Original Article
m6A RNA methylation regulators contribute to malignant development and have a clinical prognostic effect on cervical cancer

Xiaoqing Ma[1], Yan Li[2], Ji Wen[2], Yu Zhao[2]

[1]Department of Obstetrics and Gynecology, Fengrun District People's Hospital, No. 456 Caoxueqin West Road, Fengrun District, Tangshan 064000, Hebei Province, China; [2]Reproductive Medical Center, The Second Hospital Affiliated to Shandong University of Traditional Chinese Medicine, Ji'nan 250001, Shandong Province, China

Abstract: N6-methyladenosine (m6A) RNA methylation, which is related to the occurrence and development of cancer, is dynamically modulated by m6A RNA methylation regulators ("writers", "erasers" and "readers"). In this paper, we demonstrated that most of the 13 major m6A RNA methylation regulators were differently expressed in 306 cervical cancer tissues stratified according to different clinicopathological characteristics. We applied consensus clustering technique to analyze m6A RNA methylation regulators and identified two subgroups of cervical cancer, named RM1/2. Compared with the RM1, the RM2 had a poorer prognosis and lower overall survival (OS). This result suggested that m6A RNA methylation regulators were closely related to cervical cancer. Based on this result, we used m6A RNA methylation regulators to derive a risk marker that not only is an independent prognostic marker but also can predict the clinicopathological characteristics of cervical cancer. In conclusion, m6A RNA methylation regulator is a key player in the malignant progression of cervical cancer and has potential role in the stratification of prognosis and the formulation of treatment strategies.

Keywords: m6A, RNA methylation, cervical cancer, prognosis

Introduction

More than 150 RNA modifications have been identified, post-transcriptional regulation contained, which are widely distributed in various types of RNA, such as messenger RNA (mRNA), transport RNA (tRNA), ribosomal RNA (rRNA), non-coding small RNA (sncRNA) and long noncoding RNA (lncRNA).

RNA methylation accounts for more than 60% of all RNA modifications, and m6A methylation is the earliest and most common type of RNA methylation on mRNA of higher organisms. m6A methylation is a rich methylation modification of mRNA transfer, which mainly exists in the CDS region and 3'UTR region of mRNA, affecting its stability, translation efficiency, variable shear and localization. This process is in need of the participation of three types of molecules: methylase (METTL3 and METTL14), demethylase (FTO and ALKBH5), and methyla-

tion recognition enzyme (YTHDF1/YTHDF3) [4-6]. This dynamic reversible modification plays a vital role in RNA metabolism and function. Hence, the discovery of m6A RNA methylation regulators has greatly improved our understanding of the functions and mechanisms of m6A modification in the post-transcriptional regulation of gene expression.

There is increasing evidence presenting that genetic changes and expression disorders of m6A RNA methylation regulators are closely related to malignant progression of multiple cancers [7-9]. A study found that compared with the adjacent normal tissues, the level of m6A in the cervical cancer significantly decreased, and the reduction was significantly associated with FIGO staging, tumor size, differentiation, lymphatic invasion, and cancer recurrence. By manipulating the expression of m6A modulators (METTL3, METTL14, FTO and ALKBH5) to reduce m6A levels, the proliferation of cervical

cancer cells could be promoted, and increased m6A levels significantly inhibited tumor development in vitro and in vivo [10]. Other studies also have found that obesity-related protein (FTO) mediates the expression of β-catenin by lessening m6A levels in mRNA transcripts in cervical squamous cell carcinoma tissues, thus enhancing resistance to chemoradiotherapy in vivo and in vitro, and thereby increasing the activity of excision and repair of cross-complementary group 1 (ERCC1) [11]. However, there is still a lack of a comprehensive analysis of the expression, functions and prognostic value of 13 widely reported m6A RNA methylation regulators in cervical cancer with different clinicopathological characteristics.

In this study, we systematically analyzed the expression of 13 m6A RNA methylation regulators mentioned above in 306 cervical cancer tissues using RNA sequencing data from the cancer genome atlas (TCGA). We provided the expression data of each m6A modified regulator according to different clinicopathological characteristics. The findings revealed that the expression of m6A RNA methylation modulators plays a considerable role in the malignant progression of cervical cancer and we designed four markers of selective m6A RNA methylation regulators to stratify the prognosis of cervical cancer.

Materials and methods

Database

The transcriptome data and the corresponding clinical pathological information of 306 samples of cervical cancer and 3 normal samples were obtained from TCGA (https://portal.gdc.cancer.gov/).

Selection of m6A RNA methylation modulators

The list of 16 m6A RNA methylation regulators was sorted from the published literature [1-3], which was restricted to the genes with available expression data in TCGA database. Finally, 13 m6A RNA methylation regulators were obtained. Then, we systematically compared their expression in cervical cancer with different clinicopathological characteristics.

Bioinformatic analysis

To investigate the function of m6A RNA methylation regulators in cervical cancer, we divided cervical cancer into different groups (consistent clustering) using "ConsensusClusterPlus" (iteration cycles: 50%, Item resampling rate: 80%, gene resampling rate: 100% and Pearson correlation, http://www.bioconductor.org/). R package (V3.6.1) was used to study gene expression patterns in different cervical cancer groups.

