

Immune Landscape of the B7 and TNFR Families in Oral Squamous Cell Carcinoma

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Objective: To understand the immune molecular landscapes of the two major costimulatory and coinhibitory pathways (B7 and TNFR families) in oral squamous cell carcinoma.

Methods: The B7 family members (CD80, CD86, CD274, ICOSLG, CD276, VTCN1, NCR-3LG1, HHLA2 and PDCD1LG2) and TNFR family members (TNFSF4, CD40, CD70, TNFSF9, TNFRSF14 and TNFSF18) were used to analyse the costimulatory and coinhibitory pathway alterations in oral squamous cell carcinoma. The online tools UCSC Xena and cBioPortal were used to derive oral squamous cell carcinoma patients' clinical parameters, mRNA levels, mutations, DNA copy number alterations and methylation levels. The correlations between mRNA levels and methylation levels were determined using Spearman's correlation analysis. A Kaplan-Meier survival analysis was performed to examine the relationships between mRNA expression levels and overall survival.

Results: Compared with normal oral epithelial tissues, approximately 23.1% of patients showed upregulation of B7 expression and 15.3% showed upregulation of TNFR expression in oral squamous cell carcinoma, with CD274 (PD-L1) upregulation being the most common alteration. Mutations and copy number alterations were shown to have little effect on B7 and TNFR expression. The mRNA levels of B7 and TNFR genes were negatively correlated with their methylation levels. Furthermore, oral squamous cell carcinoma patients with high expression levels of CD274 showed poor overall survival, while those with high expression levels of CD276 or HHLA2 showed good clinical outcomes.

Conclusion: This study elucidated the molecular landscapes of the B7 and TNFR genes in oral squamous cell carcinoma, which could provide a novel strategy for clinical therapy.

Key words: B7, genomic alteration, oral squamous cell carcinoma, survival, TNFR
Chin J Dent Res 2020;23(2):109–117; doi: doi: 10.3290/j.cjdr.a44747

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This work was supported by grants from the Young Elite Scientist Sponsorship Program by CAST (2017QNRC001); the National Natural Science Foundation of China (81702700, 81700979); the Postdoctoral Science Foundation of China (2019M653231); the Science and Technology Program of Guangzhou city of China (201804010144). The funders had no role in the study design, data collection, analysis, decision to publish or preparation of the manuscript.

Oral squamous cell carcinoma (OSCC), which originates from the epithelial lining of the oral cavity and can take the form of tumours on the labial mucosa, alveolar ridge, gingiva, tongue (anterior two-thirds of the tongue), buccal mucosa, hard palate, floor of the mouth and retromolar trigone, is the most common type of head and neck squamous cell carcinoma (HNSCC)¹. Currently, the National Comprehensive Cancer Network (NCCN) guidelines recommend surgery as the preferred treatment for patients in the early stages of OSCC, while combined treatment with surgery and radiation or chemoradiation therapy is recommended for late-stage patients. However, the 5-year survival rate is still below

60% with little alteration in recent decades, indicating an urgent need to identify a novel strategy for the clinical management of OSCC².

Emerging evidence indicates that targeted and immune-based anticancer therapies are offering exciting results in terms of improving cancer management, such as administration of certain cytokines, engineered cell therapies and immune checkpoint inhibitors to restore T-cell function³. T-cell activation is important in regulating immune responses in two stages. First, T-cell receptors (TCRs) recognise antigens presented by the major histocompatibility complex (MHC). Then, one of the coregulators provides a second signal⁴. The coregulators include the costimulatory and coinhibitory checkpoint molecules, such as the B7 family and the tumour necrosis factor receptor (TNFR) family^{5,6}. T cells infiltrate many tumour microenvironments. However, long-term exposure to tumour antigens causes T cells to become functionally impaired (when this occurs, they are referred to as exhausted T cells), which triggers the tumour cells to acquire the ability to avoid eradication by the immune system. In past decades, T-cell signalling has been explored extensively and immune checkpoint blockade anticancer therapies have achieved exciting success, such as antagonists of programmed death receptor 1 (PD-1) and programmed death-ligand 1 (PD-L1)^{7,8}.

T cells play vital roles in the tumour microenvironment in OSCC. A meta-analysis conducted by Huang et al⁹ demonstrated that high infiltration of CD8⁺ T cells and CD45RO⁺ T cells correlates with a better prognosis in OSCC. High stromal CD8⁺ T-cell density at the tumour periphery was also linked with longer recurrence-free survival in OSCC¹⁰. However, immune surveillance induced by exhausted T cells represents one of the crucial hallmarks of OSCC, which has restricted its application in antitumour therapy. Immune checkpoints participate in immune surveillance and play a critical role in crosstalk between T cells and OSCC cells. There are multiple mechanisms that cause T-cell exhaustion, including the costimulatory and coinhibitory immune checkpoints.

