

Elevated *DKK1* expression is an independent unfavorable prognostic indicator of survival in head and neck squamous cell carcinoma

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Purpose: *DKK1* is an antagonist of the Wnt signaling pathway that has various roles in human physiology. Notably, aberrant *DKK1* expression is observed in several cancers. In this retrospective study, we assessed the association between *DKK1* expression levels and head and neck squamous cell carcinoma (HNSCC) and its prognostic value.

Materials and methods: Using RNA-seq data from HNSCC tumors (N=520) and adjacent normal tissue (N=44) in The Cancer Genome Atlas, we evaluated *DKK1* expression levels. Additionally, we evaluated the association of *DKK1* expression levels and pathophysiological features of patients with HNSCC and the value of *DKK1* expression for prediction of overall survival (OS). We also explored the correlation between *DKK1* expression and methylation of its promoter in HNSCC.

Results: *DKK1* expression was significantly upregulated in HNSCC compared with normal tissues. Moreover, *DKK1* expression was significantly associated with smoking, alcohol abuse, sex, human papillomavirus status, tumor site, tumor invasion, and pathologic stage in HNSCC patients. Kaplan–Meier curves showed that high *DKK1* expression was correlated with inferior OS. In addition, univariate and multivariate analyses showed that elevated *DKK1* expression was an independent prognostic factor for poor OS (HR: 1.85, 95% CI: 1.31–2.62, $P < 0.001$). Regression analysis identified a strong negative correlation between *DKK1* expression and methylation of its promoter.

Conclusion: These findings support the hypothesis that elevated *DKK1* expression is modulated via methylation of its promoter and indicate that *DKK1* expression is a highly informative prognostic biomarker for patients with HNSCC.

Keywords: HNSCC, *DKK1*, overall survival, prognosis, methylation

Introduction

Head and neck cancer is the sixth most common malignancy and fifth leading cause of cancer-related death worldwide, and mainly comprises head and neck cancer squamous cell carcinomas (HNSCCs) arising from the mucosal surfaces of the upper-aerodigestive tract (oral and nasal cavity, oropharynx, hypopharynx, and larynx).¹ According to recent epidemiological data from the American Cancer Society, HNSCC incidence and mortality rates have both increased in recent years.^{2,3} Despite aggressive multimodal treatment approaches, including surgical resection, chemotherapy, targeted therapy, and radiotherapy, the 5-year survival rate of patients with HNSCC is <50%,⁴ because the majority are diagnosed with advanced-stage disease⁵ and there is a high risk of local recurrence and/or distant metastatic treatment failure.⁶ Therefore, identification of potential markers to predict prognosis or

optimize therapy is a matter of clinical urgency for patients with HNSCC.

DKK1, a member of the *DKK* family, is an antagonist of Wnt signaling, which regulates diverse cellular and biological processes, including cell proliferation, migration, and apoptosis, through both β -catenin-dependent and -independent mechanisms, in various tissues and numerous cancers.⁷⁻⁹ Abnormal expression of *DKK1* is observed in numerous tumors and contributes to malignant transformation. In human renal cell carcinoma, *DKK1* acts as a tumor suppressor gene that induces apoptosis and inhibits proliferation.¹⁰ Pang et al¹¹ demonstrated that upregulation of *DKK1* in SBC-3 cells enhanced their proliferation, colony formation, cell migration, and invasion in vitro, as well as bone metastasis in vivo. In prostate cancer, levels of *DKK1* were found to increase during cancer development and were associated with inferior patient survival.¹² A recent study showed that *DKK1* expression is also elevated in the Hep-2 cell line and laryngeal squamous cell carcinoma samples and has potential as an independent unfavorable predictor of overall survival (OS) in patients with this type of cancer;¹³ however, the small sample size limited the statistical power of the study and the association of *DKK1* with HNSCC remains insufficiently investigated.

The Cancer Genome Atlas (TCGA), supervised by the National Cancer Institute's Center for Cancer Genomics and the National Human Genome Research Institute, is an open data-rich resource for biological discovery that has collated information from more than 10,000 patient samples, together with clinicopathological data, across over 30 types of human cancers.¹⁴ Genomic and phenotypic data from 528 HNSCC and normal tissues are recorded in the HNSCC-specific section of the database, TCGA-HNSC. In this retrospective study, using data from TCGA-HNSC, we explored the *DKK1* expression levels in HNSCC, their association with disease clinicopathological characteristics, and their prognostic value.