To determine the prognostic value of m6A RNA methylation regulators, univariate Cox regression analysis was performed for the expression of m6A RNA methylation regulators in TCGA database. Four identified genes significantly associated with survival (P<0.3) were selected for further functional analysis and exploration of potential risk characteristics with LASSO Cox regression algorithm. Finally, the four genes and their coefficients were determined by the minimum criteria, and the optimal penalty parameter λ associated with the smallest ten-fold cross-validation in the database was selected. Calculate the risk score of the signature using the following formula: Risk score = $\sum$ Coef i * xi, Where Coefi is the coefficient, xi is the relative expression value of z-score conversion of each selected gene. The formula was used to validate the risk score for each patient in the TCGA database.

Statistical analysis

Wilcoxon test (non-parametric test) was used for the expression differences between normal and tumor tissues of cervical cancer. Chi-square test was used for comparison of clinicopathological features between RM1 and RM2.

The Kaplan-Meier method and bilateral log-rank test were used to compare OS between RM1 and RM2 groups or the high- and low-risk groups.

Univariate Cox regression analysis was utilized to evaluate the relationship between the expression levels of 13 m6A RNA methylation regulatory genes and OS. In addition, we applied univariate and multivariate Cox regression analysis to determine the prognostic value of risk score and m6A RNA methylation regulatory genes.

We used Strawberry Perl 5.30.1.1 (https://www.perl.org), R v3.6.2 (https://www.rproject.org/), and SPSS v26.0 (IBM Corp., Armonk, NY, USA) to conduct data conversion, statistical analysis, and calculations.

Results

The expression of m6A RNA methylation regulators between normal and cervical cancer patients was significantly different

The expression level of each m6A RNA methylation modulator in normal and cervical cancer patients was shown in the heat map (**Figure 1A**), and the results showed significant differences in FTO (P<0.05), METTL3 (P<0.05), YTHDF2 (P<0.01) and RBM15 (P<0.01) between normal and cervical cancer patients. Quantitative analysis in **Figure 1B** documented that in cervical cancer patients, the expression of METTL3, YTHDF2 and RBM15 increased, while the expression of FTO decreased.

To better understand the interactions of 13 m6A RNA methylation regulators in cervical cancer samples, we further analyzed the correlations among these regulators. The significantly positive correlations between METTL14 and YTHDC1, METTL3 and HNRNPC, ZC3H13 and METTL14, as well as ZC3H13 and YTHDC1 were observed (**Figure 1C**).

Consensus clustering of m6A RNA methylation regulators identified two types of cervical cancer with different clinical outcomes and clinicopathological characteristics

According to the expression similarity of m6A RNA methylation regulators, the clustering stability increased from 2 to 10, and k=4 seemed to be an appropriate choice (**Figure 2A** and **2B**). However, we noted that 214 of the 306 cervical cancer samples clustered in one subgroup and had a high intergroup correlation (**Figure 2C**). Therefore, we chose these two subgroups clustered by k=2 for subsequent analysis, namely RM1 and RM2 groups. Subsequently, we plotted the curve of overall survival (OS) rate for cervical cancer patients, finding that the OS of the RM2 group was significantly shorter than that of the RM1 group (**Figure 2D**). As exhibited in **Figure 2E**, a heat map disclosed the relationship between RM1/2 and clinicopathological features, showing significant differences in the clinicopathological features of RM1/2.

Prognostic value of m6A RNA methylation regulators and risk characteristics established using four selected m6A RNA methylation regulators

We attempted to investigate the prognostic role of m6A RNA methylation regulators in cervical cancer. We conducted univariate Cox regression analysis of m6A expression in cervical cancer samples (**Figure 3A**). The results presented that four modulators were related to OS (P<0.3). Among the four genes, HNRNPC, KIAA1429 and ZC3H13 were the risk genes (HR>1), while YTHDF1 was the protective gene (HR<1). To better predict the clinical outcome of cervical cancer with m6A RNA methylation regulators, we applied the LASSO Cox regression algorithm to dissect the four genes associated with prognosis (**Figure 3A**). It was found that all four genes met the minimum criteria to construct risk characteristics, risk scores of which were calculated using the coefficients obtained from the LASSO algorithm. In order to investigate the prognostic effects of their risk signals, cervical cancer patients in the TCGA (n=306) database were divided into low-risk and high-risk groups based on the median risk score. There was a significant difference in the OS curve between the two groups (P=0.00112; **Figure 3B**).

The prognostic risk score was closely related with the clinicopathological features of cervical cancer

Another heat map displayed the expression patterns of the above four regulators in high-risk and low-risk groups in the TCGA database (**Figure 4A**). A significant difference in N (regional lymph node involvement) between the high-risk and low-risk groups was observed (P<0.05). The ROC curve illustrated that the risk score could predict RM1/2 subgroups of cervical cancer (AUC=0.687, **Figure 4B**). Univariate and multivariate Cox regression analyses were then carried out to determine whether risk characteristics were independent prognostic indicators. The results of univariate analysis indicated that risk score (P<0.05) and regional lymph node involvement (P<0.01) were significantly correlated with OS. When these factors were considered in the multivariate Cox regression analysis, risk score and regional lymph node involvement remained significantly correlated with OS (P<0.01, **Figure 4C**). These results confirmed that risk scores derived from m6A RNA methylation regulators independently predicted the prognosis of patients with cervical cancer.

