, Inc.) was used for reverse-transcribing the RNA into cDNA, as per the manufacturer's instructions. RT-qPCR was performed with TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (cat. no. RR820A; Takara Bio, Inc.) and was monitored using an ABI PRISM™ 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by followed by denaturation at 95°C for 5 sec; annealing at 58°C for 30 sec and elongation at 72°C for 20 sec, for 40 cycles. The following primers were used for qPCR: β-Actin, forward: 5'-AAACTGG AACGGTGAAGGTG-3' and reverse: 5'-AGTGGGGTGGCT TTTAGGAT-3'; TMC8, forward: 5'-GAACTACCCTCCCAA CACG-3' and reverse: 5'-TGCTCTTGTCTCTGCCAATG-3'. Comparative quantification was performed with either the ΔCq or the $2^{-\Delta\Delta Cq}$ method (28). RT-qPCR was used to determine the expression of TMC8 in SCC9, SCC15, SCC25 and CAL27 cell lines. Data from three independent experiments were obtained, the mean value ± standard deviation was calcualted and unpaired Student's t-test was used.
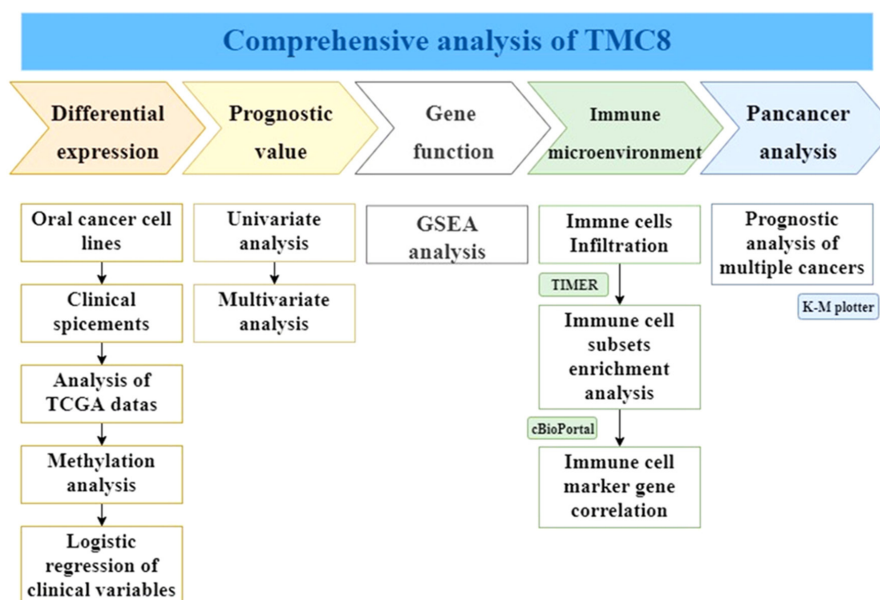
Figure 1. Workflow of the study. A comprehensive analysis of TMC8 was conducted. In the first line, from left to right, the five parts of differential expression, prognostic value of head and neck squamous cancer, gene function, immune infiltration relationship and prognostic effect on pan-cancers are analyzed and are marked with different colors. Below each part, the specific analysis content is listed. TMC8, transmembrane channel-like 8; GSEA, gene set enrichment analysis; TCGA, The Cancer Genome Atlas; K-M, Kaplan-Meier; TIMER, Tumor Immune Estimation Resource.

Acquisition of mRNA data. TMC8 gene expression and methylation data, as well as the corresponding clinical information, were downloaded from TCGA website (https://portal.gdc.cancer.gov) for HNSC and estimated as log2 (x+1) transformed RNA sequencing by expectation-maximization normalized counts (29). A total of 528 patients with HNSC were sampled, containing 44 patients with adjacent non-tumorous tissue as the control group. All data were processed using R studio software version 3.5.3 (30). The 'ESTIMATE' R package was used to predict the presence of infiltrating stromal/immune cells in tumor tissues using gene expression data (31).

Gene Expression Profiling Interactive Analysis (GEPIA) and survival analysis. The online database GEPIA (http://gepia.cancer-pku.cn/index.html) was used to analyze the differential expression of the TMC8 and its prognostic value.

Methylation and gene expression analyses. DNA methylation data from TCGA contained β-values for 485,577 CpG sites. The β-value was calculated as (M/M+U) and ranged from zero to one, where M was the frequency of the methylated allele and U was the frequency of the unmethylated allele. Therefore, higher β-values indicated higher levels of methylation. Levels of TMC8 methylation between HNSC and normal tissues were compared. In addition, the association between TMC8 expression and its DNA methylation status was investigated.

Oncomine database analysis. The Oncomine database (https://www. oncomine.org/resource/login.html) was screened for the expression of the TMC8 gene in several types of cancer. A threshold P-value of 0.001 and fold-change ≥2 was used to assess statistical significance.

Gene Set Enrichment Analysis (GSEA). To identify potential biological mechanism of TMC8, GSEA was conducted to detect whether a previously defined set of genes showed statistically significant differential expression. Firstly, the TMC8 high expression group was selected based on the median expression (cut-off value 0.453101463) of TMC8, and the genes are sorted according to expression differences to form a gene list. The annotated gene sets C2.CP (186 gene sets) and C5.BP (5,910 gene sets) MSigDB datasets from the Broad Institute (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp) were selected as the reference gene sets. These preset gene sets represented different biological processes or signal pathways. Then the GSEA algorithm could determine whether the members of this reference gene set were randomly distributed in the TMC8 high expression group gene list, or were mainly enriched at the front or end sides of the list. The third step was to calculate the enrichment score of the gene set and perform a permutation test of significance to obtain the P-value and the false discovery rate (FDR). FDR <25% and P<0.05 in the enrichment of MSigDB Collection (c2.cp.kegg.v6.2.symbols) were considered to be significantly enriched.

Tumor Immune Estimation Resource (TIMER) database analysis. The TIMER database can be used as a comprehensive resource for systematically analyzing immune infiltrates between several types of cancer (https://cistrome. shinyapps.io/timer/). Multiple deconvolution algorithms including TIMER (32), CIRBSORT (33) and xCell (34) are used to analyze the expression of TMC8 and the degree of infiltration of immune cells, including i) CD8+ T cells; ii) CD4+ T cells; iii) B cells; iv) dendritic cells (DCs); v) neutrophils; and vi) macrophages and their subgroups. The correlation between the infiltration levels of above-mentioned immune cells and the expression of TMC8 was obtained.

Co-expression analysis in cBioPortal. For cancer genomics, cBioPortal (https://www.cbioportal.org) is an open-access, open-source resource for the interactive exploration of multidimensional cancer genomics data sets. The correlation between the hub gene expression and gene markers of immune cells were explored. The gene markers of the immune cells included markers of: i) CD8+ T Cells; ii) T cells (general); iii) B cells; iv) monocytes; v) tumor-associated macrophages (TAMs); vi) M1 macrophages; vii) M2 macrophages; viii) neutrophils; ix) natural killer (NK) cells; x) DCs; xi) T helper (Th)-1 cells; xii) Th2 cells; xiii) follicular helper T (Tfh) cells; xiv) Th17 cells; xv) regulatory T cells (T regs); and xvi) exhausted T cells (34). Spearman's rank correlation coefficient method was used to identify the correlation coefficient.

Prognostic analysis of pan-cancers. Kaplan-Meier plotter (http://kmplot.com/analysis/) is capable of assessing the effect of 54,000 genes on survival in 21 types of cancer (35). The association between the hub gene expression and survival in all 21 types of cancer were analyzed using Kaplan-Meier plotter. All possible cut-off values between the lower and upper quartiles were computed and the best performing threshold was used as a cut-off value. The hazard ratio (HR) with 95% confidence intervals (CI) and log-rank P-value were also calculated.