DNA methylation is a common epigenetic modification, and aberrant promoter methylation at CpG islands in gene promoters leads to transcriptional inactivation, which is critical in cancer initiation, progression, invasion, and metastasis.¹⁵⁻¹⁷ Accumulating evidence demonstrates that transcriptional silencing of *DKK1* by methylation of its promoter is observed in hepatocellular carcinoma,¹⁸ non-small-cell lung cancer,¹⁹ colorectal cancer,²⁰ and oral and oropharyngeal cancer.²¹ In the current study, we also explored the association between *DKK1* expression and methylation of its promoter using Illumina Human Methylation 450K

BeadChip data (Illumina Inc., San Diego, CA, USA) from TCGA-HNSC.

Materials and methods

Available TCGA data

TCGA-HNSCC cohort data (Project Id: TCGA-HNSC) were downloaded from the University of California Santa Cruz Xena browser (<https://xenabrowser.net/>). Gene expression data, measured by RNA-seq, were available for 520 primary HNSCC tumor and 44 adjacent normal tissue samples; 517 primary HNSCC cases had both RNA-seq and OS data recorded. Details of the clinicopathological characteristics of patients, including age at initial pathologic diagnosis, sex, smoking history, alcohol history, histologic grade, tumor site, human papillomavirus (HPV) status, pathologic tumor category, pathologic nodal category, nodal pathologic stage, margin status, recurrence status, and OS data, were downloaded for secondary analysis. DNA methylation profiles (Illumina Human Methylation 450K BeadChip Kit) were also downloaded to explore the potential mechanisms of *DKK1* dysregulation in HNSCC.

Statistical analyses

Statistical analyses were performed using SPSS version 20.0 software (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between groups were conducted using independent Student's *t*-tests and one-way analysis of variance. For dichotomization of *DKK1* expression values, the 517 primary HNSCC patients with integrated OS data were divided into high and low *DKK1* expression groups, according to median gene expression. Survival analyses were conducted by Kaplan–Meier and Cox proportional hazard regression. The average of the β values (methylation ratios) of seven Illumina Human Methylation 450K BeadChip probes (cg25454948, cg27411220, cg04932230, cg11931116, cg09445939, cg07684796, and cg27591349) on the *DKK1* promoter region was calculated and used for subsequent analysis. Correlation between *DKK1* expression and methylation was tested using the Spearman's rank correlation coefficient. *P*-values lower than 0.05 were considered significant.

Results

High *DKK1* expression levels in HNSCC tumor samples

In the current study, *DKK1* RNA-seq data from 520 patients with histologically confirmed HNSCC and 44 adjacent normal tissues were available from TCGA database. Comparison of *DKK1* expression, as determined by RNA-seq,

demonstrated that *DKK1* levels were significantly elevated in HNSCC tissues compared with the adjacent normal samples ($P<0.001$; Figure 1).

Association of *DKK1* expression with clinicopathological parameters

We also explored the association of *DKK1* expression with the clinicopathological parameters in patients with HNSCC (Table 1). Significantly elevated *DKK1* expression levels were found in patients with histories of smoking ($P=0.020$) and alcohol consumption ($P=0.024$) in the TCGA-HNSCC cohort. We also identified a difference in *DKK1* expression between patients who were HPV– and those who were HPV+, with the latter group having significantly lower *DKK1* expression levels ($P<0.001$). Additionally, we found that hypopharynx and larynx cancers presented with significantly higher *DKK1* expression levels compared with oral cavity and oropharynx cancers ($P=0.045$). Advanced tumor (T) category ($P=0.006$) and pathologic stage ($P=0.048$) were also associated with significantly higher *DKK1* expression levels compared with early-stage disease. No differences in *DKK1* expression were observed according to sex, age, histologic grade, nodal (N) category, surgical margin status, or recurrence status.

High *DKK1* expression level as an independent prognostic factor for unfavorable OS

We constructed survival curves to investigate whether *DKK1* expression can act as a prognostic biomarker for HNSCC

(Figure 2). The median OS for both the *DKK1* high and *DKK1* low expression groups were 32.83 and 70.67 months, respectively. Kaplan–Meier analysis and log-rank testing demonstrated that the group with high *DKK1* expression had significantly inferior outcome (log-rank $P<0.001$).

In univariate Cox proportional hazards analysis (Table 2), we found that age ≥ 60 years, female, advanced T, lymphatic metastasis, advanced stage, positive surgical margin, and increased *DKK1* expression were significantly associated with inferior OS of patients with HNSCC. To avoid overfitting of the results, univariate Cox regression analysis was also performed using *DKK1* expression as a continuous variable. The result confirmed that there was a significantly increased risk of death for patients with high *DKK1* expression. Multivariate Cox proportional hazard analysis was conducted including only variables that showed significance in univariate analysis (Table 2). The results confirmed that advanced T, lymphatic metastasis, positive surgical margin, and increased *DKK1* expression were independent prognostic indicators of unfavorable OS for patients with HNSCC.

