



The methylation modification of m6A regulators contributes to the prognosis of head and neck squamous cell carcinoma

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Background: N6-methyladenosine (m6A) regulation is a common type of messenger ribonucleic acid (mRNA) modification, and has been proven to contribute to the malignant behavior of tumors. However, the expression pattern and the prognostic role of m6A RNA methylation regulators in head and neck squamous cell carcinoma (HNSCC) remains unclear.

Methods: We downloaded the data of 422 patients from The Cancer Genome Atlas (TCGA) database. The relationship between the expression level of m6A RNA methylation regulators and clinicopathological variables in HNSCC was analyzed by R language.

Results: The m6A gene alteration was significantly correlated with tumor grade and tumor stage. Next, a least absolute shrinkage and selection operator (LASSO) Cox regression model was used to identify three m6A RNA methylation regulators [i.e., *methyltransferase-like 14 (METTL14)*, *methyltransferase-like 3 (METTL3)*, and *heterogeneous nuclear ribonucleoproteins C1/C2 (HNRNPC)*] to construct a risk signature. Based on the risk signature, the patients were classified into high- and low-risk groups. The overall survival (OS) rate of the low-risk group was significantly higher than that of the high-risk group. Additionally, the risk panel was an independent prognostic marker in HNSCC patients.

Conclusions: The m6A RNA methylation regulators are involved in HNSCC cancer progression. Further and more importantly, the risk signature comprising the three selected m6A RNA methylation regulators could serve as a potential marker to predict HNSCC patient outcomes.

Keywords: N6-methyladenosine (m6A); head and neck squamous cell carcinoma (HNSCC); *heterogeneous nuclear ribonucleoproteins C1/C2 (HNRNPC)*; *Wilms' tumor 1-associating protein (WTAP)*; prognosis

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Introduction

Head and neck cancer, ranked as the 7th most common cancer worldwide in 2018, accounted for 3% of all cancer cases (1). Head and neck squamous cell carcinoma (HNSCC) refers to squamous cell carcinoma arising from mucosal surfaces of the sinonasal cavity, oral cavity, larynx, and

pharynx, but does not include nasopharyngeal cancer (2). Improvements in surgery and radiotherapy techniques and curative multidisciplinary therapies have substantially enhanced patient outcomes (3). However, recurrent or metastatic disease occurred in almost more than 65% HNSCC patients results in dismal prognosis (4). Thus, the introduction of new treatment options targeted at vital new

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regulators of carcinogenesis is urgent.

N6-methyladenosine (m6A) modification, which is defined as the addition of a methyl group at the N6 site of adenosine, is the most prevalent internal chemical modification in eukaryotes (5). Consisting of a group of proteins referred to as the “writer”, “eraser”, and “reader”, m6A methylation can affect multiple aspects of ribonucleic acid (RNA) metabolism, including messenger RNA (mRNA) splicing, stability, localization, and translation (6). The methylation of m6A mRNA is mainly accomplished by “writer” proteins, including *methyltransferase-like 14* (*METTL14*), *Wilms’ tumor 1-associating protein* (*WTAP*) and *methyltransferase-like 3* (*METTL3*) (7). Recently, *RNA-binding motif protein 15* (*RBM15*), *KIAA1429*, and *zinc finger CCCH domain-containing protein 13* (*ZC3H13*) were added to the methyltransferase complex (8). Conversely, the demethylation procedure is mainly conducted by “eraser” proteins, including *AlkB homolog 1* (*ALKBH1*), *AlkB homolog 5* (*ALKBH5*), and the *fat mass and obesity-associated protein* (*FTO*) (9,10). More importantly, the exertion of m6A modification in the degradation and translation of downstream RNA mainly relies on the recruitment of “reader” proteins, including five YTH domain family members (*YTHDF1–3* and *YTHDC1–2*), and heterogeneous nuclear ribonucleoproteins (11,12). The “writer”, “eraser”, and “reader” genes collaboratively control reversible m6A modification, and thus play vital roles in physiological activities and human diseases, especially cancer (13).