Statistical analysis. Survival curves were created using GEPIA and Kaplan-Meier plots. Three independent repeated unpaired and paired t-tests were carried out in GraphPad Prism (GraphPad Software, Inc. version 7.04) and R software (30) (version 3.5.3), and the results were shown as mean ± standard deviation. The Oncomine results were presented with P-values, fold-changes and ranks. The Kaplan-Meier plots and GEPIA results were displayed with the HR and P-values obtained using log-rank tests. Correlation between TMC8 expression and the clinicopathological characteristics were analyzed using logistic regression. Potential prognostic factors were determined with a univariate Cox analysis and the associations between TMC8 expression and survival in conjunction with other clinical features were verified with a multivariate Cox analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of TMC8 in oral cancer specimens and adjacent normal tissue. HNSC specimens from 25 patients were analyzed in the present study, including 17 males and 8 females. The age of this group ranged from 43 to 81 years with a median age of 67. Immunohistochemical staining of OSCC tissue specimens and adjacent normal tissues revealed that TMC8 was strongly stained in tumor tissues, but the staining intensity was lower in adjacent tissues (Fig. 2A-D).

Expression of TMC8 is increased in oral cancer cell lines. Fig. 2E shows that, compared with HOK, the expression levels of TMC8 in SCC9, SCC15, SCC25 and CAL27 cell lines were significantly upregulated.

TMC8 is upregulated in HNSC of TCGA data. As shown in Fig. 3A, TMC8 expression was higher in colorectal, breast and head and neck cancer compared with that of normal tissues. However, lower TMC8 expression was also observed in colorectal and breast cancer in addition to lung, leukemia and lymphoma cancer in some data sets. The differential level of TMC8 expression between tumor and normal tissues in HNSC for TCGA data is shown in Fig. 3B and C. The results indicated that TMC8 was overexpressed in HNSC samples (P<0.001) and paired samples (P<0.05).

Methylation analysis. In order to explore the possible reasons for the upregulation of TMC8 in HNSC, the relationship between its methylation status and its expression was further analyzed. Overall, 26 methylation sites out of a total of 31 were found to be significantly hypomethylated. Among them, cg00447208, cg01246266, cg03190661, cg08470991, cg19056418 and cg20943461, which were all located in the promoter region, showed significantly decreased levels of methylation in HNSC compared with normal samples. Additionally, they were significantly negatively correlated with the expression of TMC8 (all P<0.05). The relationship between methylation sites and expression of TMC8 is shown in Table I.

Association with TMC8 expression and clinicopathologic variables. As shown in Table II, the increased expression of TMC8 was significantly correlated with the tumor origin (oropharynx vs. oral cavity; P=0.003), histological grade (G3/G4 vs. G1/G2; P=0.04), HPV infection status (positive vs. negative; P=0.001), immune-score (high vs. low, P<0.001), as well as the stromal-score (high vs. low; P<0.001). However, no significant differences between TMC8 expression and i) age; ii) alcohol use; iii) metastasis; iv) lymph node infiltration; v) T classification; vi) clinical stage; vii) lymphovascular invasion; viii) perineural invasion; or ix) nodal extra-capsular spread were observed.

Survival outcomes and multivariate analysis. As shown in Fig. 4A, the analysis of HNSC cases in TCGA showed that the 5-year OS time of the TMC8-high group was significantly greater compared with that of the low-expression group (P<0.05). Fig. 4B-H further shows the relationship between TMC8 expression and clinical features. In the cases of histopathological grade II-III and clinical stage III-IV, overexpression of TMC8 was significantly correlated with improved OS time. This result suggested that in advanced HNSC cases, high expression of TMC8 may improve the prognosis.

Univariate analysis (Table III) revealed that TMC8-overexpression was significantly associated with an improved OS time (HR: 0.80; 95% CI: 0.70-0.90; P=0.003). Other clinicopathological variables were associated with increased survival including advanced age, positive marginal status, B cell infiltration and immune score. In the multivariate analysis, TMC8 remained independently associated with OS, with an HR of 0.80 (CI: 0.68-0.95; P=0.01), In conjunction with the advanced age, clinical stage and positive marginal status.

GSEA identifies a TMC8-associated signaling pathway. TMC8-associated pathway enrichment analysis results showed that 28 gene sets were significantly enriched when the adjusted P-value was <5%. As shown in Table IV, the following
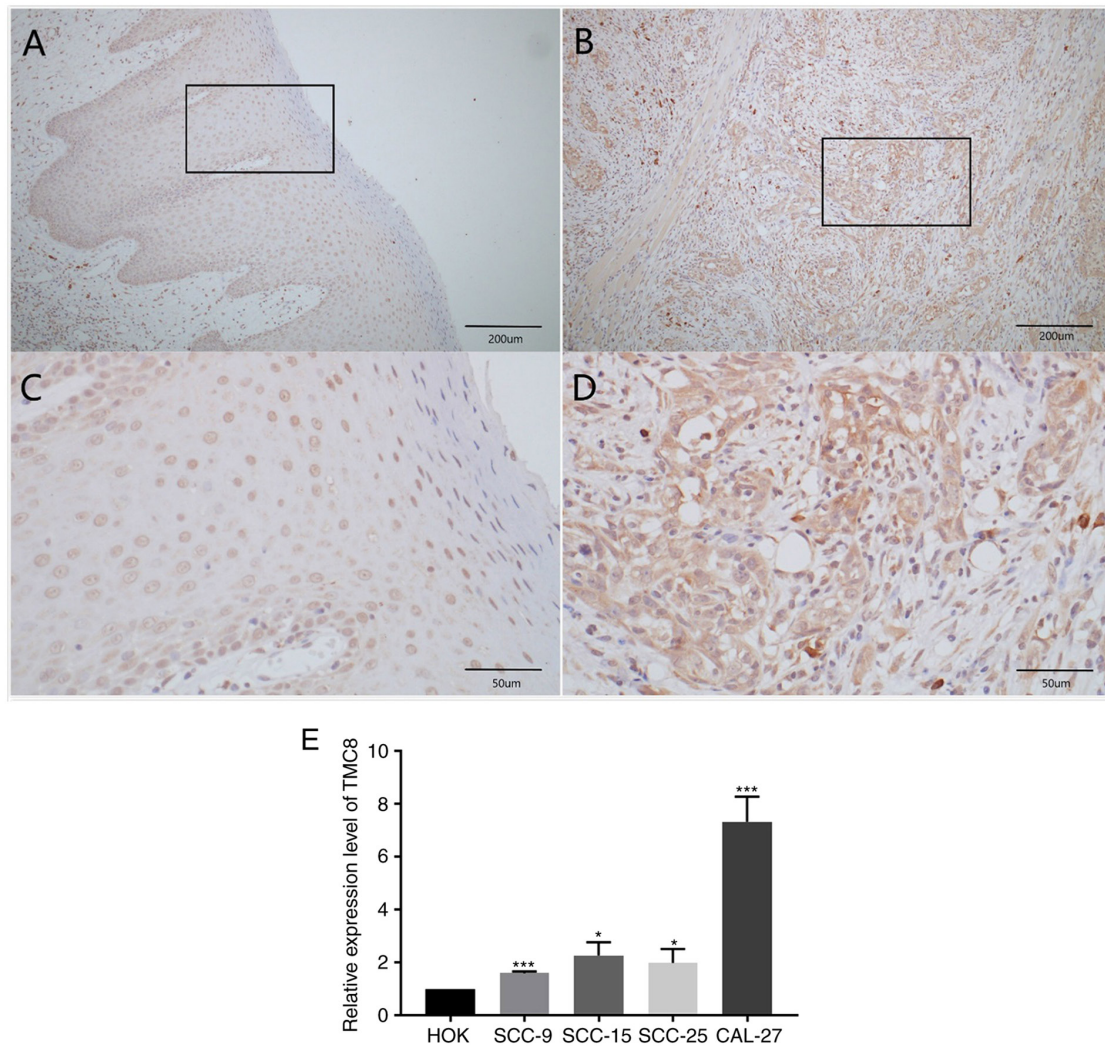
Figure 2. TMC8 is upregulated in OSCC. Representative immunohistochemical staining performed for detecting the expression of TMC8 from tumor-tissue specimens and adjacent normal tissues of patients with OSCC. (A and B) Normal mucosal epithelial cells, lightly stained with TMC8 antibody. (magnification, x100 or 400, respectively). (C and D) Oral cancer mucosal epithelial cells, lightly stained with TMC8 antibody. (magnification, x100 or 400, respectively). (E) Compared with HOK, the expression levels of TMC8 in SCC9, SCC15, SCC25 and CAL27 cell lines were significantly upregulated. *P<0.05 and ***P<0.001 vs. HOK. TMC8, transmembrane channel-like 8; OSCC, oral squamous cell carcinoma; HOK, human oral keratinocyte.