In this study, we focused on alterations in the immune molecular landscape of the two major families: B7 and TNFR genes. We found that most of the B7 and TNFR genes were upregulated in OSCC. Methylation was identified to be associated with alterations in their expression. Furthermore, we identified 5 molecules that were associated with OSCC patients' survival. This study provides a better understanding of the immune coregulators in OSCC and may offer several novel immunotherapy targets for OSCC patients.

Materials and methods

Clinical specimens

The HNSC dataset from The Cancer Genome Atlas (TCGA) database¹¹ and OSCC dataset from the MD Anderson Cancer Center¹² which provided detailed clinical information were included in this study. For the HNSC dataset from TCGA database, the normal tissues and OSCC tissues were identified using biopsies of the floor of the mouth, oral cavity, tongue, alveolar ridge, buccal mucosa, hard palate and lip. Thus, 30 normal oral cavity epithelial tissues and 320 OSCC tumour tissues from the TCGA database and 40 OSCC tumour tissues from the MD Anderson Cancer Center dataset were selected.

Bioinformatics analyses

The online analysis tool UCSC Xena (<http://xena.ucsc.edu/>) was used to derive the mRNA and methylation data (level 3) of TCGA database. The Illumina HiSeq 2000 RNA Sequencing platform was applied to detect mRNA expression in genes. DNA methylation levels were measured using the Illumina Infinium HumanMethylation450 platform. The cBioportal (<https://www.cbioportal.org/>) was used to derive the mutations and DNA copy number alterations (CNAs). The mutation data were generated by whole-exome sequencing on an IlluminaHiSeq system. The CNAs were generated from the whole genome microarray and calculated using GISTIC2.

Statistical analyses

The IBM SPSS statistics 20.0 software (IBM Corp, Armonk, NY, USA) was used for statistical analysis. Student's *t* test was performed to compare the differences between groups. Spearman's rank correlation coefficient was used to test the relationship between the mRNA levels and methylation levels. Receiver operating characteristic (ROC) curve analysis was used to divide OSCC patients into groups with low and high expression of B7 and TNFR. The relationship between gene expression levels and OSCC patients' overall survival (OS) was estimated using the Kaplan-Meier method and univariate Cox regression analysis. The data are represented as mean \pm standard deviation (SD). $P < 0.05$ was considered as statistically significant.