More and more evidence suggest that m6A RNA methylation regulators play a vital role in cancer development and prognosis prediction; however, little is known about the relationship between m6A modification and HNSCC. Two previous studies have developed two different two-gene panel to predict the patient outcome in HNSCC. However, the relationship between m6A RNA methylation modulators signature and prognosis of HNSCC still needs further verification. To further investigate the precise m6A regulation pattern in HNSCC, we systematically analyzed the expression of those well studied m6A regulators in HNSCC and studied the correlation between these regulators and the clinicopathological features of patients using patient data from The Cancer Genome Atlas (TCGA) database. More importantly, we constructed a risk signature comprising three regulators rather than different two gene panel to classify the prognosis of HNSCC patients. We present the following article in accordance with the REMARK reporting checklist (available at <https://dx.doi.org/10.21037/>

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Methods

Data collection

The RNA-seq transcriptome data of 422 HNSCC patients and their corresponding clinical and prognosis information were acquired from TCGA database (<https://cancergenome.nih.gov/>). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

m6A RNA methylation regulators selection

Thirteen genes are recognized as vital m6A methylation regulators, including *METTL3*, *METTL14*, *WTAP*, *ALKBH5*, *FTO*, *YTHDC1*, *YTHDC2*, *YTHDF1*, *YTHDF2*, *HNRNPC*, *KIAA1429*, *RBM15*, and *ZC3H13*. However, due to the *KIAA1429* data missing, we could only investigate the relationship among the other 12 m6A-related genes and the clinicopathological characteristics as well as the overall survival (OS) of HNSCC patients based on TCGA dataset.

Bioinformatics analysis

The relationship between the expressions of m6A RNA methylation regulators and clinicopathological variables in HNSCC was analyzed by the Limma package (<http://www.bioconductor.org/packages/release/bioc/html/limma.html>) with a cut-off P value of 0.05. Next, *vioplot* was used to visualize the expression of the 12 regulators in 381 tumor tissues and 41 normal tissues. A spearman analysis was conducted to explore the correlations among these regulator genes. Next, the Consensus Cluster Plus package was used (<https://www.bioconductor.org/packages/release/bioc/html/ConsensusClusterPlus.html>) to divide tumor samples into two groups, and a principal component analysis (PCA) was conducted to verify the grouping results. The analysis of the survival of the two clusters was processed by a survival package. To explore the prognostic role of m6A methylation regulators in HNSCC patients, we performed a univariate Cox analysis and developed a risk signature by employing the least absolute shrinkage and selection operator (LASSO) Cox regression algorithm. Three genes were identified as powerful prognostic factors. The risk score of each patient was calculated using the following formula:

$$\text{Risk score} = \sum_{i=1}^n \text{Coef}_i \times x_i \quad [1]$$

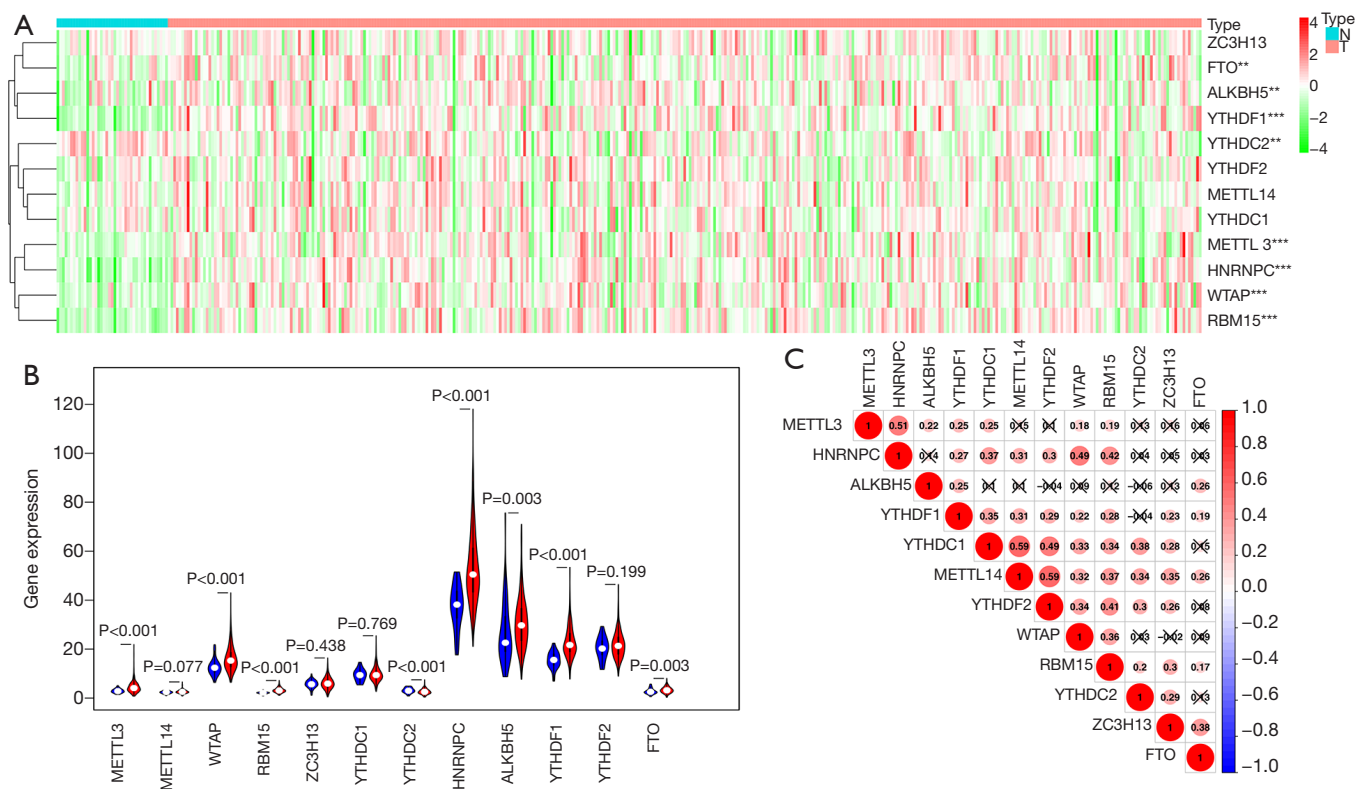


Figure 1 Expression pattern of m6A RNA methylation regulators in HNSCC. (A) Comparison of expression levels of 13 m6A RNA methylation regulators between normal tissues and tumor tissues. N represents normal tissues and T represents tumor tissues. (B) Violin plot visualizing the differentially expressed regulators in HNSCC. The normal tissues were marked blue, and the cancer tissues were marked red. (C) Spearman correlation analysis of 13 m6A RNA methylation regulators in HNSCC. ** represents for P<0.01; *** represents for P<0.001. m6A, N6-methyladenosine; HNSCC, head and neck squamous cell carcinoma.

Coeffi is the coefficient value and x_i is the expression value of each selected gene.

Statistical analysis

The Wilcoxon's test was used to compare the expression level of the 12 m6A RNA methylation regulators between the tumor and normal tissues. A one-way analysis of variance was used to analyze the relationship between the m6A regulators and the clinicopathological features of HNSCC patients. The median risk score was set as the cut-off value to divide patients into the high- or low-risk group. To further analyze the OS difference between the two groups, the Kaplan-Meier method was used. R software (version 3.5.1) was used for all the statistical analyses. P values of less than 0.05 were considered statistically significant.

Results

The expression landscape of m6A RNA methylation regulators in HNSCC

To better understand the role of m6A RNA methylation regulators in carcinogenesis, we first compared the m6A RNA methylation regulators expression between cancer tissues and normal tissues based on the extracted RNA data from TCGA database, and found eight differentially expressed regulators. The heatmap and the violin plot both showed that *FTO* (P<0.01), *ALKBH5* (P<0.01), *YTHDF1* (P<0.001), *METTL3* (P<0.001), *HNRNPC* (P<0.001), *WTAP* (P<0.001), and *RBM15* (P<0.001) were significantly upregulated in cancer tissues (see *Figure 1A,1B*). Additionally, *YTHDC2* expression was significantly lower in cancer tissues than normal tissues (P<0.01; see *Figure 1A,1B*). The analysis of these m6A-related genes revealed that the strongest correlations were between

METTL14 and YTHDC1, and METTL14 and YTHDF2 (see *Figure 1C*).

Relationship between the expression of the m6A RNA methylation regulators and the clinicopathological features of HNSCC patients

To examine the relationship between the m6A RNA methylation regulators and the clinicopathological features of HNSCC patients, we analyzed the clinical significance of these regulators individually. The results showed that the expression of *HNRNPC* ($P < 0.001$), *YTHDC1* ($P < 0.05$), *RBM15* ($P < 0.001$), and *WTAP* ($P < 0.001$) was significantly correlated with grade (see *Figure 2A-2E*). Additionally, the expression of *HNRNPC*, *YTHDC1*, *RBM15*, and *WTAP* were elevated as the grade increased. Further, the expression of *WTAP* and *HNRNPC* were also significantly correlated with grade and tumor (T) stage (see *Figure 2F-2I*). However, *METTL3* expression was negatively correlated with nodes (N) stage ($P < 0.01$; see *Figure 2f*).

Cluster classification based on m6A RNA methylation expression

The Consensus Cluster Plus package was used to group the 381 HNSCC cancer tissues. Based on the cumulative distribution function value, we tried to divide these samples into two or three groups. We found that dividing these samples into two groups ensured the significant difference between groups (see *Figure 3A,3B*). We then used PCA to verify the classification. The results showed that Clusters 1 and 2 gathered together respectively (see *Figure 3C*). To further understand the relationship between clustering and clinical outcomes, we analyzed the OS data of these two clusters. We found that the Cluster 1 subgroup had a higher OS than the Cluster 2 subgroup (see *Figure 3D*).