immune-associated biological processes are enriched in response to the increased TMC8 phenotype: i) 'Intestinal immune network for IgA production'; ii) 'primary immuno-deficiency'; iii) 'leishmania infection'; iv) 'cytokine-cytokine receptor interaction'; v) 'natural killer cell-mediated cyto-toxicity'; vi) 'hematopoietic cell lineage'; vii) 'autoimmune thyroid disease'; viii) 'T cell receptor signaling pathway'; ix) 'antigen processing and presentation'; and x) 'cell-adhesion molecules'. These signaling pathways may be the mechanisms involved in TMC8 function.

Relationship between TMC8 and immune cells infiltration. There was a significant negative correlation between TMC8 expression and tumor purity (Fig. 5A). In addition, the level of TMC8 expression was significant correlated with high levels of CD4^{+} T cell and CD8^{+} T cell infiltration in HNSC. Similarly, there were weak to moderate positive correlations with the level of infiltrating lymphocytes in HPV-positive HNSC samples, as well as in HPV-negative HNSC samples (correla-tion coefficient 0.142 to 0.639, all P<0.05; Fig. B and C).

To avoid bias, the results of six other algorithms were listed, each of which provided the correlation coefficient between infiltration of different subsets of immune cells and expression of TMC8. As shown in Table V, different algorithms acquired similar results. TMC8 was positively correlated with T cell subsets, such as Th1, Th17, T regs and Tfh.

TMC8 expression and immune marker correlation analysis. To further verify the relationship between TMC8 and immune cells, the correlation between marker genes and TMC8 expression in different cells was calculated. As shown in Fig. 6A-C, TMC8 is strongly correlated with T cell (general) marker genes, including CD3D, CD3E and CD2 (all coefficients >0.6 and P<0.05). TMC8 was significantly correlated with marker genes of CD8^{+} cells (all P<0.05 and coefficients >0.3; Fig. 6D-F) and B cells (all coefficients >0.4 and P<0.05; Fig. 6G-I). In addition, the level of expression for a majority of markers on M1 (nitric oxide synthase 2 and interferon regulatory factor 5), M2 macrophages (CD163, V-set and immunoglobulin domain-containing protein 4 and
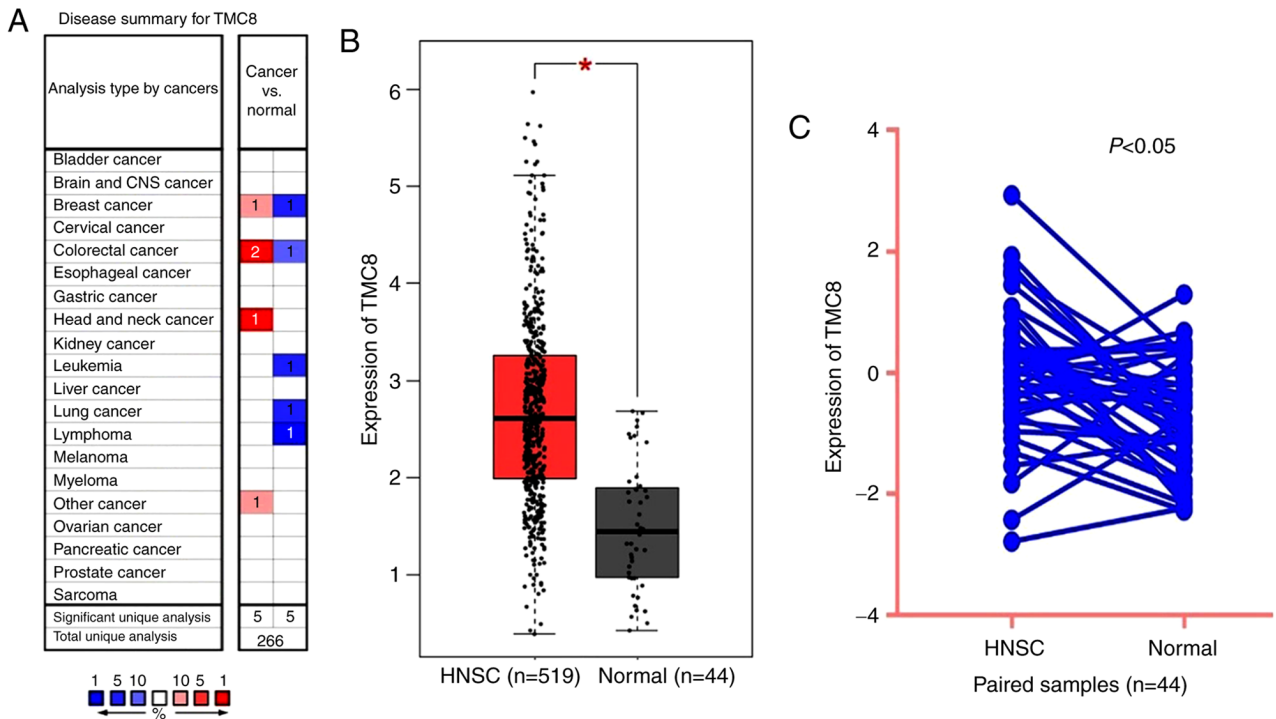
Figure 3. Levels of TMC8 expression in HNSC and other types of cancer. (A) Increased or decreased TMC8 expression in datasets of different types of cancer in the Oncomine database. Cell color is determined by the best gene rank percentile for the analyses within the cell. Blue indicates that TMC8 gene expression is downregulated and red indicates that the gene is upregulated. (B) TMC8 expression was compared between normal tissues and HNSC tissues based on The Cancer Genome Atlas database. (C) Levels of TMC8 expression was compared in the paired samples. *P<0.05 vs. HNSC. HNSC, head and neck squamous cancer; TMC8, transmembrane channel-like 8.
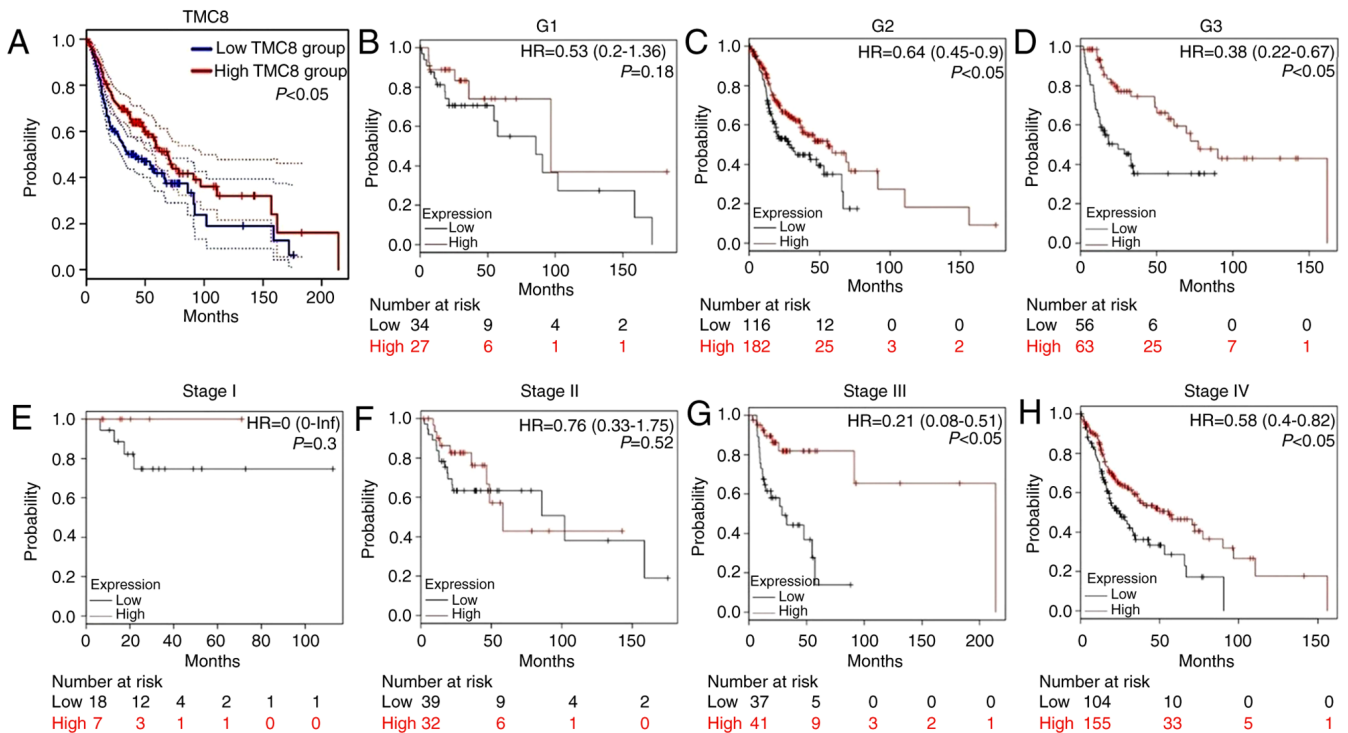
Figure 4. Relationship between the level of TMC8 expression and OS time. (A) Patients with high expression of TMC8 had better prognosis. (B-D) Kaplan-Meier curves for OS time in HNSC cases of histological grades G1, G2 and G3. (E-H) Kaplan-Meier curves for OS time in HNSC cases of clinical stages I-IV. HNSC, head and neck squamous cell cancer; OS, overall survival; TMC8, transmembrane channel-like 8; HR, hazard ratio.