Discussion

Cervical cancer is one of the most common malignant tumors of the reproductive system in
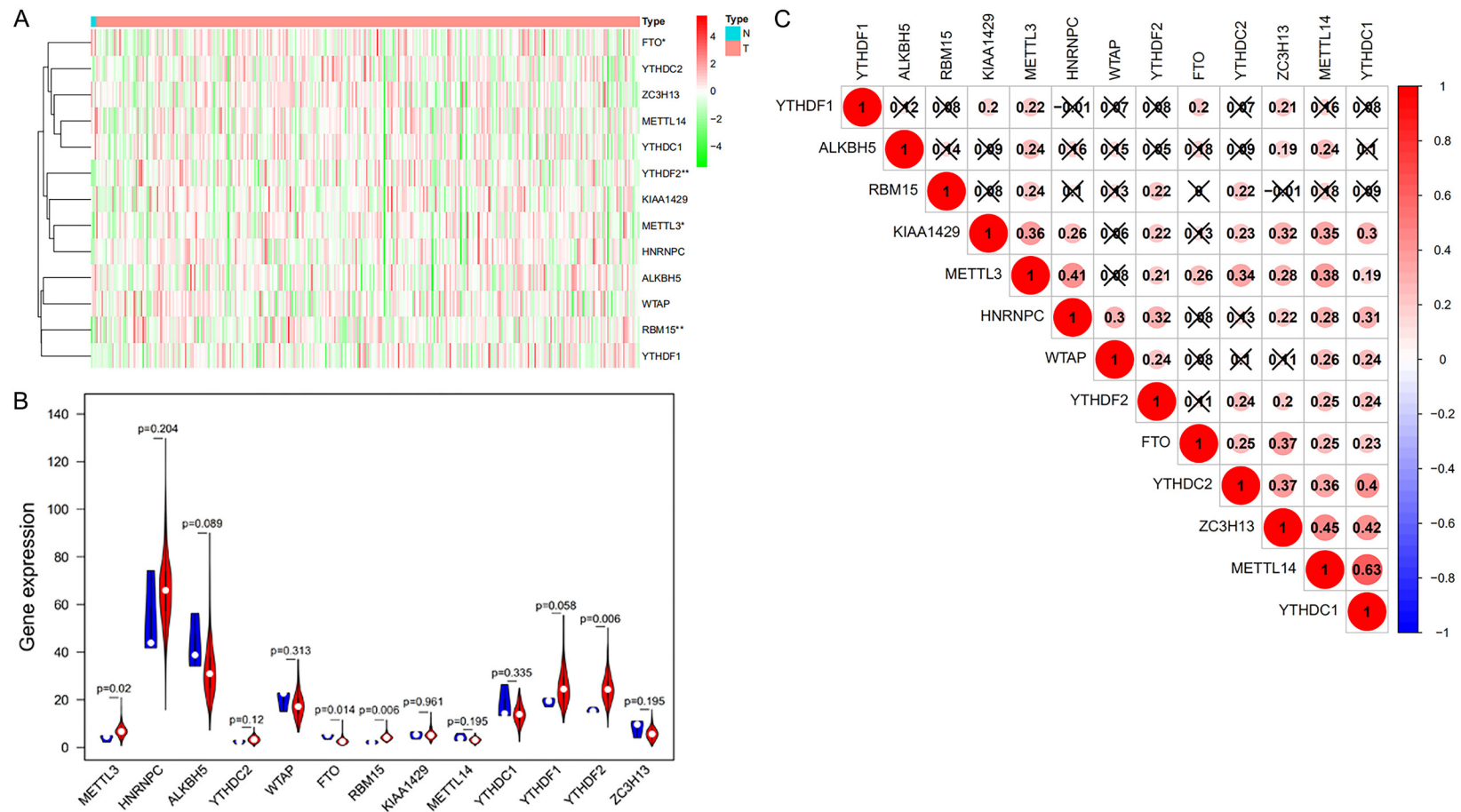
Figure 1. The expression of m6A RNA methylation regulators between normal and cervical cancer patients are significantly different. A. The expression of each m6A RNA methylation regulator such as METTL3, YTHDF2, RBM15 and FTO in both normal and cervical cancer patients. B. Quantitative analysis of m6A RNA methylation regulators in cervical cancer. C. Spearman's correlation analysis was performed to analyze the correlation between m6A RNA methylation regulators. (A fork indicated that the correlation between two regulators did not accord with P<0.05).