Prognostic role of m6A RNA methylation regulators in HNSCC

Next, we conducted a Cox univariate analysis to explore the prognostic role of m6A RNA methylation regulators in HNSCC (see *Figure 4A*). Notably, the high expression of *METTL14* resulted in worse survival in HNSCC patients [hazard ratio (HR) = 1.323, 95% confidence interval (CI) = 1.077–1.624]. Among these m6A regulators, *METTL3*, *METTL14*, and *HNRNPC* were selected to build the risk signature to predict prognosis according to the P value from

the previous univariate analysis. To verify the risk signature prediction role, the cancer patients were divided into high- and low-risk groups based on the median score. The OS curve indicated that the high-risk group had a worse survival rate than the low-risk group (see *Figure 4B*). We next used the coefficients of the three regulators obtained from the LASSO regression algorithm to calculate the risk scores of HNSCC patients from TCGA dataset (see *Figure 4C-4E*).

The risk score was closely related to the clinical outcomes of HNSCC patients

We also generated a heat map to investigate the correlation between the risk score and clinicopathological features of HNSCC patients from TCGA datasets. We only found a significant difference between the high- and low-risk group in living status ($P < 0.01$; see *Figure 5*). Further, the high-risk group had a higher proportion of *METTL14* and *HNRNPC* and a lower proportion of *METTL3* than the low-risk group (see *Figure 5*).

Discussion

There is evidence that up to 60% of patients with HNSCC are diagnosed at an advanced stage despite improvements in screening and epidemiology changes. Advanced stage HNSCC is characterized by local invasion, metastases to the regional nodes or even distant metastasis. Thus, advanced stage HNSCC carries a high local recurrence rate and poor prognosis, especially in patients with laryngeal and hypopharyngeal cancer (14). Patients in the same late stage could have different reactions to the same treatment strategy, which could result in distinct outcomes (15). In this case, the tumor, nodes, metastases (TNM) stage alone could not sufficiently predict patient outcomes, or direct personalized targeted therapy. Thus, new gene regulators in tumorigenesis urgently need to be identified to group patients and ensure the optimal selection of treatments.

In recent years, the role of m6A modification in many biological processes such as immune regulation, metabolism, maintenance and differentiation of cell dryness has been proved. In addition, numerous studies have found that m6A modification of RNA also plays an important regulatory role in cancer development by regulating oncoprotein expression, intriguing cell proliferation and tumor progression (16,17). In HNSCC, the regulatory role of m6A modification in cancer pathogenesis has also been proved. METTL3 and METTL 14 mediated m6A