membrane-spanning 4-domains subfamily A member 4A), TAMs (C-C motif chemokine 2, CD68 and interleukin 10), monocytes, DCs (human leukocyte antigen [HLA]-DPB1, HLA-DRA and HLA-DPA1) and other subclasses of T cells

Table I. Relationship between methylation sites and expression of transmembrane channel-like 8.

Methylation site	Cor.	P-value
cg00447208[a]	-0.24	5.79×10^{-08}
cg01246266[a]	-0.16	2.63×10^{-04}
cg03190661[a]	-0.26	3.10×10^{-09}
cg08470991[a]	-0.19	1.74×10^{-05}
cg19056418[a]	-0.28	1.39×10^{-10}
cg20943461[a]	-0.31	1.73×10^{-12}
cg01125010	0.13	2.20×10^{-03}
cg01791634	-0.18	4.01×10^{-05}
cg02909991	-0.17	6.01×10^{-05}
cg02911077	-0.20	4.09×10^{-06}
cg03596178	0.06	0.21
cg03742808	-0.24	5.20×10^{-08}
cg04947157	-0.24	3.02×10^{-08}
cg05637296	-0.29	2.67×10^{-11}
cg06248406	-0.04	0.35
cg06643271	-0.17	1.16×10^{-04}
cg08852879	0.09	0.04
cg09413013	-0.10	0.03
cg11493223	-0.18	2.17×10^{-05}
cg12798338	-0.24	5.76×10^{-08}
cg14210726	0.29	1.72×10^{-11}
cg16214492	-0.23	8.22×10^{-08}
cg16301617	-0.26	3.79×10^{-09}
cg16935597	-0.16	1.55×10^{-04}
cg18901278	-0.17	6.72×10^{-05}
cg21282054	-0.26	1.11×10^{-09}
cg22563987	0.13	4.16×10^{-03}
cg22833809	-0.16	2.60×10^{-04}
cg24109860	-0.06	0.17
cg24988684	-0.24	3.65×10^{-08}
cg26003388	-0.22	4.63×10^{-07}

[a]Promoter.

were significantly correlated with TMC8 expression, details shown in Table SI.

Prognostic analysis of TMC8 on pan-cancers. As shown in Fig. 7, in five types of tumors: i) Bladder cancer; ii) cervical squamous cell carcinoma; iii) pheochromocytoma and paraganglioma; iv) sarcoma; and v) thymoma, high expression of TMC8 predicted a significantly increased OS time and improved prognosis (all P<0.05).

Discussion

The present study analyzed the levels of TMC8 expression in HNSC and its prognostic association. The expression of TMC8 was upregulated in patients with HNSC and the methylation level in the promoter was significantly lower compared with that in normal tissues, which may be the reason for the change in TMC8 expression. The increased expression level of TMC8 in HNSC was further validated using tissue specimens. GSEA analysis was used to clarify the function of TMC8 and the results showed that it was involved in immune-associated pathways.

TMC8 expression was found to be significantly positively correlated with the infiltration of CD4+ T cells, CD8+ T cells, B cells, macrophages and DCs and their respective subgroups. These results indicated that TMC8 may enhance the infiltration of cytotoxic T cells and B lymphocytes by affecting CD4+ T cells. Finally, the impact of TMC8 on the prognosis of various malignancies was evaluated and the results showed that TMC8 could significantly affect the OS of various types of cancer, including HNSC. The findings of the present study showed that TMC8, as one of the genes involved in HPV immune responses (36) may play a very complex role in the immune network and ultimately inhibit the development of tumors.