DKK1 expression was negatively correlated with methylation of its promoter in HNSCC

We examined the association of *DKK1* expression with methylation of its promoter using data from 520 patients with HNSCC from TCGA. Regression analysis revealed a strong negative correlation (Pearson's $r=-0.515$, $P<0.001$ between *DKK1* expression and methylation of its promoter) (Figure 3).

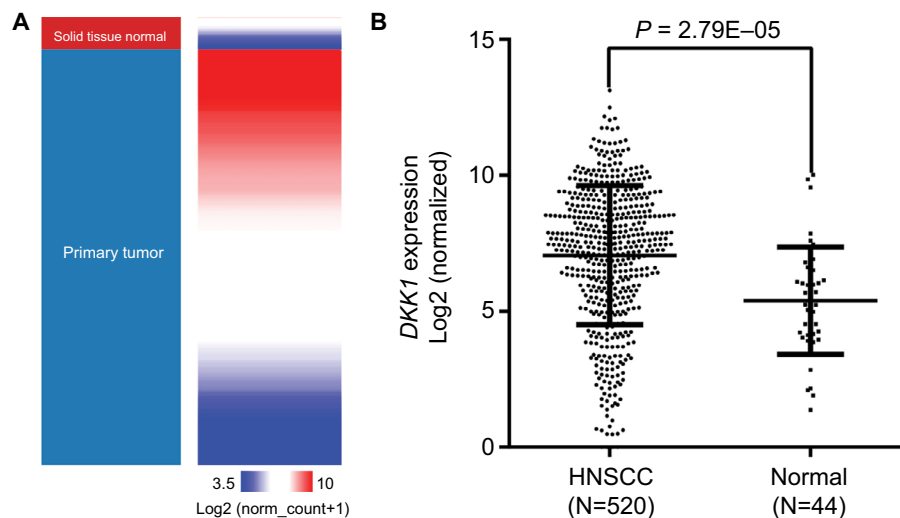


Figure 1 HNSCC tissues have significantly elevated *DKK1* expression levels compared with adjacent normal tissues.

Note: Heatmap (A) and plot (B) showing *DKK1* expression in HNSCC tissue (T=520) and normal tissue (N=44).

Abbreviation: HNSCC, head and neck squamous cell carcinoma.

Table 1 Association of *DKK1* expression with clinicopathological characteristics of patients with HNSCC

Characteristics	N	Mean ± SD	P-value
Sex			
Female	136	7.01±2.55	0.772
Male	384	7.08±2.57	
Age (years)			
<60	233	6.88±2.67	0.137
≥60	286	7.21±2.47	
Smoking behavior			
No	117	6.58±2.67	0.020
Yes	391	7.21±2.50	
Alcohol history			
No	162	6.70±2.61	0.024
Yes	347	7.25±2.51	
Histologic grade			
G1+2	366	7.08±2.42	0.756
G3+4	132	7.16±2.88	
Tumor site			
Oral cavity	385	6.88±2.61	0.045
Oropharynx	9	7.08±1.30	
Hypopharynx	10	8.05±2.06	
Larynx	116	7.57±2.45	
HPV status			
Positive	41	5.12±2.43	3.66×10 ⁻⁵
Negative	73	7.21±2.44	
Pathologic tumor category			
Tis/T1/T2	185	6.78±2.45	0.006
T3/T4	273	7.45±2.59	
Pathologic nodal category			
No	176	7.00±2.46	0.197
Yes	244	7.33±2.60	
Pathologic stage			
I + II	101	6.76±2.42	0.048
III + IV	347	7.33±2.59	
Margin			
No	351	7.16±2.58	0.724
Yes	59	7.29±2.48	
Recurrence status			
No	329	6.93±2.60	0.246
Yes	108	7.27±2.71	

Abbreviations: HNSCC, head and neck squamous cell carcinoma; HPV human papillomavirus.

Discussion

The Wnt signaling pathway is a well-conserved and critical regulatory cascade that contributes to the development and homeostasis of the human body. *DKK1* is a canonical Wnt signaling pathway inhibitor that has various functions in numerous human diseases, particularly cancers.^{22–24} A previous study showed that *DKK1* expression is elevated

in laryngeal squamous cell carcinoma, which is the second most common malignant HNSCC neoplasm;¹³ however, the relationship between *DKK1* and HNSCC remains unclear. In the current study, through characterization of *DKK1* expression based on data from TCGA-HNSC, we confirmed that *DKK1* expression is significantly upregulated in HNSCC.