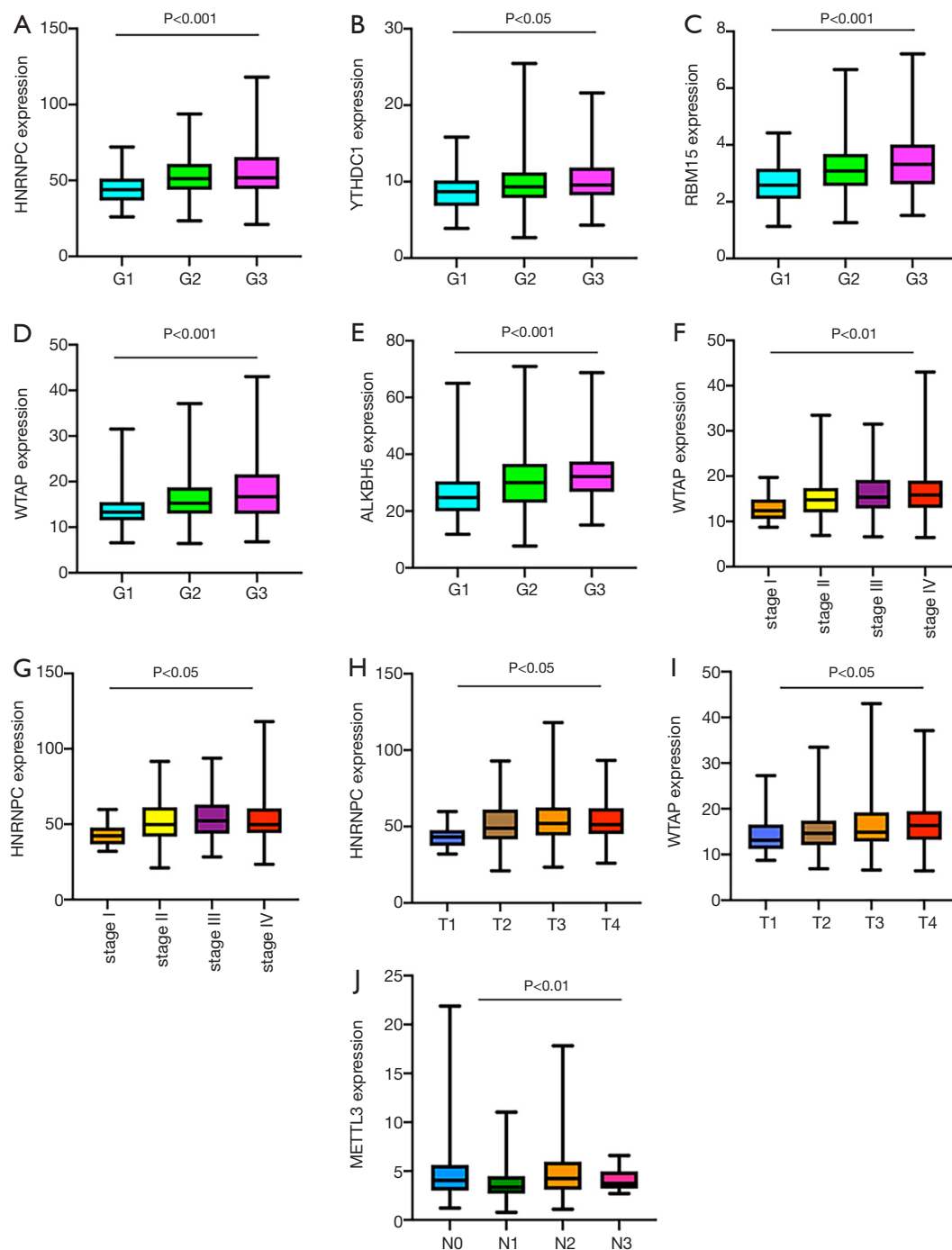


Figure 2 Correlation between m6A RNA methylation regulators and the clinicopathological features of HNSCC patients. (A-E) Relationship between m6A RNA methylation regulators and grade. (F,G) Relationship between m6A RNA methylation regulators and tumor stage. (H,I) Relationship between m6A RNA methylation regulators and T stage. (J) Relationship between METTL3 and N stage. m6A, N6-methyladenosine; HNSCC, head and neck squamous cell carcinoma.