The relationship between HPV and skin malignancies has been found in patients with EV (37). These patients usually develop invasive skin squamous cell carcinoma (38). In a previous study (38) germline mutations of two genes, EVER1 and EVER2, also known as TMC6 and TMC8, were found in patients with EV. The inability of patients with EV to effectively clear HPV from their own skin is mainly due to cell-mediated immunodeficiency (39). Defects in the TMC8 gene have been previously suggested to promote an environment favorable to HPV replication in addition to the persistence of skin cancer-prone lesions (23). A study investigating a common polymorphism (rs7208422) in the TMC8 gene in patients with skin cancer has found that the polymorphism is not only associated with positive serological tests for skin HPV types, but also with an increased risk of skin squamous cell carcinoma (40). In the present study, TMC8 was found to be highly expressed in HNSC. At present, no study has been conducted to detect the changes in the expression level of TMC8 in HNSC, to the best knowledge of the authors. Two previous studies have revealed that common single nucleotide polymorphisms in TMC8 increase the risk of HNSC (36,41). In the current study, the TIMER algorithm showed a negative correlation between TMC8 expression and tumor purity, suggesting that TMC8 was expressed more frequently in tumor-infiltrating lymphocytes and mesenchymal cells compared with in squamous cancer cells. The mesenchymal cells around the tumor mainly include immune cells, for example, macrophages, T cells and neutrophils (42). Previously published data indicate that TMC8 is highly expressed in various types of hematopoietic cells, including CD4+ and CD8+ T lymphocytes, B cells and NK cells (25,26,43). Therefore, the high expression of TMC8 in mesenchymal cells may contribute to its upregulation in HNSC. To determine whether TMC8 is highly expressed in tumor cells, immunohistochemical staining and detection of TMC8 expression in SCC cell lines using qPCR was used in the present study. These results confirmed that TMC8-upregulation was not only due to the enrichment of T cells in tumor stroma, but also synchronously upregulated in tumor cells as the SCC cell line tested by qPCR avoids the interstitial cells contained in the tumor tissue.

The present study then explored the underlying causes of the observed transcriptional changes and focused on the

Table II. Correlation between TMC8 expression and the clinicopathological characteristics of patients with head and neck squamous cancer (logistic regression).

Clinical characteristics	Total, n	Odds ratio in TMC8 expression hazard ratio (CI)	P-value
Age, continuous	520	1.01 (0.99-1.02)	0.23
Drinking, yes vs. no	509	0.96 (0.65-1.43)	0.84
Smoke, yes vs. no	520	1.10 (0.78-1.56)	0.60
Tumor origin, oropharynx vs. oral cavity	520	1.74 (1.20-2.51)	3.20×10^{-3}[a]
Distance metastasis, positive vs. negative	496	0.94 (0.51-1.74)	0.85
Lymph nodes, positive vs. negative	498	0.82 (0.58-1.17)	0.28
T classification, T1/T2 vs. T3/T4	504	0.71 (0.49-1.02)	0.07
Stage, I/II vs. III/IV	506	0.75 (0.28-1.98)	0.57
HPV, positive vs. negative	121	3.98 (1.78-9.39)	1.10×10^{-3}[a]
Lymphovascular invasion, positive vs. negative	348	1.39 (0.89-2.16)	0.15
Perineural invasion, positive vs. negative	362	0.75 (0.49-1.13)	0.17
Nodal extracapsular spread, positive vs. negative	356	0.77 (0.49-1.21)	0.25
Histological grade, G3/G4 vs. G1/G2	498	1.51 (1.02-2.27)	0.04
Immune score, high vs. low	520	5.17 (3.57-7.55)	6.81×10^{-18}[a]
Stromal score, high vs. low	520	1.95 (1.38-2.77)	1.72×10^{-4}[a]

[a]P<0.05. TMC8, transmembrane channel-like 8; CI, confidence interval.

Table III. Univariate and multivariate analyses of OS time using the Cox proportional hazard regression model (n=415).

Parameter	Univariate analysis			Multivariate analysis		
	HR	CI, 95%	P-value	HR	CI, 95%	P-value
Age	1.02	1.01-1.04	9.94×10^{-4}[a]	1.02	1.006-1.035	4.00×10^{-3}[a]
Alcohol-use	0.96	0.71-1.29	0.77	1.03	0.741-1.425	0.87
Sex	0.81	0.59-1.10	0.18	0.87	0.614-1.222	0.41
Clinical stage	1.32	0.93-1.88	0.12	1.45	1.005-2.078	0.47×10^{-2}[a]
Margin status	1.61	1.11-2.34	0.01[a]	1.69	1.146-2.480	0.01[a]
Histological grade	1.08	0.78-1.49	0.63	1.24	0.881-1.752	0.22
B cell	0.09	0.01-0.64	0.02[a]	0.16	0.011-2.298	0.18
CD4+ T cell	0.28	0.05-1.61	0.16	0.03	0.008-7.332	0.42
CD8+ T cell	0.55	0.20-1.49	0.24	0.77	0.130-4.509	0.77
Neutrophil	0.81	0.15-4.52	0.81	3.93	0.229-67.382	0.35
Macrophage	1.43	0.32-6.39	0.64	3.49	0.328-36.985	0.30
Dendritic cell	0.88	0.47-1.66	0.70	3.75	0.745-18.908	0.11
Immune-score	1.00	9.998×10^{-3}-9.99×10^{-3}	0.04[a]	1.00	9.997×10^{-3}-1.000×10^{-3}	0.14
Stromal-score	1.00	9.998×10^{-3}-1.000×10^{-3}	0.13	1.00	9.999×10^{-3}-1.000×10^{-3}	0.15
TMC8	0.80	0.70-0.90	3.00×10^{-4}[a]	0.80	0.68-0.95	0.0[1a]

[a]P<0.05. TMC8, transmembrane channel-like 8.

frequency of methylation changes. The downregulation of gene expression caused by hypermethylation of CpG islands in gene promoter regions is a common gene silencing mechanism in epigenetic regulation (44). A total of six methylation sites of TMC8 located in the promoter region showed significant hypomethylation in patients with HNSC, which was a notable reason for TMC8-downregulation. The DNA modification of different structural elements, such as the promoter, coding region or distal enhancer region of the gene, as well as the combined action of transcription factors and microRNA, constitute a complex regulatory system for gene transcription (45,46). Whether other epigenetic modifications, such as

Table IV. Gene sets enriched analysis of upregulated TMC8 in head and neck squamous cancer.

| Gene sets	NES	Adjusted P-value
Intestinal immune network for IgA production	2.19	0.01
Primary immunodeficiency	2.13	0.01
Leishmania infection	2.08	0.01
Cytokine-cytokine receptor interaction	2.01	0.01
Natural killer cell mediated cytotoxicity	2.01	0.01
Hematopoietic cell lineage	2.01	0.01
Autoimmune thyroid disease	1.98	0.01
T cell receptor signaling pathway	1.99	0.01
Antigen processing and presentation	1.96	0.01
Cell-adhesion molecules	1.97	0.01
Type I diabetes mellitus	1.88	0.03
Chemokine signaling pathway	1.89	0.03
Systemic lupus erythematosus	1.86	0.03
B cell receptor signaling pathway	1.86	0.03
Asthma	1.85	0.03
Tryptophan metabolism	1.83	0.04