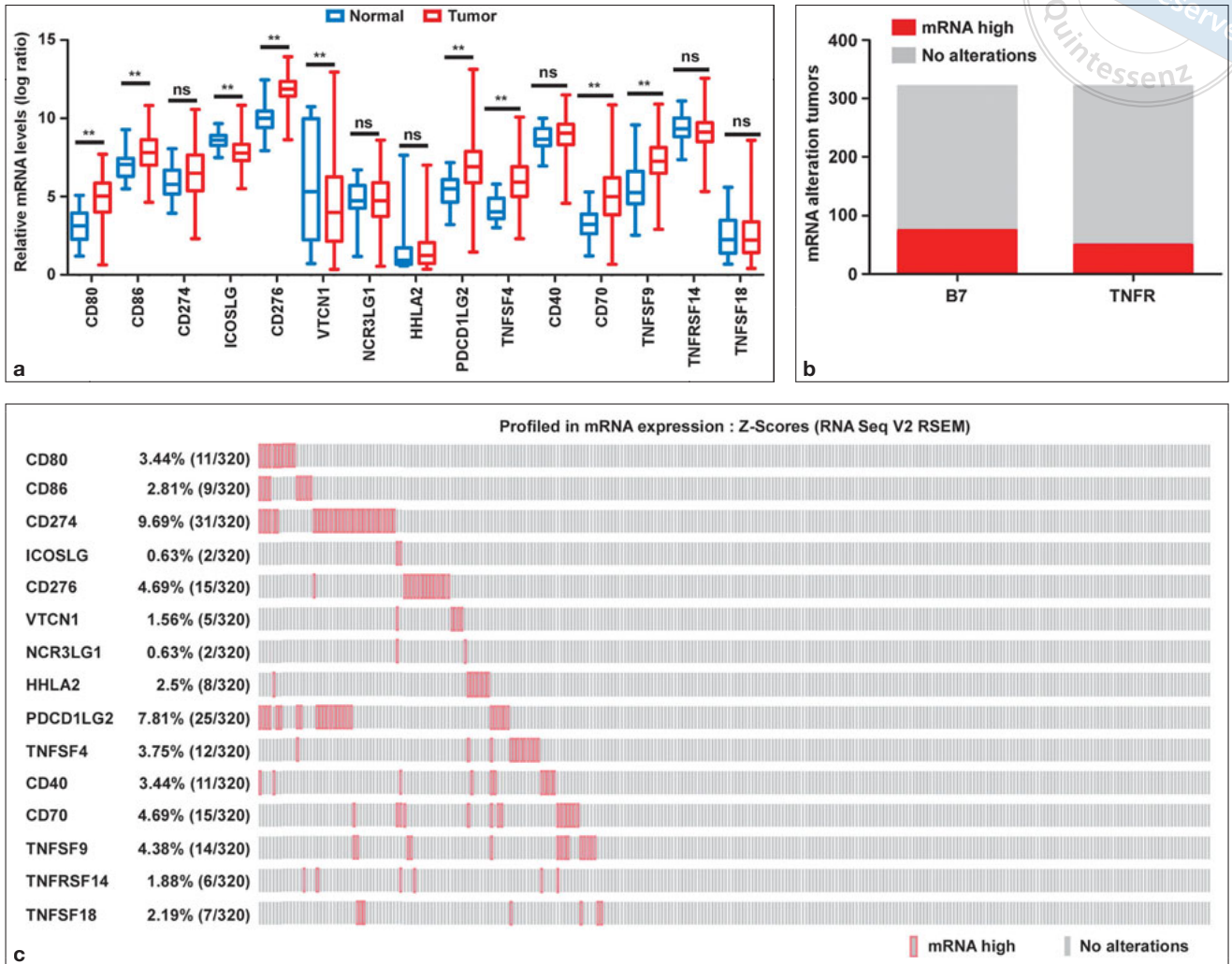


Fig 1 mRNA level alterations of B7 and TNFR genes in OSCC: **(a)** average, **(b)** total and **(c)** individual mRNA alterations in normal tissues (n = 30) and OSCC tissues (n = 320). Each box along the X-axis represents each individual. Grey: no alteration; Red: high mRNA; Student's t test; Mean ± SD; **, P < 0.01.

Results

Alterations in mRNA levels in B7 and TNFR genes in OSCC

To determine the expression levels of B7 and TNFR genes in OSCC, the RNA sequencing data of the HNSC cohort in TCGA database was used. People with normal oral cavity epithelial tissues (n = 30) and primary tumours on the floor of the mouth, tongue, alveolar ridge, buccal mucosa, hard palate and lip and in the oral cavity (n = 320) were included in this study. Compared with the normal oral cavity epithelial tissues, CD80, CD86, CD276, VTCN1, PDCD1LG2 of the B7 fam-

ily and TNFSF4, CD70, TNFSF9 of the TNFR family were significantly upregulated (fold change > 1.5, P < 0.05), while ICOSLG was downregulated (Fig 1a). To further examine the mRNA dysregulation of each patient, cBioPortal was deployed. The results showed that 23.1% (74/320) of patients showed upregulation of B7 genes and 15.3% (49/320) showed upregulation of TNFR genes in OSCC (Fig 1b). Interestingly, the average mRNA level of CD274 (PD-L1) showed no significant alteration in OSCC tumours compared with normal tissues, however CD274 mRNA was most frequently upregulated, in 9.69% (31/320) of patients (Fig 1c), indicating its essential role in the immunotherapy of OSCC patients.

B7 and TNFR genomic alterations in OSCC

The mutation rates and CNAs were considered to contribute to the alterations in mRNA levels in B7 and TNFR genes in OSCC patients from TCGA database. We found that the total frequencies of genomic alterations of B7 (below 15%) and TNFR (below 5%) genes were quite low in OSCC patients. The genomic alterations of the two families in the MD Anderson Cancer Center cohort were also examined, and showed similar results (Fig 2).

Methylation may be responsible for alterations in mRNA levels in B7 and TNFR genes in OSCC

To further explore the mechanism of alterations in B7 and TNFR family expression, Spearman's correlation analysis was performed to assess the relationships between mRNA levels and methylation levels. The methylation probes which were most significantly correlated with mRNA levels are shown in Fig 3. Furthermore, all the genes showed a negative correlation with mRNA levels. Notably, CD274 mRNA levels showed the strongest correlation with methylation (Spearman: -0.343) (Fig 3a). These findings demonstrated that the expression of B7 and TNFR family members might be epigenetically regulated in OSCC patients.