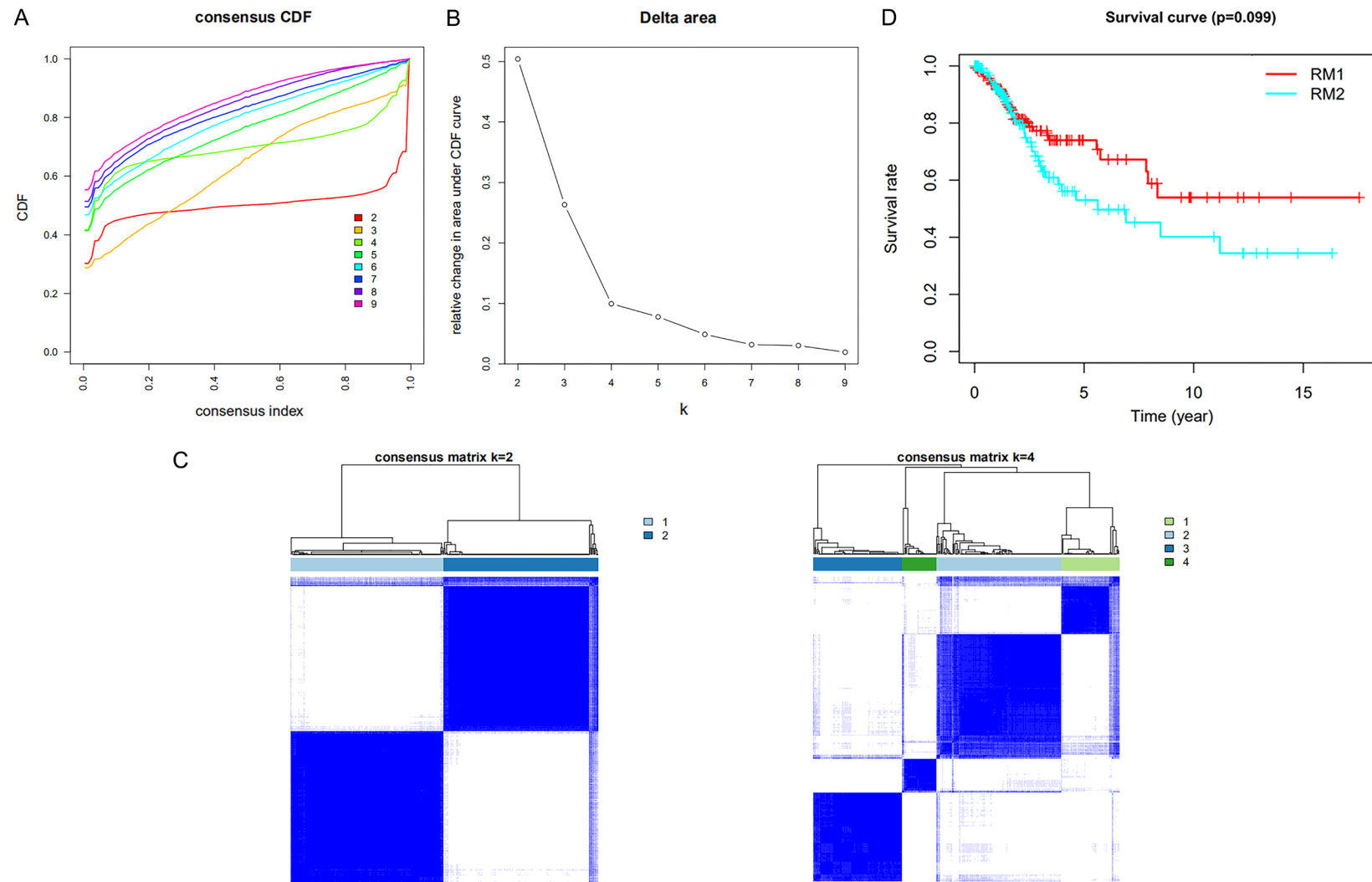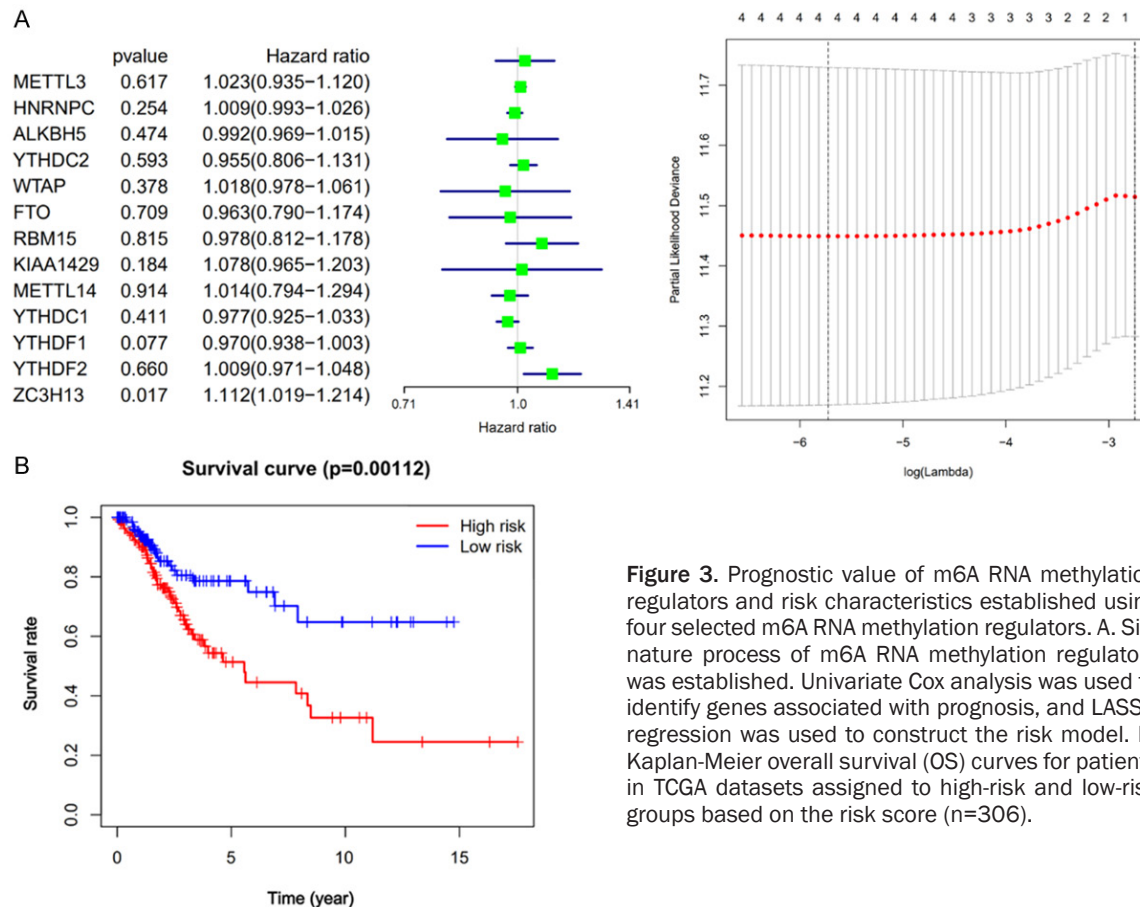
Role of m6A RNA methylation regulators in cervical cancer
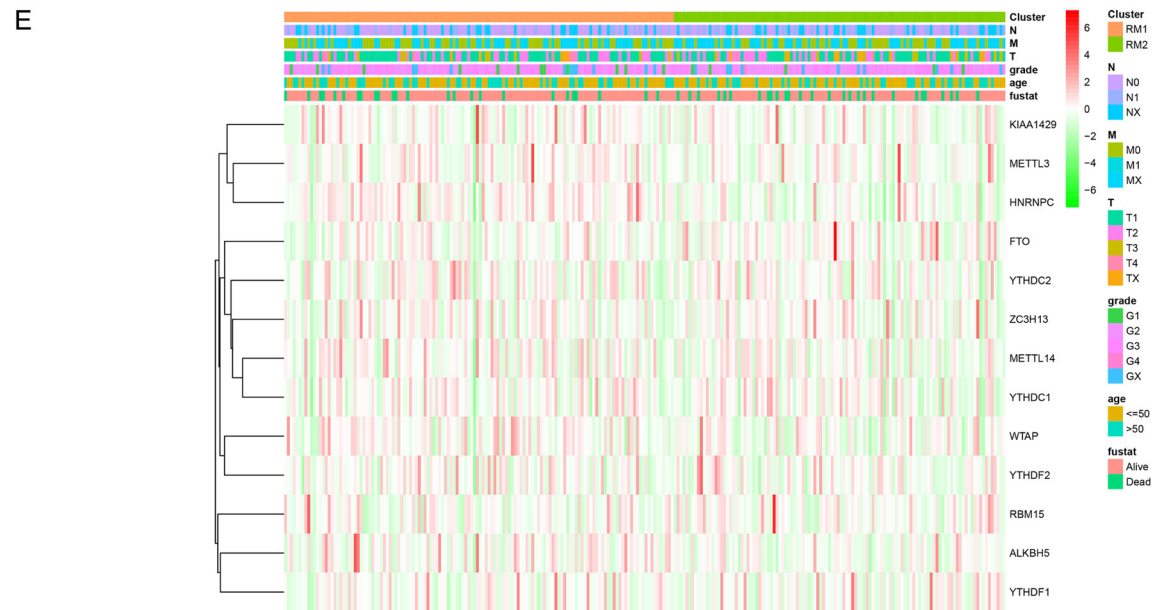
Figure 2. Consensus clustering of m6A RNA methylation regulators identified two types of cervical cancer with different clinical outcomes and clinicopathological characteristics. A. Consensus clustering cumulative distribution function (CDF) for k=2 to 10. B. Relative change in area under CDF curve for k from 2 to 10. C. K=2 and k=4 consensus clustering matrix. D. Kaplan-Meier overall survival (OS) in patients with cervical cancer. E. Heatmap and clinicopathologic features of the two clusters (RM1/2) defined by the m6A RNA methylation regulators consensus expression.