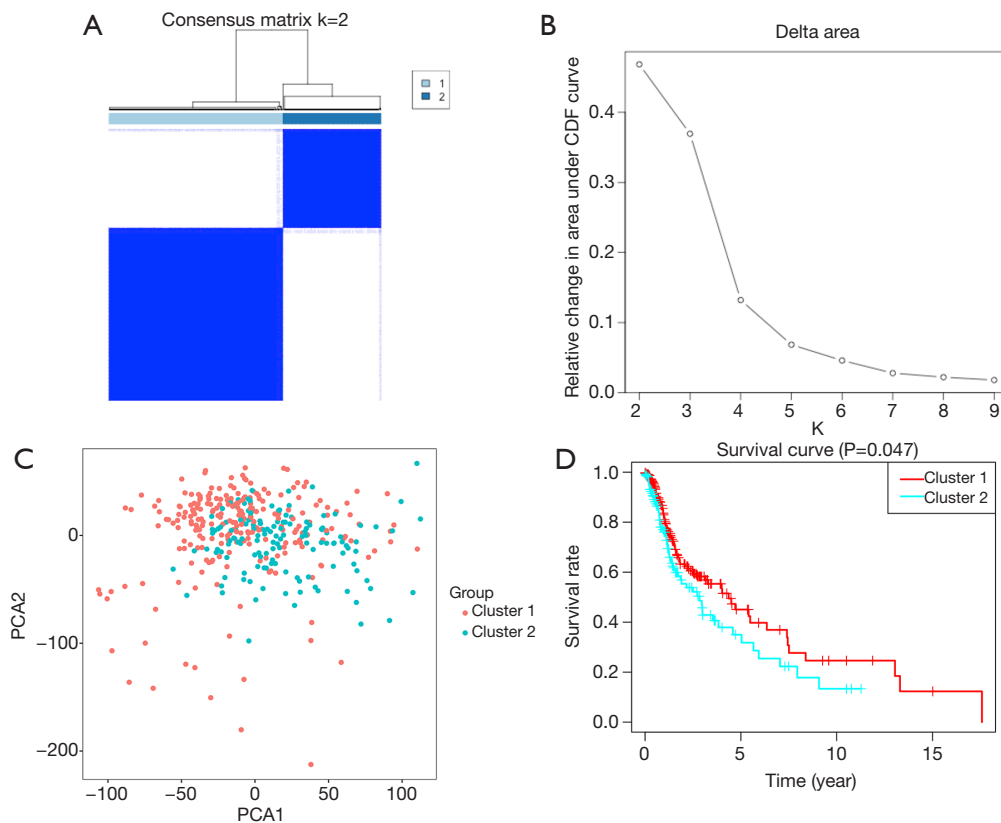


Figure 3 Consensus cluster classification by m6A RNA methylation regulators. (A) Consensus clustering matrix for $k=2$. (B) Relative area change under the cumulative distribution function curve for $k=2$ to 9 . (C) PCA of the total RNA expression profile of two clusters in TCGA database. Clusters 1 and 2 were marked red and blue, respectively. (D) Kaplan-Meier OS curves of HNSCC patients. Clusters 1 and 2 were marked red and blue, respectively. m6A, N6-methyladenosine; PCA, principal component analysis; TCGA, The Cancer Genome Atlas; OS, overall survival; HNSCC, head and neck squamous cell carcinoma.

modification can stabilize lncAROD, which protected YBX1 from proteasomal degradation in HNSCC, thus helping lncAROD to exert its oncogenic role (18). What's more, METTL3 can promote EZH2 expression and NPC progression (19). METTL3 can interact with IGF2BP1, which promotes BMI-1 expression and accelerates OSCC proliferation and metastasis (20). Since HNSCC contains multiple cancer sites and some genes showed contradictory roles in different cancer type, bioinformatics analysis turns to be an effective way to explore the core genes of HNSCC and provide potential target for tumor treatment. In our study, we used TCGA dataset to extract patients' complete data with detailed information. What's more, we went through all data carefully and delete some patients for important data missing to ensure the fidelity of our bioinformatics analysis. We developed a three gene panel as the prognostic characteristic based on their relationship

with tumor grade, clinical stage, T stage, N stage and OS, thus offered three important candidate gene for further investigation.

We first analyzed the different expression pattern between cancer tissues and normal tissues and the relationship among the regulators. The results showed that eight of the 12 regulators (i.e., *ALKBH5*, *FTO*, *YTHDF1*, *YTHDC2*, *METTL3*, *HNRNPC*, *WTAP*, and *RBM15*) were significantly differently expressed in cancer tissues compared to normal tissues. With the exception of *YTHDC2*, all the other seven regulators exhibited higher expression levels in cancer tissues. However, a TCGA data analysis of 508 HNSCC cancer patients, indicated that the *YTHDC2* expression in tumors was higher compared to normal ones. In addition, relatively high *YTHDC2* expression was correlated with poorer survival (21). *YTHDC2* has been recognized as a frequently altered regulator in different