B7 and TNFR genes provide potential prognostic biomarkers for OSCC patients

The relationships between OS and B7 and TNFR mRNA levels in OSCC patients were investigated using a Kaplan-Meier survival analysis and univariate Cox

regression analysis. The results showed that patients with high CD274 mRNA levels had shorter OS than those with low expression levels (Fig 4a). In contrast, patients with high CD276 and HHLA2 mRNA levels showed better clinical outcomes (Fig 4b).

We also analysed the relationships between clinical characteristics and CD274, CD276 and HHLA2 mRNA levels in OSCC patients. CD274 mRNA levels were correlated with patients' age and gender. CD276 and HHLA2 showed no significant correlation with patients' age, gender, tumour-node-metastasis (TNM) stage, smoking and alcohol consumption (Table 1).

Discussion

This study provided an overview of the immune molecular landscapes of the two major costimulatory and coinhibitory pathways (B7 and TNFR families) in OSCC. We found that most of the genes were upregulated in OSCC, with CD274 (PD-L1) upregulation being the most frequent event. Mutations and CNAs of B7 and TNFR genes were rare. The mRNA levels of these two families were all negatively correlated with their methylation levels. Moreover, patients with high CD274 levels showed poor OS, while patients with high CD276 or HHLA2 levels showed good OS, indicating that these could be potential prognostic and predictive biomarkers and therapeutic targets in OSCC.

OSCC is an aggressive tumour with an annual incidence of $> 300,000$, and remains one of the most common cancers worldwide. The routine clinical and therapeutic decisions of OSCC patients made by medical professionals are based on TNM staging. Surgery com-

Table 1 Correlations between CD274, CD276 and HHLA2 mRNA levels and clinical features in OSCC patients.

Characteristic		CD274			CD276			HHLA2		
		Low n = 196	High n = 119	P value	Low n = 257	High n = 52	P value	Low n = 29	High n = 212	P value
Age (median)	≤ 60	104	45	0.009*	119	30	0.137	14	100	0.911
	> 60	92	74		143	23		15	112	
Gender	Female	51	51	0.002*	86	16	0.721	12	60	0.144
	Male	146	68		177	37		17	153	
Pathological tumour stage	Stage I–II	42	33	0.150	65	10	0.523	6	47	0.904
	Stage III–IV	143	76		183	36		19	158	
Smoking	Non-smoker	52	34	0.840	69	17	0.346	4	61	0.094
	Former or current smoker	137	85		188	34		24	147	
Alcohol	No	58	48	0.077	96	10	0.012	8	71	0.463
	Yes	132	71		161	42		21	135	