Subsequently, we also determined the association between *DKK1* expression and the clinicopathological characteristics of patients with HNSCC. The etiologic risk of developing HNSCC is associated with multiple factors, including geographical region, dietary habits, and genetic background, with cigarette smoking and abusive consumption of alcohol representing the most important risk factors for HNSCC development and exhibiting a synergistic effect.²⁵ By analysis of TCGA data, we found that *DKK1* expression was significantly elevated in HNSCC patients with smoking and alcohol abuse, suggesting that smoking and alcohol consumption may be involved in the progression of HNSCC via regulation of *DKK1*. Over the past decade, clear molecular and epidemiologic evidence has emerged supporting a vital role for HPV in a subset of HNSCC tumors, particularly oral and oropharynx squamous cell carcinomas.^{26,27} Also, consistent with prior reports, our analysis showed that *DKK1* expression significantly decreased in the HPV associated tumors in TCGA-HNSCC, which may be due to the fact that integration of HPV into the genome results in altered mRNA transcript abundance and splicing.²⁸ Tumor invasion and TNM stage are vital factors for assessment of prognosis in patients with cancer and are among the most common tools used for that purpose.^{29–31} Our analyses revealed that *DKK1* expression was higher in HNSCC patients with advanced tumor and clinical stage compared with those with early-stage disease, supporting a possible role for *DKK1* in the development of HNSCC.

HNSCC is a common type of cancer with a dismal prognosis, and the rates of local or distant recurrence are up to 30% and 25%, respectively.³² TNM staging remains a vital tool for predicting cancer prognosis;^{33,34} however, the latest edition of the TNM classification is unable to absolutely justify clinical application, due to the heterogeneous molecular mechanisms and clinical behaviors of HNSCC. Consequently, reliable prognostic biomarkers are urgently needed to aid the risk stratification of patients and inform personalized clinical treatment decision-making processes and subsequent surveillance for HNSCC.

DKK1 upregulation has prognostic value in various cancers, including chondrosarcoma,³⁵ bladder cancer,³⁶ pancreatic cancer,³⁷ and laryngeal squamous cell carcinoma.¹³ In this study, using the large TCGA dataset, Kaplan–Meier

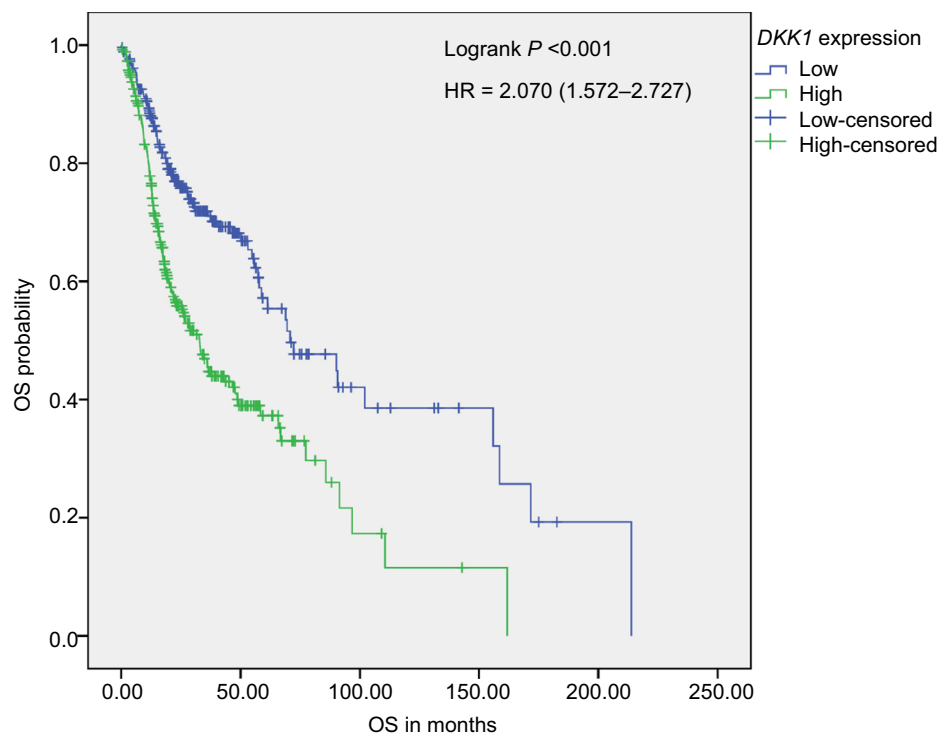


Figure 2 Kaplan–Meier survival analysis.