A

	pvalue	Hazard ratio
METTL3	0.617	1.023(0.935-1.120)
HNRNPC	0.254	1.009(0.993-1.026)
ALKBH5	0.474	0.992(0.969-1.015)
YTHDC2	0.593	0.955(0.806-1.131)
WTAP	0.378	1.018(0.978-1.061)
FTO	0.709	0.963(0.790-1.174)
RBM15	0.815	0.978(0.812-1.178)
KIAA1429	0.184	1.078(0.965-1.203)
METTL14	0.914	1.014(0.794-1.294)
YTHDC1	0.411	0.977(0.925-1.033)
YTHDF1	0.077	0.970(0.938-1.003)
YTHDF2	0.660	1.009(0.971-1.048)
ZC3H13	0.017	1.112(1.019-1.214)

B

Survival curve (p=0.00112)

Figure 3. Prognostic value of m6A RNA methylation regulators and risk characteristics established using four selected m6A RNA methylation regulators. A. Signature process of m6A RNA methylation regulators was established. Univariate Cox analysis was used to identify genes associated with prognosis, and LASSO regression was used to construct the risk model. B. Kaplan-Meier overall survival (OS) curves for patients in TCGA datasets assigned to high-risk and low-risk groups based on the risk score (n=306).

women, which seriously threatens women's physical and mental health. The development of cervical cancer is closely associated with epigenetic modification, such as DNA methylation, histone modification and RNA editing [12].