NES, normalized enrichment score.
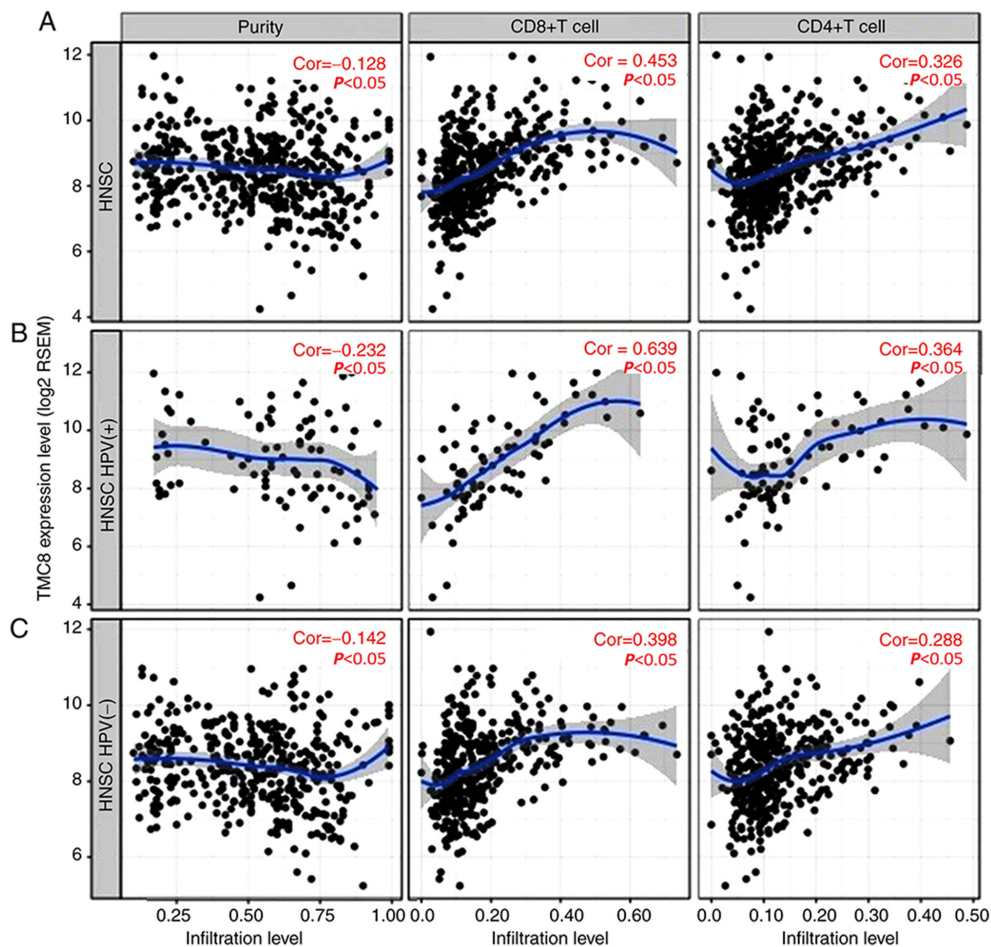
Figure 5. Correlation between the level of TMC8 expression and T lymphocyte infiltration in HNSC obtained from the Tumor Immune Estimation Resource database. (A) TMC8 expression was negatively correlated with tumor purity in HNSC, but was significantly positively correlated with infiltration of CD8^{+} T cells and CD4^{+} T cells (all P<0.05). (B and C) Similar positive correlations were observed with the level of infiltrating lymphocytes in both HPV-positive and -negative HNSC samples (all P<0.05). HNSC, head and neck squamous cancer; TMC8, transmembrane channel-like 8; Cor, correlation; HPV, human papillomavirus; RSEM, RNA sequencing by expectation-maximization.

Table V. Relationship between transmembrane channel-like 8 and immune cell subgroups by different algorithm.

Subsets of immune cells	Correlation coefficients					
	CIBERSORT	Xcell	TISIDB	EPIC	MCPCOUNTER	QUANTISEQ
CD4^{+} T cell	-	-	-	-	-	-
CD4^{+} T cell	-	-	-	0.18	-	-0.30[a]
CD4^{+} naive	-0.17	0.37[a]		-	-	-
CD4^{+} Th1	-	0.13	0.42[a]	-	-	-
CD4^{+} Th2	-	0.11	0.05	-	-	-
CD4^{+} Th17	-	-	0.33[a]	-	-	-
Tfh	0.67[a]	-	0.36[a]	-	-	-
Treg	0.44[a]	0.16	0.26[a]	-	-	0.52[a]
CD4^{+} memory	-	0.25[a]	-	-	-	-
CD4^{+} memory resting	0.22[a]	-	-	-	-	-
CD4^{+} central memory	-	-0.10	0.04	-	-	-
CD4^{+} memory activated	0.26[a]	-	-	-	-	-
CD4^{+} effector memory	-	0.00	0.17	-	-	-
$\gamma\delta$	0.10	0.18	-	-	-	-
CD8^{+} T cell	-	-	-	-	-	-
T cell CD8^{+}	0.60[a]	0.41[a]	-	0.13	0.58[a]	0.54[a]
T cell CD8^{+} naive	-	0.02	0.50[a]	-	-	-
T cell CD8^{+} central memory	-	0.52[a]	-0.06	-	-	-
T cell CD8^{+} effector memory	-	0.35[a]	-	-	-	-
B cell	-	-	-	-	-	-
B cell	-	-	-	0.41[a]	0.48[a]	0.53[a]
B cell naive	0.21	0.27[a]	-	-	-	-
B cell plasma	0.24[a]	0.25[a]	-	-	-	-
B cell memory	0.22[a]	0.39[a]	0.12	-	-	-
Macrophage	-	-	-	-	-	-
Macrophage	-	0.26[a]	0.34[a]	0.43[a]	0.27[a]	-
Macrophage M1	0.49[a]	0.31[a]	-	-	-	0.17
Macrophage M2	0.45[a]	0.20	-	-	-	0.40[a]
Dendritic cell	-		-	-	-	-
Myeloid dendritic cell	-	0.33[a]	-	-	0.51[a]	0.08
Myeloid dendritic cell activated	-0.05	0.48[a]	0.25[a]	-	-	-
Myeloid dendritic cell resting	0.19	-	-	-	-	-
NK cell	-	-	-	-	-	-
NK cell	-	-0.13	0.34[a]	0.19	0.52[a]	0.32[a]
NK cell resting	-0.09	-	-	-	-	-
NK cell activated	0.37[a]	-	-	-	-	-
Neutrophil	-	-	-	-	-	-
Neutrophil	-0.09	0.22[a]	-0.05	-	-0.03	0.06
Monocyte	-	-	-	-	-	-
Monocyte	0.11	-0.02	0.19	-	0.27[a]	0.11
Mast cell	-	-	-	-	-	-
Mast cell	-	-0.03	0.27[a]	-	-	-
Mast cell resting	-0.10	-	-	-	-	-
Mast cell activated	0.25[a]	-	-	-	-	-

[a]P<0.05. Tfh, follicular T helper; Treg, regulatory T cell; NK, natural killer T cell.

histone deacetylation or chromatin remodeling, affects the expression or function of TMC8 requires further study.