Notes: Kaplan–Meier analysis of OS in 517 HNSCC patients stratified according to median *DKK1* expression. OS in patients with high *DKK1* expression was significantly reduced compared with that of patients with low *DKK1* expression.

Abbreviations: HNSCC, head and neck squamous cell carcinoma; OS, overall survival.

Table 2 Univariate and multivariate analyses of overall survival in HNSCC patients

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (≥ 60 vs < 60 years)	1.32	1.00–1.73	0.047	1.16	0.831–1.63	0.377
Sex (male vs female)	1.35	1.01–1.80	0.040	1.43	0.979–2.08	0.640
Smoking history (yes vs no)	1.12	0.803–1.57	0.498			
Alcohol history (yes vs no)	0.942	0.709–1.25	0.680			
Histologic grade (G3/4 vs G1/2)	0.867	0.637–1.18	0.419			
Pathologic T (T3/4 vs T1/2)	1.91	1.40–2.61	< 0.001	1.89	1.12–3.19	0.017
Pathologic N (N1/2/3 vs N0)	1.86	1.34–2.58	< 0.001	1.59	1.05–2.42	0.029
Pathologic stage (III/IV vs I/II)	1.75	1.20–2.56	0.004	1.14	0.512–2.55	0.746
Surgical margin (positive vs negative)	1.74	1.22–2.49	0.002	1.71	1.14–2.57	0.010
<i>DKK1</i> expression (continuous)	1.14	1.08–1.21	< 0.001			
<i>DKK1</i> expression (dichotomized)	2.07	1.57–2.73	< 0.001	1.85	1.31–2.62	< 0.001

Abbreviations: HNSCC, head and neck squamous cell carcinoma.

survival analysis demonstrated that patients with high *DKK1* expression had remarkably shorter OS. In addition, our univariate and multivariate analyses showed that elevated *DKK1* expression was an independent prognostic factor for poor OS (HR: 1.85, 95% CI: 1.31–2.62, $P < 0.001$), after adjustment for age, sex, tumor invasion, lymphatic metastasis, pathologic stage, and surgical margin status. Therefore, we infer that

DKK1 expression is a potential biomarker of unfavorable OS in HNSCC patients and that implementation of *DKK1* expression screening into prospective clinical trials would be highly desirable to provide further support for these findings.

Development of HNSCC is a multistep process that includes various molecular alterations during carcinogenesis. Both genetic and epigenetic alterations can influence

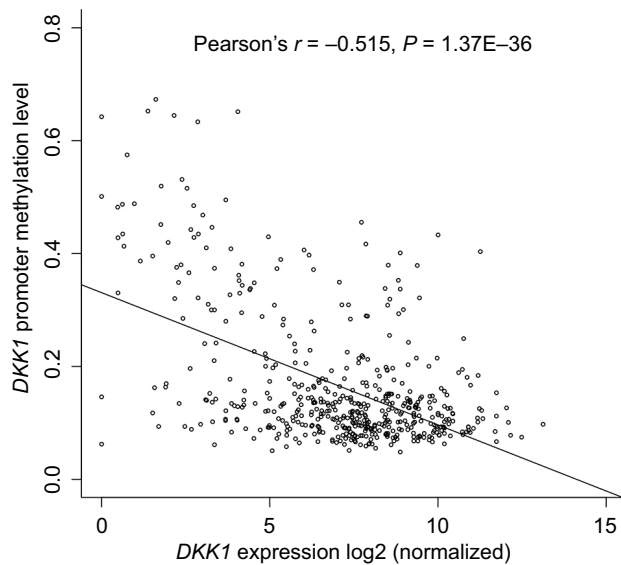


Figure 3 The correlation between *DKK1* expression and DNA methylation of its promoter in HNSCC tissues.

Note: *DKK1* expression was negatively correlated with methylation of its promoter in HNSCC.

Abbreviation: HNSCC, head and neck squamous cell carcinoma.

gene expression and modulate cancer cell behavior. As one of the most important epigenetic alterations, aberrant DNA methylation in the promoter region can directly affect gene transcription in numerous cancers, including HNSCC.^{15,16} Regression analysis confirmed a significant negative correlation between *DKK1* expression and methylation of its promoter, suggesting that DNA methylation may be an important mechanism influencing *DKK1* expression in HNSCC.

Conclusion

In the current study, we took advantage of public data and identified that high *DKK1* expression may be mediated by methylation of its promoter and that this gene is a highly informative prognostic biomarker for patients with HNSCC. However, a well-designed prospective large sample size study and further cell experiments are urgently needed to ascertain our results.

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Disclosure

The authors report no conflicts of interest in this work.

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