m6A is a common internal trim of RNA molecules, and proteins modified by m6A function in a variety of cancers. The modification level of transcript m6A is dynamically regulated by methyltransferase (encoder), binding protein (reader), and demethylase (decoder). The discovery of m6A RNA methylation regulators has greatly improved our understanding of the functions and mechanisms of m6A modification in the post-transcriptional gene regulation [13].

m6A methylation regulators may have similar effects on different types of cancers and also play different roles in similar types of cancers [14, 15]. Although the roles of m6A methylation regulators in cervical cancer has been widely studied, there is neither a comprehensive analysis of the expression of the 13 common m6A

methylation regulators in cervical cancer with different clinicopathological characteristics, nor a study on their functions in the malignant progression of cervical cancer and their prognostic values. In this paper, we discovered that the expression of m6A RNA methylation regulators was closely associated with the prognosis of cervical cancer. This is the first comprehensive study to investigate the characterization and prognostic effects of 13 m6A methylation regulators in cervical cancer. In our study, it was proved that the expression levels of m6A methylation regulators were closely related to the malignancy and prognosis of cervical cancer. Based on the expression of m6A RNA methylation regulators, two cervical cancer subtypes were divided by consensus clustering. These subtypes not only affected the clinico-pathological features of patients, but also were closely related to the prognosis. In addition, four m6A RNA methylation regulators were selected to obtain prognostic risk characteristics for classifying cervical cancer patients into
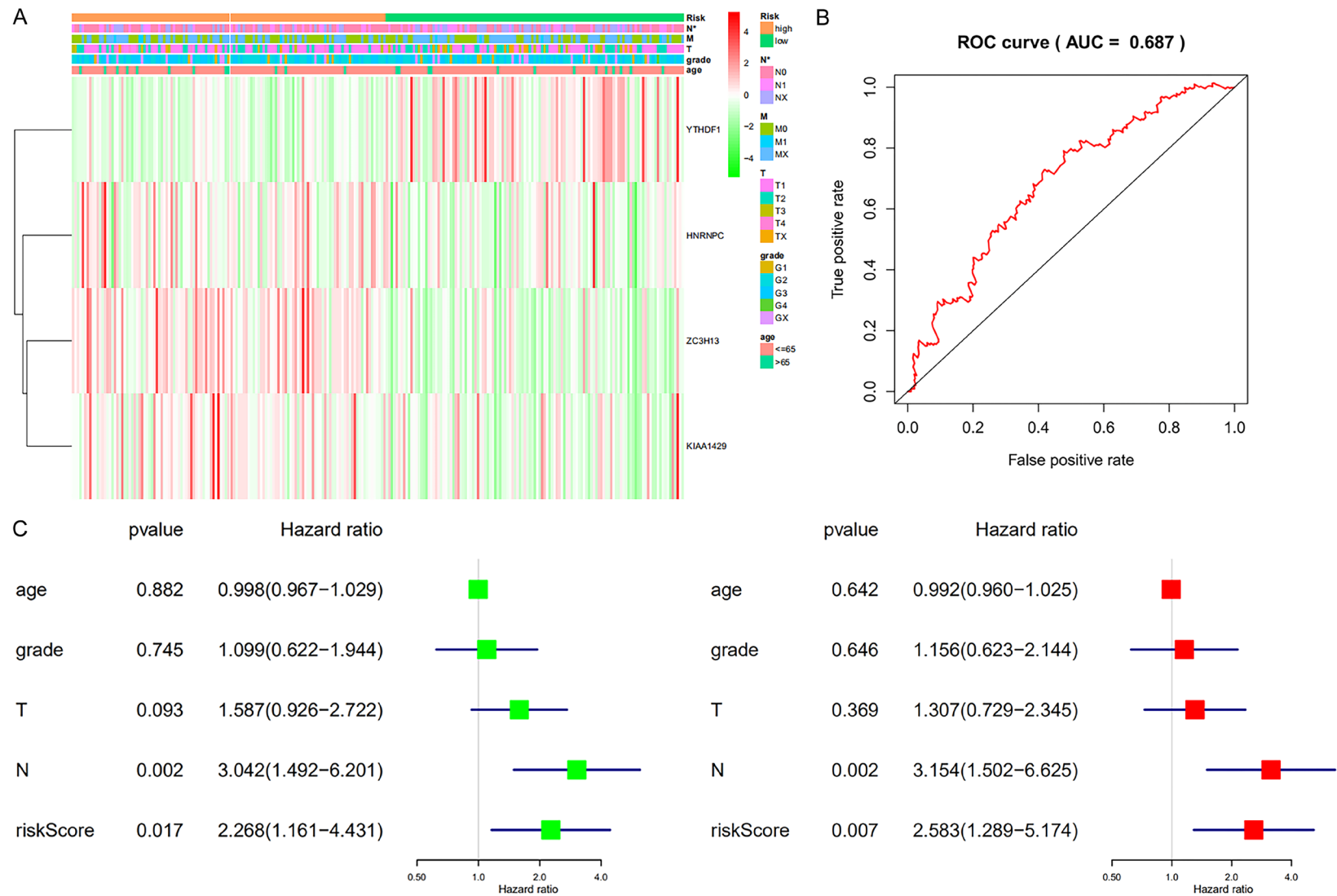
Figure 4. The prognostic risk score was closely related to the clinicopathological features of cervical cancer. A. The heatmap showed the expression levels of four m6A RNA methylation regulators in low- and high-risk cervical cancer. The distribution of clinicopathological features in low- and high-risk populations was compared. B. The ROC curve showed the predictive efficiency of the risk signature on mesenchymal subtype. C. Univariate and multivariate Cox regression analyses of the correlation between clinicopathological factors (including risk score) and overall patient survival.

high-risk and low-risk groups based on the median risk score.