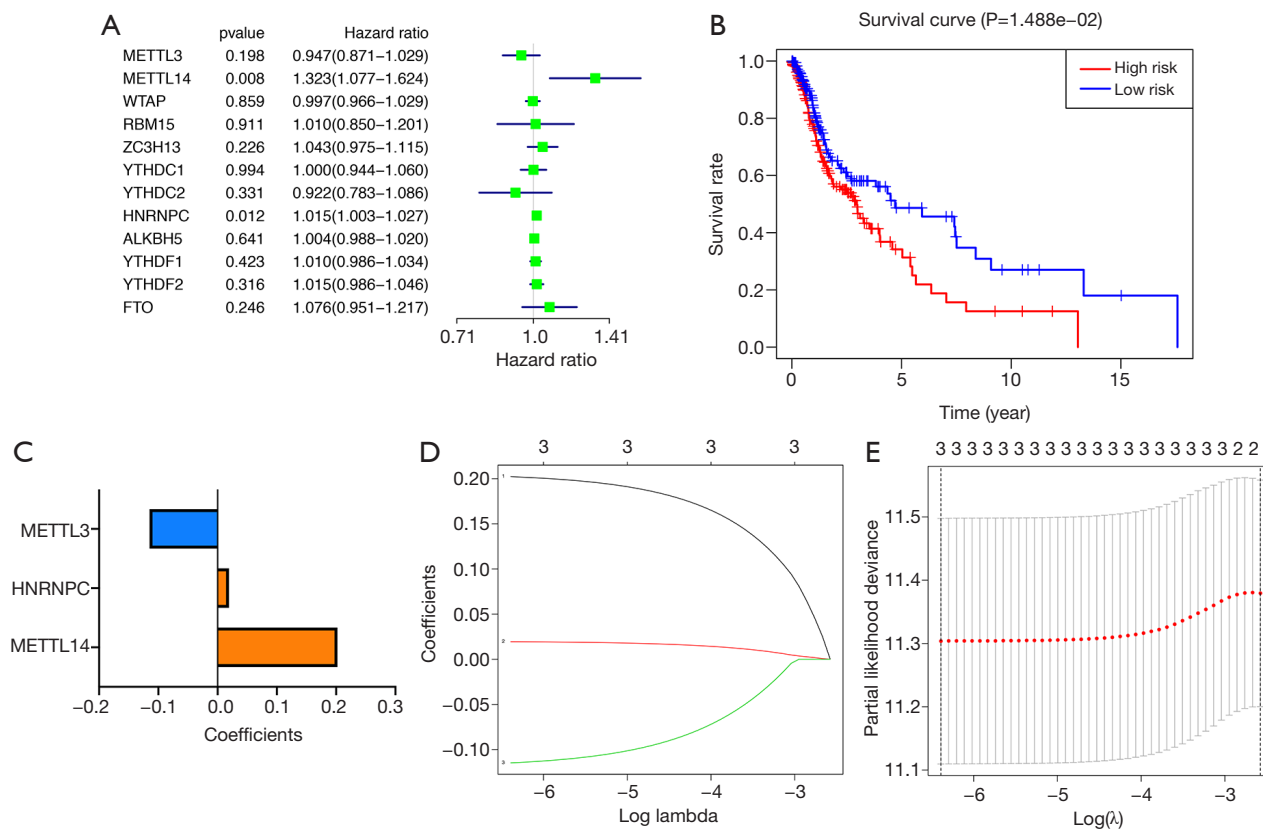


Figure 4 The risk signature comprising three m6A RNA methylation regulators. (A) Univariate Cox analysis of 12 m6A RNA methylation regulators in HNSCC patients. HRs and 95% CIs were calculated. (B) Kaplan-Meier OS curves of HNSCC patients assigned to the high- and low-risk groups. (C-E) The coefficients value of three selected m6A RNA methylation regulators. m6A, N6-methyladenosine; HNSCC, head and neck squamous cell carcinoma; HR, hazard ratio; CI, confidence interval; OS, overall survival.

cancer types, and is mainly involved in carcinogenesis by increasing hypoxia-inducible factor-1 α translation (22). These controversial results may be attributable to the heterogeneity of cancers arising from different sites.

We also found that some of the m6A RNA methylation regulators were significantly associated with different clinicopathological features in HNSCC. HNRNPC is a RNA-binding protein responsible for pre-mRNA processing that has been reported to promote chemoresistance in gastric cancer and facilitate colorectal cancer progression (23,24). Our results showed that HNRNPC was not only highly expressed in cancer tissues, but was also significantly associated with tumor grade, tumor clinical stage, and T stage. Consistent with HNRNPC oncogenic function, higher expression of HNRNPC showed in late stage. WTAP is recognized as a m6A writer that contributes to the *METTL3*-*METTL14* methyltransferase localization to the nuclear and executes translation and post-translation

regulations (25,26). It can facilitate metastasis in pancreatic cancer and promote the progression of hepatocellular carcinoma (HCC) (27,28). In renal cell carcinoma, *WTAP* has also been shown to play an oncogenic role (29). In HNSCC, we observed that *WTAP* expression was correlated with tumor grade, tumor clinical stage, and tumor T stage, and mainly functioned as a tumor promoter.

To examine the prognostic value of the m6A RNA methylation regulators, a Cox univariate analysis and LASSO regression analysis were undertaken and three regulators were selected to construct a risk signature. Next, we classified the patients into high- or low-risk groups according to their risk scores. The OS curve verified that the risk signature could help distinguish the patients' outcomes. The heatmap showed that *METTL14* tended to have a higher level of expression in the high-risk group than the low-risk group. However, *METTL3* in the high-risk group exhibited a lower level expression pattern. *METTL3*