The relationship between TMC8 and lymphocytes is not completely clear. Research by Lazarczyk *et al* (25) showed that
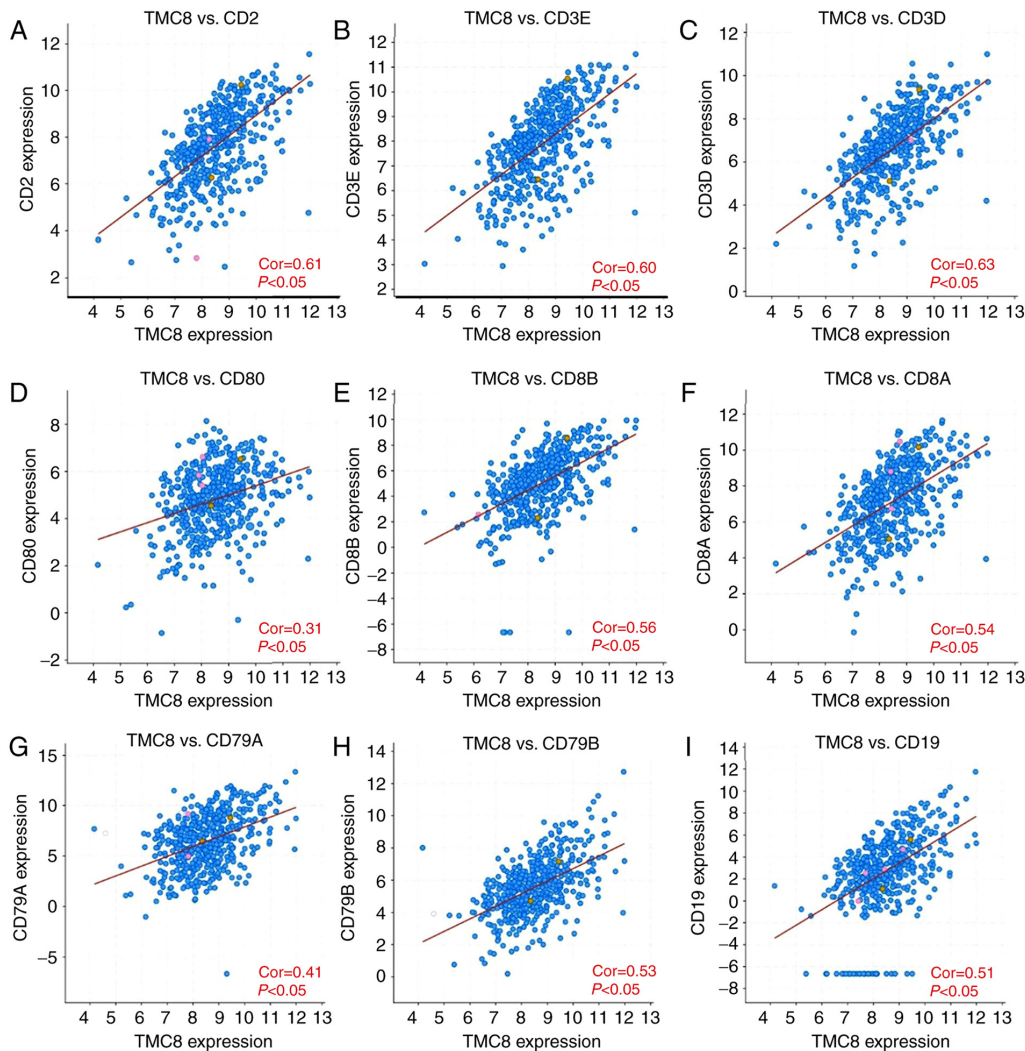
Figure 6. Correlation analysis between TMC8 and lymphocyte marker genes in cBioPortal. (A-C) T cell marker genes, including CD2, CD3E and CD3D, had a significant positive correlation with TMC8. (D-F) CD80, CD8B and CD8A are marker genes of CD8+ T cells. (G-I) Marker genes of B cells, including CD79A, CD79B and CD19, had a significant negative correlation with TMC8. TMC8, transmembrane channel-like 8; Cor, correlation.

TMC8 is also involved in the maintenance of lymphocyte zinc homeostasis. Moreover, mutations in the TMC8 gene result in an excess of zinc ions, which in turn blocks the activation and proliferation of T cells. Crequer *et al* (26) studied the lymphocytes of three adult patients with EV and TMC8 mutations and found that the number of CD4+ and CD8+ T cells and the response to stimulation were normal. However, the number of memory CD4+ T cells and effector memory CD8+ T cells increased significantly. This finding suggests that patients with TMC8 dysfunction have mild T cell dysfunction. The significant positive correlation between TMC8 and T cells is due to its high expression in T cells in the current study, which also shows that it can be used as an effective indicator of T cell infiltration and function. However, different subpopulations of lymphocytes exert different effects (47,48), and whether the expression of TMC8 can reflect the enrichment of specific subpopulations has not yet been reported to the authors' knowledge. Therefore, the present study further explored the role of TMC8 in immune network through correlation analysis.

Various immune deconvolution methods (49) can be used to estimate the abundance of immune infiltrates, including TIMER, CIBERSORT, quanTIseq, xCell, MCPCounter and EPIC methods. To avoid bias, multiple algorithms were used for cross-validation in the present study. The results showed that different algorithms had almost consistent results. The primary CD4+ T cells differentiate into Th1 cells, Th2 cells, Th7 cells, T reg cells and follicular helper T cells under the action of different cytokines and transcription factors. Among them, the body's antitumor and anti-virus effect is dominated by Th1-mediated cellular immunity. IFN-γ secreted by Th1 can significantly promote the expression level of MHC class I molecules in tumor cells, thereby activating the function of specific CD8+ T lymphocytes. IFN-γ can also increase the phagocytic activity of macrophages and inhibit the formation of tumor blood vessels (47,50). Previous research also shows that the Th1 cell immune response plays a very important role in controlling and eliminating HPV infection and determines the outcome of HPV infection (51,52). In the current study, the expression of TMC8 and Th1 infiltration were significantly positively correlated, suggesting that TMC8-upregulation is an important reference indicator of Th1 cell function. The expression of TMC8 in patients with HPV-positive HNSC was
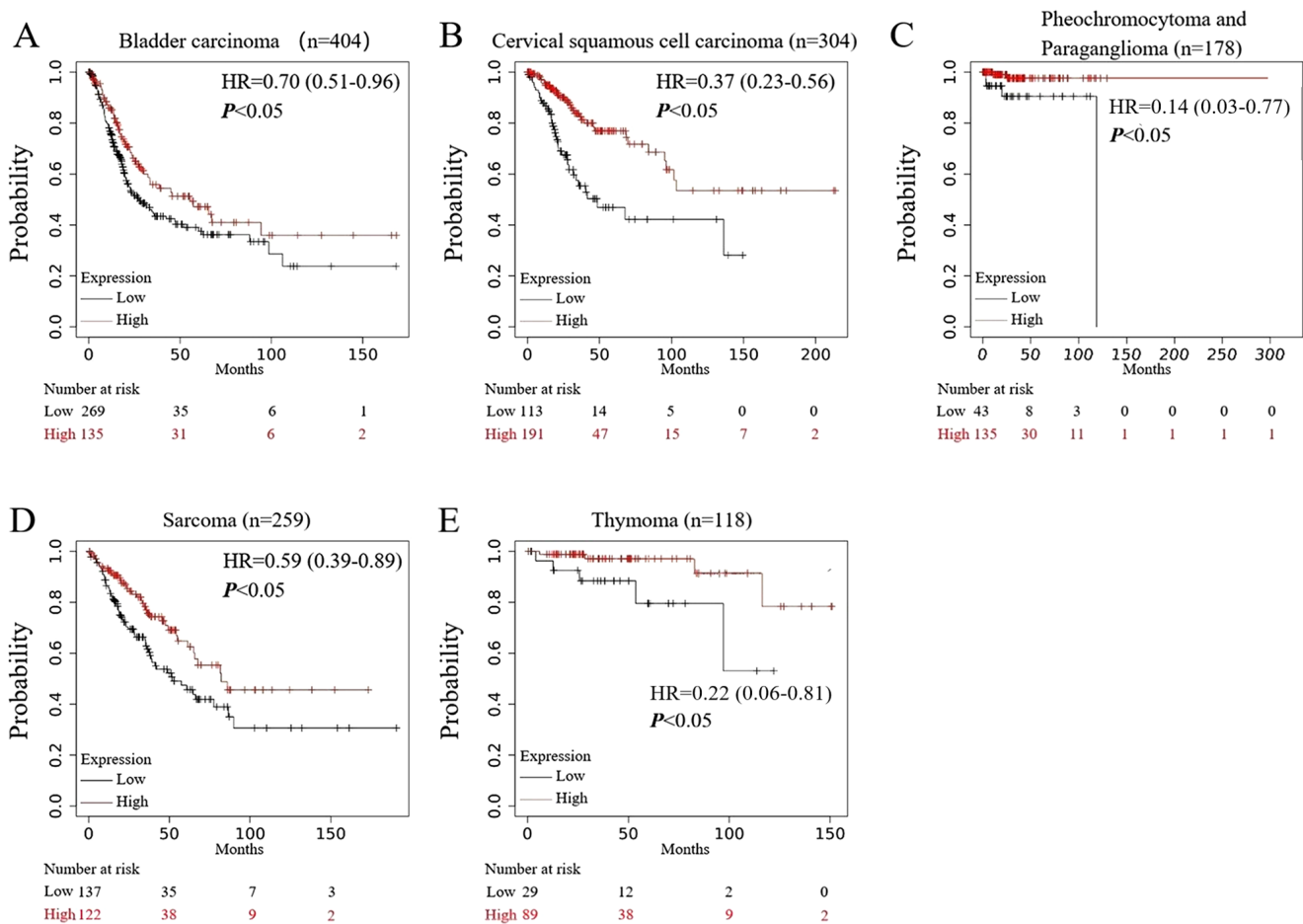
Figure 7. Prognostic analysis of TMC8 in multiple types of cancer. (A-E) In bladder carcinoma, cervical squamous cell carcinoma, pheochromocytoma and paraganglioma, sarcoma and thymoma, high expression of TMC8 was associated with good prognosis ($P=2.7 \times 10^{-2}$, $P=1.6 \times 10^{-5}$, $P=8.2 \times 10^{-3}$, $P=0.01$ and $P=1.3 \times 10^{-2}$, respectively). TMC8, transmembrane channel-like 8; HR, hazard ratio.