Three risk genes (HNRNPC, KIAA1429, ZC3H13) and one protective gene (YTHDF1) of cervical cancer were identified in the current study. Through literature review, we found out that few studies have been conducted on the roles of these m6A methylation mediators in cervical cancer. The activation of LBX2-AS1 promotes the development of esophageal squamous cell carcinoma through the interaction with HNRNPC [16]. Knockout of the HNRNPC in two specific human breast cancer cell lines, MCF7 and T47D, can inhibit cell proliferation [17]. Moreover, KIAA1429 can contribute to liver cancer progression through N6-methyladenosine-dependent post-transcriptional modification of GATA3 [18]. And KIAA1429 acts as an oncogenic factor in breast cancer by regulating CDK1 in an N6-methyladenosine-independent manner [19]. In oocytes, KIAA1429 deletion leads to abnormal RNA metabolism in cells and decreases the level of m6A in oocytes, indicating that KIAA1429-mediated RNA metabolism serves an important role in follicular development and oocyte function maintenance [20]. ZC3H13 regulates nuclear RNA m6A methylation and mouse embryonic stem cell self-renewal [21]. Moreover, ZC3H13 suppresses colorectal cancer proliferation and invasion via inactivating Ras-ERK signaling [22]. YTHDF1 regulates tumorigenicity and cancer stem cell-like activity in human colorectal carcinoma [23]. YTHDF1 augments the translation of EIF3C in an m6A-dependent manner by binding to m6A-modified EIF3C mRNA and concomitantly promotes the overall translational output, thereby facilitating tumorigenesis and metastasis of ovarian cancer [24].

In our article, we comprehensively analyzed the expression of the 13 most common m6A methylation regulators in cervical cancer tissues, which uncovered that the expression of METTL3, YTHDF2 and RBM15 obviously increased, while the expression of FTO distinctly decreased. Through literature review, lncRNA GAS5-AS1 is found to inhibit the growth and metastasis of cervical cancer by increasing the stability of GAS5 in an ALKBH5-m6A-YTHDF2-dependent manner [25]. FTO exerts important oncogenic role in regulating cervical cancer cells' effort and migration via controlling m6A

modification of E2F1 and Myc transcripts [26]. In conclusion, the expression of m6A RNA methylation regulators is in close relation with the occurrence and development of cervical cancer. These findings are also useful for developing novel therapeutic methods through characterizing the expression of each m6A methylation regulator in cervical cancer, as chemicals targeting m6A methylation are considered a new method for cancer therapy [8, 27].

Whether the expression of m6A RNA methylation regulators can predict the prognosis of tumors is an important research hot spot [7]. In this study, we found four m6A methylation regulators related to OS, which were also used to construct risk scores to predict OS in patients with cervical cancer. Patients in the high-risk group have higher risk scores and regional lymph node involvement in the TCGA database. It is worth noting that the risk scores of patients with cervical cancer in TCGA are independently related to OS.

In summary, our results systematically demonstrated the expression, potential function and prognostic value of m6A RNA methylation regulators in cervical cancer. Our study provides important evidence for further study of the role of m6A RNA methylation in cervical cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Yu Zhao, Reproductive Medical Center, The Second Hospital Affiliated to Shandong University of Traditional Chinese Medicine, 1#, Jingba Road, Ji'nan 250001, Shandong Province, China. E-mail: zhaoyunv123@163.com

References

[1] Yang Y, Hsu PJ, Chen YS and Yang YG. Dynamic transcriptomic m(6)A decoration: writers, erasers, readers and functions in RNA metabolism. Cell Res 2018; 28: 616-624.

[2] Wang S, Sun C, Li J, Zhang E, Ma Z, Xu W, Li H, Qiu M, Xu Y, Xia W, Xu L and Yin R. Roles of RNA methylation by means of N(6)-methyladenosine (m(6)A) in human cancers. Cancer Lett 2017; 408: 112-120.

[3] Kwok CT, Marshall AD, Rasko JE and Wong JJ. Genetic alterations of m(6)A regulators predict poorer survival in acute myeloid leukemia. J Hematol Oncol 2017; 10: 39.

[4] Cui Q, Shi H, Ye P, Li L, Qu Q, Sun G, Sun G, Lu Z, Huang Y, Yang CG, Riggs AD, He C and Shi Y. m(6)A RNA methylation regulates the self-renewal and tumorigenesis of glioblastoma stem cells. Cell Rep 2017; 18: 2622-2634.

[5] Huang Y, Yan J, Li Q, Li J, Gong S, Zhou H, Gan J, Jiang H, Jia GF, Luo C and Yang CG. Meclofenamic acid selectively inhibits FTO demethylation of m6A over ALKBH5. Nucleic Acids Res 2015; 43: 373-384.

[6] Meyer KD and Jaffrey SR. Rethinking m(6)A readers, writers, and erasers. Annu Rev Cell Dev Biol 2017; 33: 319-342.

[7] Pan Y, Ma P, Liu Y, Li W and Shu Y. Multiple functions of m(6)A RNA methylation in cancer. J Hematol Oncol 2018; 11: 48.