*Chi-square test or Fisher's exact test

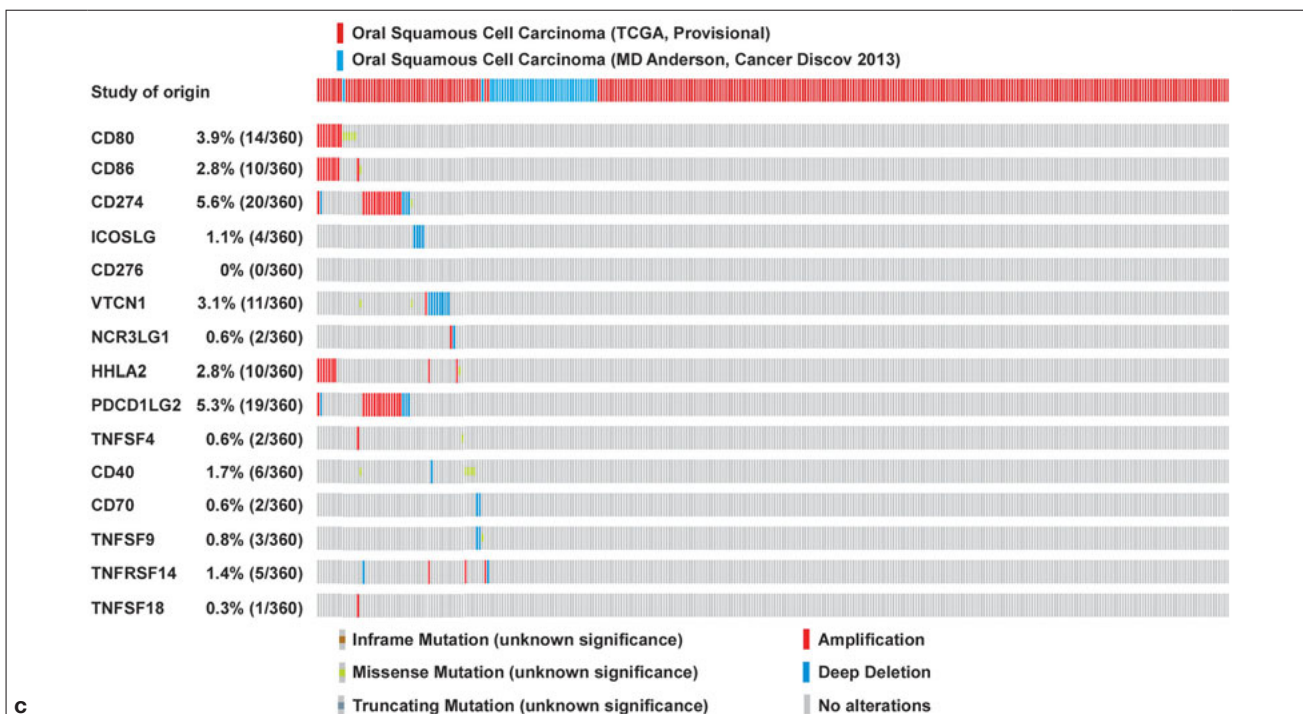
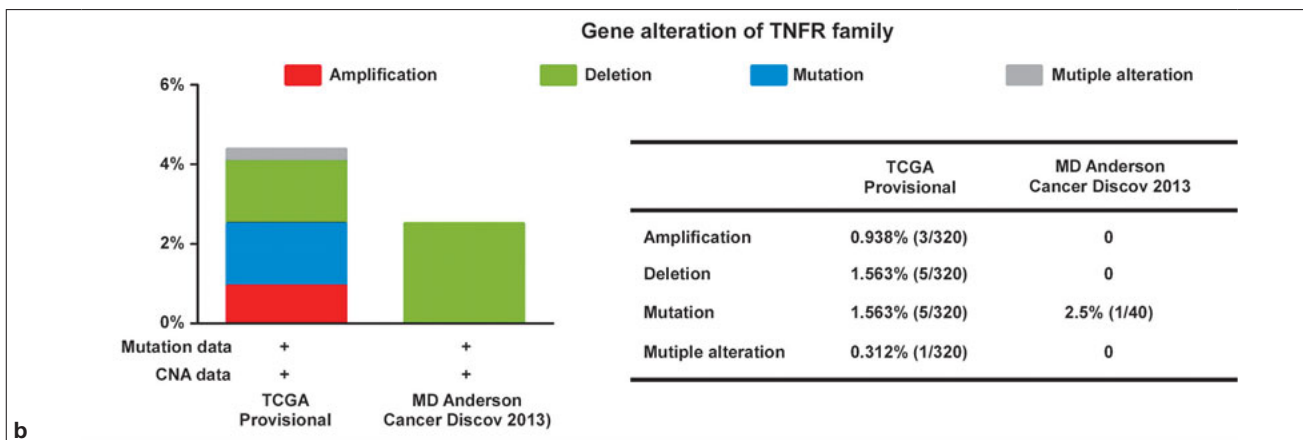
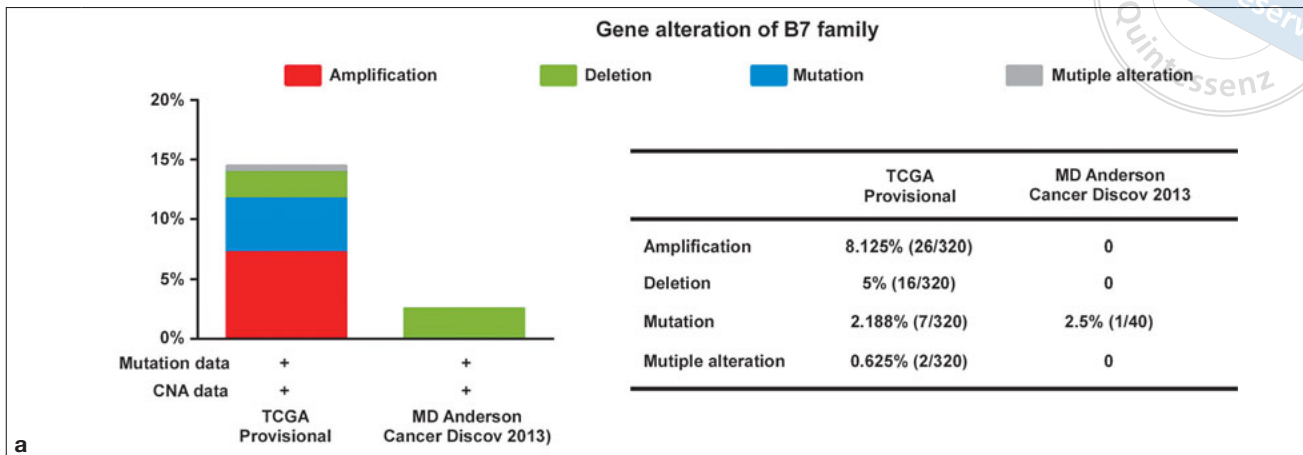


Fig 2 Genomic alterations of B7 and TNFR genes in OSCC: total mutation and CNAs of (a) B7 genes and (b) TNFR genes in OSCC tissues (n = 360). Each box along the X-axis represents each individual. Grey: no alteration; red: amplification; blue: deep deletion; green: missense mutation. (c) The individual genomic alterations of B7 and TNFR families in OSCC tissues (n = 360).