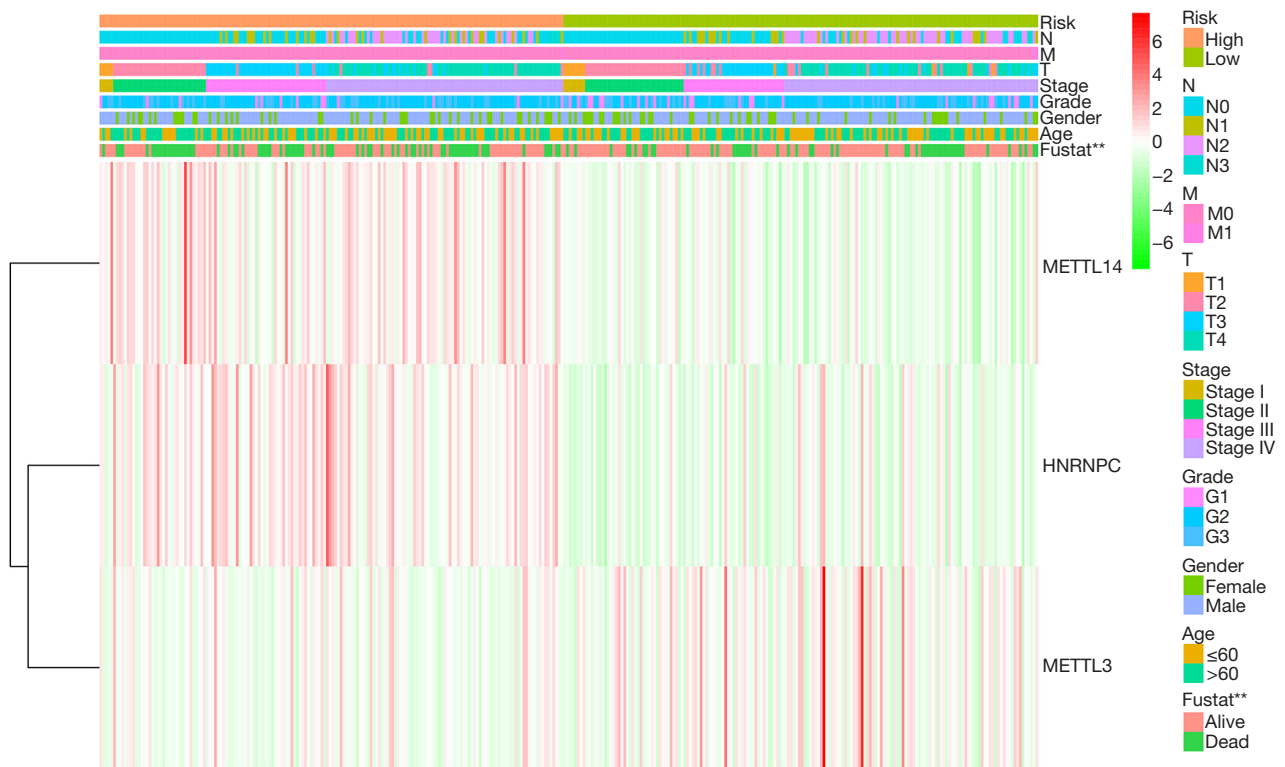


Figure 5 A heatmap of the expression of three m6A RNA methylation regulators and the distribution of the clinical pathological features between the low- and high- risk groups. ** represents for $P < 0.01$. m6A, N6-methyladenosine.

is the main component of the “writer” complex that has been proven to be closely related to cancer progression. *METTL3* has been reported to exhibit significantly higher levels of expression in HCC compared to normal tissues. Moreover, it is critical to epithelial mesenchymal transition in HCC (30). In gastric cancer, *METTL3* has also been shown to have a higher expression pattern in cancer tissues than normal tissues, and to be a poor prognostic indicator in gastric cancer patients (31). Conversely, of all the m6A gene regulators we analyzed, *METTL3* was the sole 1 associated with lymph node stage, and was also negatively correlated to N stage, which is inconsistent with the oncogene role it has been verified to have in other cancer types. The favorable prognostic role of *METTL3* in HNSCC needs to be verified in cancer tissues and other public databases.

More importantly, the landmark discovery of the role of immune check point in immune evasion of cancer cells brought immunotherapy era to the cancer treatment. The OS benefit from immunotherapy (i.e., pembrolizumab, nivolumab, camrelizumab) has been proved in multiple phase II to III clinical trial especially in recurrent or metastatic HNSCC. To better understand the tumor

response to immune therapy, tumor microenvironment is the key part to draw the whole immune map. Tumor microenvironment is formed from dynamic changes involving multiple immunosuppressive signal pathways and values a lot in predicting patient prognosis and therapeutic response (32). Previous study indicated that lower level of *METTL3* and *METTL14* contributes to the T cells differentiation (33). What’s more, CD8+ T cells and NK cells showed increased level in YTHDF1-deficient mouse tumors (34). In HNSCC, tumor infiltrating lymphocytes related immune score has been proved to potentially predict the treatment efficacy. Yi *et al.* demonstrated that a risk score consisted of seven m6A genes was positively correlated with dendritic cells, neutrophils infiltration and negatively correlated with CD4+ T, CD8+ T and B cells infiltration. They also suggested that up-regulated m6A modulators was positively correlated with PD-L1 in HNSCC tumor immune microenvironment, which indicated m6A modulators as potential immunotherapy target in HNSCC (35). Our study provided three potential target genes for further immunotherapy investigation for HNSCC treatment.

Conclusions

Our study showed that there was a close relationship between the expression of m6A RNA methylation regulators and the clinicopathological features of HNSCC patients. Additionally, we constructed a risk signature comprising three regulators that could serve as the prognostic predictor in HNSCC. Our results provide vital evidence that can be used to further predict cancer prognosis and tumor response to cancer treatment of HNSCC.

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Footnote

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