[8] Dai D, Wang H, Zhu L, Jin H and Wang X. N6-methyladenosine links RNA metabolism to cancer progression. Cell Death Dis 2018; 9: 124.

[9] Zhang C, Cheng W, Ren X, Wang Z, Liu X, Li G, Han S, Jiang T and Wu A. Tumor purity as an underlying key factor in glioma. Clin Cancer Res 2017; 23: 6279-6291.

[10] Wang X, Li Z, Kong B, Song C, Cong J, Hou J and Wang S. Reduced m(6)A mRNA methylation is correlated with the progression of human cervical cancer. Oncotarget 2017; 8: 98918-98930.

[11] Zhou S, Bai ZL, Xia D, Zhao ZJ, Zhao R, Wang YY and Zhe H. FTO regulates the chemo-radiotherapy resistance of cervical squamous cell carcinoma (CSCC) by targeting beta-catenin through mRNA demethylation. Mol Carcinog 2018; 57: 590-597.

[12] Fang J, Zhang H and Jin S. Epigenetics and cervical cancer: from pathogenesis to therapy. Tumour Biol 2014; 35: 5083-5093.

[13] Reichel M, Koster T and Staiger D. Marking RNA: m6A writers, readers, and functions in Arabidopsis. J Mol Cell Biol 2019; 11: 899-910.

[14] Liu ZX, Li LM, Sun HL and Liu SM. Link between m6A modification and cancers. Front Bioeng Biotechnol 2018; 6: 89.

[15] Sun T, Wu R and Ming L. The role of m6A RNA methylation in cancer. Biomed Pharmacother 2019; 112: 108613.

[16] Zhang Y, Chen W, Pan T, Wang H, Zhang Y and Li C. LBX2-AS1 is activated by ZEB1 and promotes the development of esophageal squamous cell carcinoma by interacting with HNRNPC to enhance the stability of ZEB1 and ZEB2 mRNAs. Biochem Biophys Res Commun 2019; 511: 566-572.

[17] Wu Y, Zhao W, Liu Y, Tan X, Li X, Zou Q, Xiao Z, Xu H, Wang Y and Yang X. Function of HNRNPC in breast cancer cells by controlling the dsRNA-induced interferon response. EMBO J 2018; 37: e99017.

[18] Lan T, Li H, Zhang D, Xu L, Liu H, Hao X, Yan X, Liao H, Chen X, Xie K, Li J, Liao M, Huang J, Yuan K, Zeng Y and Wu H. KIAA1429 contributes to liver cancer progression through N6-methyladenosine-dependent post-transcriptional modification of GATA3. Mol Cancer 2019; 18: 186.

[19] Qian JY, Gao J, Sun X, Cao MD, Shi L, Xia TS, Zhou WB, Wang S, Ding Q and Wei JF. KIAA1429 acts as an oncogenic factor in breast cancer by regulating CDK1 in an N6-methyladenosine-independent manner. Oncogene 2019; 38: 6123-6141.

[20] Hu Y, Ouyang Z, Sui X, Qi M, Li M, He Y, Cao Y, Cao Q, Lu Q, Zhou S, Liu L, Liu L, Shen B, Shu W and Huo R. Oocyte competence is maintained by m(6)A methyltransferase KIAA1429-mediated RNA metabolism during mouse follicular development. Cell Death Differ 2020; [Epub ahead of print].

[21] Wen J, Lv R, Ma H, Shen H, He C, Wang J, Jiao F, Liu H, Yang P, Tan L, Lan F, Shi YG, He C, Shi Y and Diao J. Zc3h13 regulates nuclear RNA m(6)A methylation and mouse embryonic stem cell self-renewal. Mol Cell 2018; 69: 1028-1038, e1026.

[22] Zhu D, Zhou J, Zhao J, Jiang G, Zhang X, Zhang Y and Dong M. ZC3H13 suppresses colorectal cancer proliferation and invasion via inactivating Ras-ERK signaling. J Cell Physiol 2019; 234: 8899-8907.

[23] Bai Y, Yang C, Wu R, Huang L, Song S, Li W, Yan P, Lin C, Li D and Zhang Y. YTHDF1 regulates tumorigenicity and cancer stem cell-like activity in human colorectal carcinoma. Front Oncol 2019; 9: 332.

[24] Liu T, Wei Q, Jin J, Luo Q, Liu Y, Yang Y, Cheng C, Li L, Pi J, Si Y, Xiao H, Li L, Rao S, Wang F, Yu J, Yu J, Zou D and Yi P. The m6A reader YTHDF1 promotes ovarian cancer progression via augmenting EIF3C translation. Nucleic Acids Res 2020; 48: 3816-3831.

[25] Wang X, Zhang J and Wang Y. Long noncoding RNA GAS5-AS1 suppresses growth and metastasis of cervical cancer by increasing GAS5 stability. Am J Transl Res 2019; 11: 4909-4921.

[26] Zou D, Dong L, Li C, Yin Z, Rao S and Zhou Q. The m(6)A eraser FTO facilitates proliferation and migration of human cervical cancer cells. Cancer Cell Int 2019; 19: 321.

[27] Deng X, Su R, Weng H, Huang H, Li Z and Chen J. RNA N(6)-methyladenosine modification in cancers: current status and perspectives. Cell Res 2018; 28: 507-517.