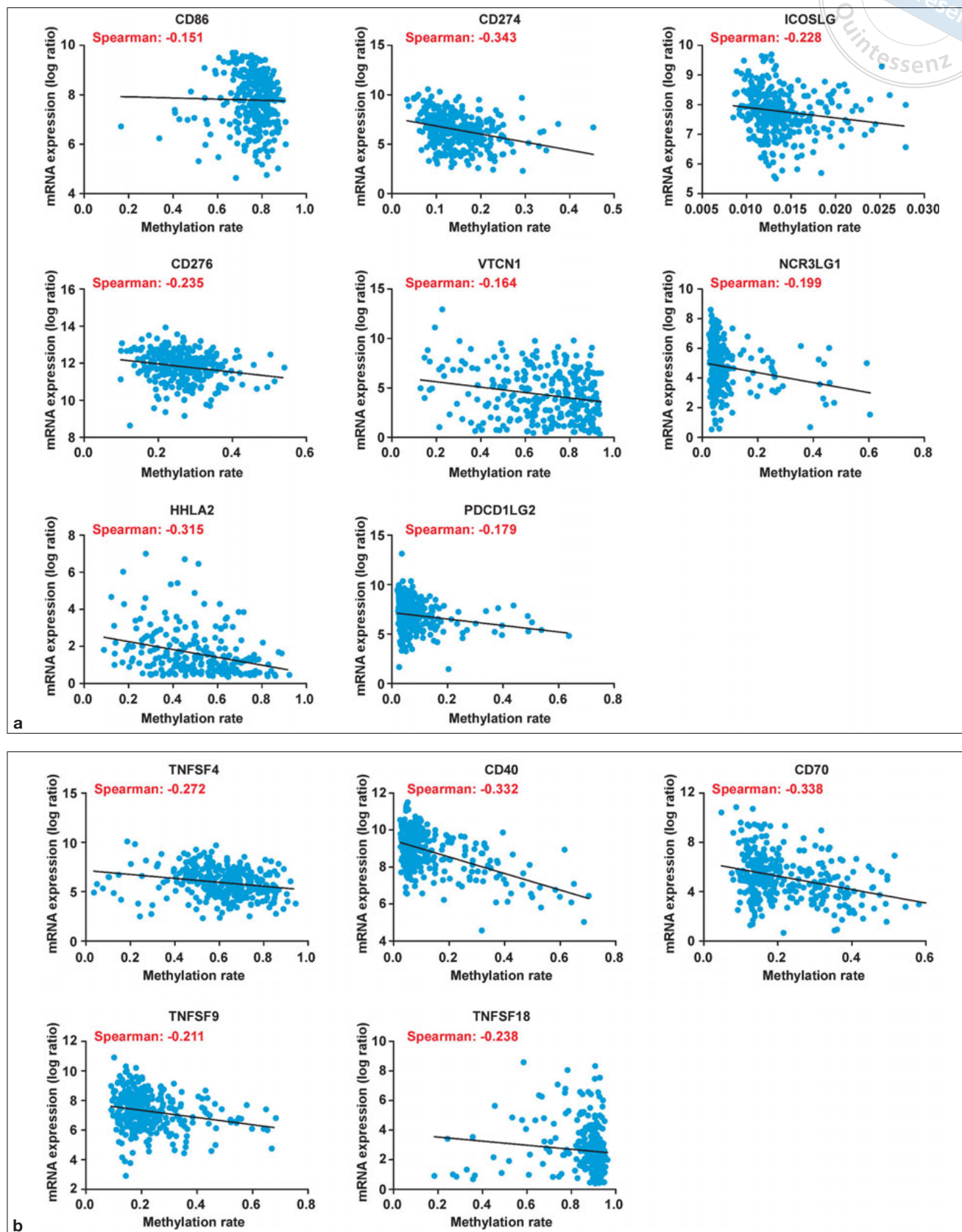


Fig 3 Relationship between mRNA levels and methylation levels of B7 and TNFR genes in OSCC. The mRNA levels of **(a)** B7 genes and **(b)** TNFR genes showed a negative correlation with the methylation levels. Spearman's correlation; $P < 0.05$.