significantly upregulated compared with that of patients who were HPV-negative, suggesting that TMC8 may play the role of a HPV barrier through Th1 cells.

Tfh cells are key Th cells that can activate naive B cells to differentiate into plasma cells to produce antibodies and produce a humoral immune response (53). However, the direct effect of Tfh on tumors has not yet been clarified. At present, Tfh is closely associated with the etiology in angioimmunoblastic T-cell lymphoma (AITL) (54), peripheral T-cell lymphomas with follicular growth pattern (PTCL-F) (55) and B cell lymphoma 6 (BCL-6) (53). Th2 stimulates B cell proliferation by secreting interleukin (IL)-4, which mediates the humoral immune response (56). The current study showed that there was a significant positive correlation between TMC8 expression and Tfh, Th2 infiltration and B cell enrichment, suggesting that TMC8 may further regulate B cell activation through Tfh and Th2.

Th17 cells have opposite functions at different stages of the tumor. In the microenvironment of solid tumors, it exerts anticancer effects by secreting IL-17 (57,58). However, as the tumor progresses, the infiltration of T reg cells will induce Th1 to secrete a large amount of IL-10 and promote tumor angiogenesis. The results of the present study indicated that in advanced HNSC (grades G3 and G4), TMC8 expression was upregulated, while TMC8 and Th17 were significantly co-expressed. This is consistent with the change of Th17

function, suggesting that TMC8 plays a complex role in the tumor microenvironment.

In addition to lymphocytes, TMC8 was also found to be significantly positively correlated with infiltration of mesenchymal cells, including macrophages and DCs. Macrophages account for >50% of the tumor stroma (59). M1 and M2 macrophages play opposite roles in the tumor microenvironment (60), but TMC8 was upregulated in both types of macrophages, indicating that TMC8 is not involved in macrophage polarization. To further clarify the possible function of TMC8 expression in immunity, previously reported gene markers were compared. Co-expression of markers for TAMs, M1 phenotype and M2 phenotype with TMC8 remained consistent with the results of the previous algorithm results. DC markers also showed a significant correlation with TMC8 expression, indicating a close relationship between TMC8 and DC penetration. The combination of DC and T cells can secrete IL-12 and IL-18 to activate T cell proliferation, induce CTL production and the Th1 type immune response, which is conducive to tumor clearance (61). The co-expression of TMC8 and marker genes of specific immune cells further verified the results of the different immune algorithms.

Tumors of different cancer types may share underlying similarities (62). Thus, pan-cancer analysis of large-scale datasets has the potential to improve disease modeling by

exploiting these similarities. In addition to HNSC, the results of the Kaplan-Meier database also showed that in bladder cancer, cervical squamous cell carcinoma, pheochromocytoma and paraganglioma, thymoma and sarcoma, TMC8-upregulation and improved OS time were significantly correlated. It was hypothesized that TMC8 was responsible for the resistance to HPV infection, thereby increasing cancer susceptibility. In the present study, TMC8-overexpression was associated with the improved prognosis of a variety of cancer types; however, the mechanism of how these different types of cancer are associated with HPV infection still needs further confirmation. In pathogen-associated human malignancies, up to 35% are caused by HPV and the carcinogenic potential of different HPV species varies widely (63). It is well known that cervical cancer and HNSC have also been shown to be associated with HPV infection (64). Studies have reported that mutations in TMC6 and TMC8 increases the risk of developing cervical cancer from persistent HPV infection (24,65). Liang *et al* (36) reported that the common genetic variations in TMC8 are associated with the etiology of high-risk HPV infection and HNSC. These results show that the TMC8 gene is a key component of human keratinocyte HPV barrier that may affect the biological behavior of cells through immune-mediated pathways and ultimately affect disease prognosis. However, the opposite outcome was observed in other cancer types. Yamada *et al* (66) suggested that TMC8 is one of the numerous downstream genes of microRNA-144-5p/oncogenic syndecan-3 axes, which are associated with a poor prognosis of renal clear cell carcinoma. In addition, Lu *et al* (67). Reported that a higher level of TMC8 expression is associated with a poorer prognosis for hepatocellular carcinoma.

The present study had certain limitations. The correlation strength between TMC8 and the infiltration level of some immune cells was only weak to moderate. Further research, including deep sequencing, is needed to identify the full spectrum of variability and any functional variants of TMC8. It is also necessary to further explore the specific molecular mechanism of TMC8 in HNS carcinoma cells.

In summary, the present study reported that variations in the level of TMC8 expression correlated with HNSC prognosis. High levels of TMC8 expression were associated with improved OS time, which suggested that TMC8 expression could predict tumor prognosis. Furthermore, the findings demonstrated that the extent of immune cell infiltration and the diversity of immune marker expression were correlated with TMC8 expression in HNSC. Therefore, these results provided insight into the potential function of TMC8 in tumor immunology and its potential as a biomarker for HNSC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. Additional datasets generated and/or analyzed during the current study are available in the following repositories: The Cancer Genome Atlas (https://portal.gdc.cancer.gov), Gene Expression Profiling Interactive Analysis (http://gepia.cancer-pku.cn/index.html), Oncomine database (https://www. oncomine.org/resource/login.html) Tumor Immune Estimation Resource (https://cistrome. shinyapps.io/timer/), cBioPortal (https://www.cbioportal.org) and Kaplan-Meier plotter (http://kmplot.com/analysis/).

Authors' contributions

HYY and YHS designed the research and reviewed the writing. BL analyzed the data and prepared the original draft. SJW and YDY performed the statistical calculations and experiments. All authors read and approved the manuscript. BL and YHS confirmed the authenticity of all raw data.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Peking University Shenzhen Hospital (Shenzhen, China). Signed informed consent were obtained from the patients and/or guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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