bined with radiochemical therapy is still the preferred strategy at present. However, approximately 140,000 OSCC-related deaths have occurred per year in the past twenty years^{13,14}. There is still a lack of accurate therapeutic targets and classifiers to identify patients at high risk of treatment failure. Given the encouraging results of the immunotherapy clinical trials with immune checkpoint antibodies in human cancers, like PD-L1 inhibitor pembrolizumab (Keytruda), several other drug target coregulators (the B7 and TNFR family members) are being developed and are in clinical trials^{15,16}. Thus, identifying the target immune coregulators is a rational approach to improving OSCC survival. In the present study, we provided an overview of the immune molecular landscapes of the two major costimulatory and coinhibitory pathways in OSCC, including 9 B7 family members and 6 TNFR family members.

The B7 family has been well known for its role in regulating immune homeostasis⁵. The first coinhibitory receptor cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and the first costimulatory receptor CD28 and their shared ligands CD80 (B7-1) and CD86 (B7-2) serve as a paradigm for other coregulatory pathways. CD28-CD80/CD86 interactions deliver costimulatory signals to T cells, while CTLA-4 can outcompete CD28 for CD80/CD86 binding due to its higher affinity receptor than CD28 for CD80/CD86, which results in reduced T-cell activation^{17,18}. Both CD274 (B7-H1, PD-L1) and PDCD1LG2 (B7-DC, PD-L2) are ligands of PD-1. Together, they not only regulate the balance between T-cell activation and tolerance, but also inhibit PI3K activity, reducing Bcl-xL and leading to upregulation of the pro-apoptotic molecule BIM, which ultimately induces, which ultimately induce T-cell exhaustion^{19,20}. The interaction of ICOSLG (B7-H2) and ICOS is reported to be critically involved in TGN1412-mediated T-cell activation. ICOSLG is also an essential costimulatory ligand for CD28 in T cells' primary responses²¹. Inducible expression of CD276 (B7-H3) is demonstrated on T cells and its upregulation is associated with several cancers. CD276 can act as both costimulator and coinhibitor, seemingly depending on its binding partner^{22,23}. VTCN1 (B7-H4) is expressed in several cancers and its upregulation is correlated with poor clinical outcomes. The receptor for VTCN1 is currently unknown, however its putative receptor was induced on activated T cells to suppress their proliferation, cytokine production and cytotoxicity²⁴. HHLA2 (B7-H5, B7-H7) has limited expression in normal tissues, but is expressed at higher levels in multiple forms of cancer. HHLA2 is reported to function as either costimulator via binding to CD28H or coinhibitor

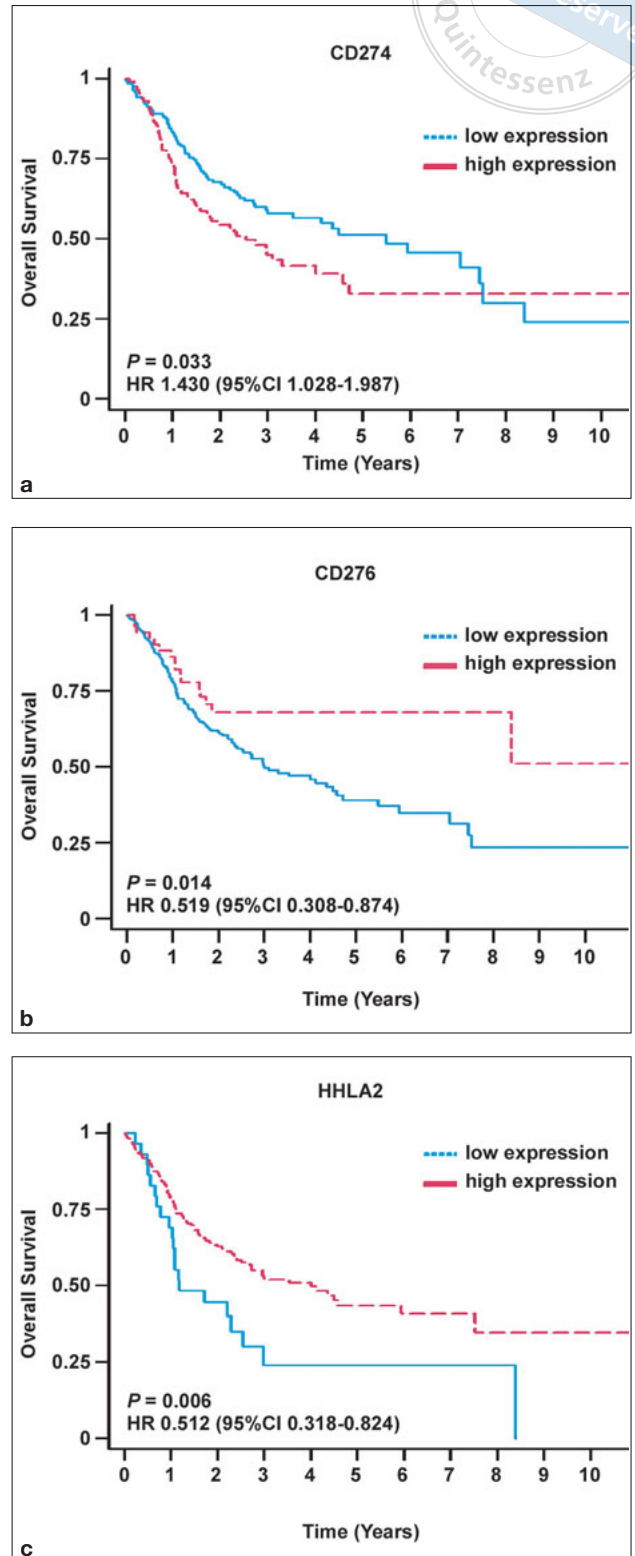


Fig 4 Relationship between mRNA levels of B7 genes and OS in OSCC patients: **(a)** High levels of CD274 are associated with poor OS. High levels of **(b)** CD276 and **(c)** HHLA2 are associated with good OS. Kaplan-Meier survival analysis; P value and HR ratio were calculated by univariate Cox regression analysis.



via the putative ligands²⁵. NCR3LG1 (B7-H6) is not detected in normal human tissues at steady state but is expressed in a broad range of haematopoietic and solid cancers. Nkp30 is identified as a ligand of NCR3LG1, the combination of which could activate naive T cells^{26,27}. In the present study, to identify the promising immune targets for OSCC, we overviewed B7 gene alterations. We found that 5 out of 9 B7 genes (CD80, CD86, ICOSLG, CD276, VTCN1) were abnormally expressed in contrast to their expression in normal tissues. ICOSLG and VTCN1 were downregulated, while CD80, CD86 and CD276 were upregulated in OSCC. Overall, CD274 showed little alteration in OSCC patients, however around 10% of patients showed high PD-L1 expression levels. Patients with high CD274 expression levels showed poor OS, while those with high CD276 or HHLA2 expression levels exhibited the opposite results, indicating that CD274, CD276 and HHLA2 might be promising biomarkers for selecting OSCC patients at high risk of treatment failure.

The subset of the TNFR superfamily can activate diverse cellular functions from the production of type 1 interferons to the regulation of the survival of antigen-activated T cells⁶. TNFSF4 (OX40L) is the monospecific signalling partner of OX40 (TNFRSF4), which can provide costimulatory signals for T-cell activation²⁸. CD40 (TNFRSF5) activation can activate the dendritic cells and turn cold tumours into hot tumours, which plays a critical role in generating T-cell immunity²⁹. The interaction of CD70 (TNFSF7) with its unique receptor CD27 can induce proliferation and cytokine production by T cells and promote cytotoxic T-cell responses³⁰. TNFSF9 acts as a T-cell costimulatory receptor in promoting activated T-cell survival, expansion and enhanced effector function, making it a promising target for tumour immunotherapy³¹. TNFRSF14 (HVEM) can mediate bidirectional signalling and be involved in positive or negative immunological reactions via binding to different receptors, like BTLA, CD160 or LIGHT³². TNFSF18 (GITRL) acts as a costimulatory molecule through interaction with GITR³³. The present study overviewed 6 members of the TNFR family and found that TNFSF4, CD70 and TNFSF9 were upregulated. However, no TNFR gene showed predictive value in OSCC patients' clinical outcomes.

In conclusion, all the B7 and TNFR family members showed alterations in expression at different frequencies in OSCC patients. Importantly, patients with high CD274 levels or low CD276 or HHLA2 levels showed poor clinical outcomes, indicating that CD274, CD276 and HHLA2 may be potential prognosis biomarkers and therapeutic targets for immunotherapy to treat OSCC.

Declaration

The authors reported no conflicts of interest related to this study.

Author contribution

Drs Xian Yue REN and Bin CHENG designed the study and wrote the manuscript; Drs Xi Juan CHEN, Xiao Bing CHENG, Chun Yang WANG, Qin LIU, Xue PAN, Si Yuan ZHANG and Wei Lin ZANG conducted the experiments and analysed the data. All authors read and approved the final manuscript.

(Received Sep 24, 2019; accepted Jan 07, 2020)

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