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Original Article

Expression patterns and prognostic value of m^{6}A-related genes in colorectal cancer

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Abstract: Colorectal cancer (CRC), including colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ), is one of the most prevalent malignancies worldwide. N^6-methyladenosine (m^{6}A) is a ubiquitous RNA modification that plays a vital role in human tumors, but its expression patterns and prognostic value in CRC have not yet been determined. Here, we first used the Cancer Genome Atlas (TCGA), the Gene Expression Omnibus (GEO) and the Human Protein Atlas (HPA) databases and a tissue microarray (TMA) cohort to verify the expression of m^{6}A-related genes at the mRNA and protein levels. We found that most m^{6}A-related genes were substantially upregulated in tumor tissues compared with normal tissues, but METTL14, YTHDF3 and ALKBH5 were downregulated in CRC. There was no obvious difference in FTO. In addition, WTAP, METTL16, HNRNPC and YTHDC1 were abundantly expressed in COAD but not in READ. Moreover, immunofluorescence (IF) analyses of SW480 and HCT116 cells showed that most of the m^{6}A-related proteins were expressed in the nucleus and cytoplasm. Survival analysis demonstrated that the expression levels of METTL3, METTL14, METTL16, FTO and ALKBH5 were associated with the clinical outcomes of CRC patients. Taken together, all the results revealed that m^{6}A-related genes were dysregulated in CRC and might play a significant role in the progression of CRC.

Keywords: Colorectal cancer, m^{6}A, TCGA, TMA, prognosis

Introduction

Colorectal cancer (CRC) ranks third in terms of incidence (10.2% of total cases) and is the second cause of cancer-related death (9.2% of all cases) worldwide [1]. CRC includes colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ). Although considerable advancements in therapeutic strategies have been achieved, the survival rate of CRC remains far from satisfactory due to its late diagnosis, rapid development and easy metastasis [2, 3]. Therefore, extensive and in-depth studies are needed for improvements in the diagnosis and treatment of CRC and for the prediction of its recurrence.

N^6-methyladenosine (m^{6}A) is the most abundant and evolutionarily conserved modification [4], occurring in nearly all types of RNAs and in most organisms, from bacteria to animals [5]. Many studies have established that the m^{6}A modification is reversible and involves adenosine methyltransferases, demethylases, and RNA-binding proteins, which can add, remove, or recognize m^{6}A-modified sites and alter important biological processes accordingly [6]. Adenosine methyltransferases, known as the "writer" complex, consist of METTL3/14/16, RBM15/15B, WTAP, and KIAA1429, which aid the deposition of m^{6}As at the DRACH (D=A/G/U, R=A/G, H=A/C/U) consensus site on RNA polymerase II (pol II)-transcribed RNAs [7]. FTO and ALKBH5, which are considered m^{6}A "erasers", are selective demethylases capable of regulating gene expression and cell fate through oxidative removal of the methyl group in m^{6}A-containing substrates. Furthermore, RNA-binding proteins, which are considered "readers" and incorporate the YTH and hnRNP domains, can selectively recognize mRNA m^{6}A sites to mediate the degradation of mRNA [8].

Recent studies have demonstrated that m6A is associated with various human diseases and is particularly found in tumors. The linkages between m6A and human cancer types have been previously demonstrated in various cancers, including cervical cancer [9], prostate cancer [10], breast cancer [11], pancreatic cancer [12], and hepatocellular carcinoma [13]. For example, Tang found that Wilms' tumor 1-associating protein promoted renal cell carcinoma proliferation by regulating CDK2 mRNA stability [14]. Zhao *et al* determined that the overexpression of YTHDF1 was associated with poor prognosis in patients with hepatocellular carcinoma [15]. However, the specific expression patterns and clinical value of m6A-related genes in CRC are largely unknown.

In the present study, we investigated two types of colorectal cancer, namely, COAD and READ. And analyses of The Cancer Genome Atlas (TCGA), the Gene Expression Omnibus (GEO), the Human Protein Atlas (HPA) databases and the tissue microarray (TMA) cohort revealed that m6A-related proteins were frequently dysregulated in COAD and READ patients at the mRNA and protein levels. Immunofluorescence (IF) analyses were performed to determine the localization of m6A-related genes in CRC cells. Furthermore, the correlation between m6A-related gene expression and many molecular/clinicopathological parameters was explored in CRC patients. The survival analysis and univariate and multivariate Cox regression analyses established that the expression of m6A-related genes had a critical influence on the overall survival (OS) and recurrence-free survival (RFS) of CRC patients (**Figure 1**). These results emphasized the significance of m6A-related genes in colorectal cancer.

Materials and methods

CRC dataset acquisition and process

The TCGA-COAD and TCGA-READ datasets and all corresponding clinical data used in our study were downloaded from the TCGA data portal (http://gdc-portal.nci.nih.gov/). Seven sets of microarrays (GSE20916, GSE41258, GSE41328, GSE19249, GSE33113, GSE68204 and GSE87211) were extracted from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). Their characteristics, including cohort ID, RNA-Seq platform, number of samples (normal and tumor samples), publication year and country, are summarized in Table S1. Mutation data were obtained from cBioPortal (https://www.cbioportal.org/). In addition, validation of the translation of m6A-related genes was performed using the Human Protein Atlas database (http://www.proteinatlas.org/).

Tissue samples

For TMA, tumor tissues including 22 COAD tissue specimens and 21 READ specimens with corresponding normal adjacent tissue specimens, were obtained from April 2016 to December 2016 at the First Affiliated Hospital of Zhengzhou University, Zhengzhou University, China. None of the patients was administered any chemotherapy, immunotherapy, or radiotherapy prior to surgery. Our study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University, and all the patients provided signed informed consent.

Immunohistochemistry (IHC)

An IHC analysis of m6A-related genes was performed using formalin-fixed, paraffin-embedded tissues according to the manufacturer's instructions [16]. Briefly, the TMA sections were deparaffinized, and 0.3% hydrogen peroxide was applied to block endogenous peroxidase activity. After antigen retrieval, the sections were incubated overnight with primary antibody at 4°C and then with secondary antibody at room temperature. Subsequently, the Signal-Stain® DAB Substrate Kit (CST, USA) and Hematoxylin QS (Vector Laboratories) were used for the detection of immunoreactive cells. Two pathologists who were blind to the clinical parameters assessed the staining intensity of the reactions. The samples were scored based on the proportion of positive cells as follows: 0-none, 1 - <25%, 2 - 25-50%, 3 - 50-75%, and 4 - 75-100%. The staining intensity was evaluated as follows: 0 - none, 1 - weak, 2 - medium and 3 - strong. A total score was then calculated by multiplying the two sub-scores, and the samples with total scores of 0-6 and 7-12 were classified as low and high expression, respectively. The characteristics of the antibodies used in this study are summarized in Table S2.

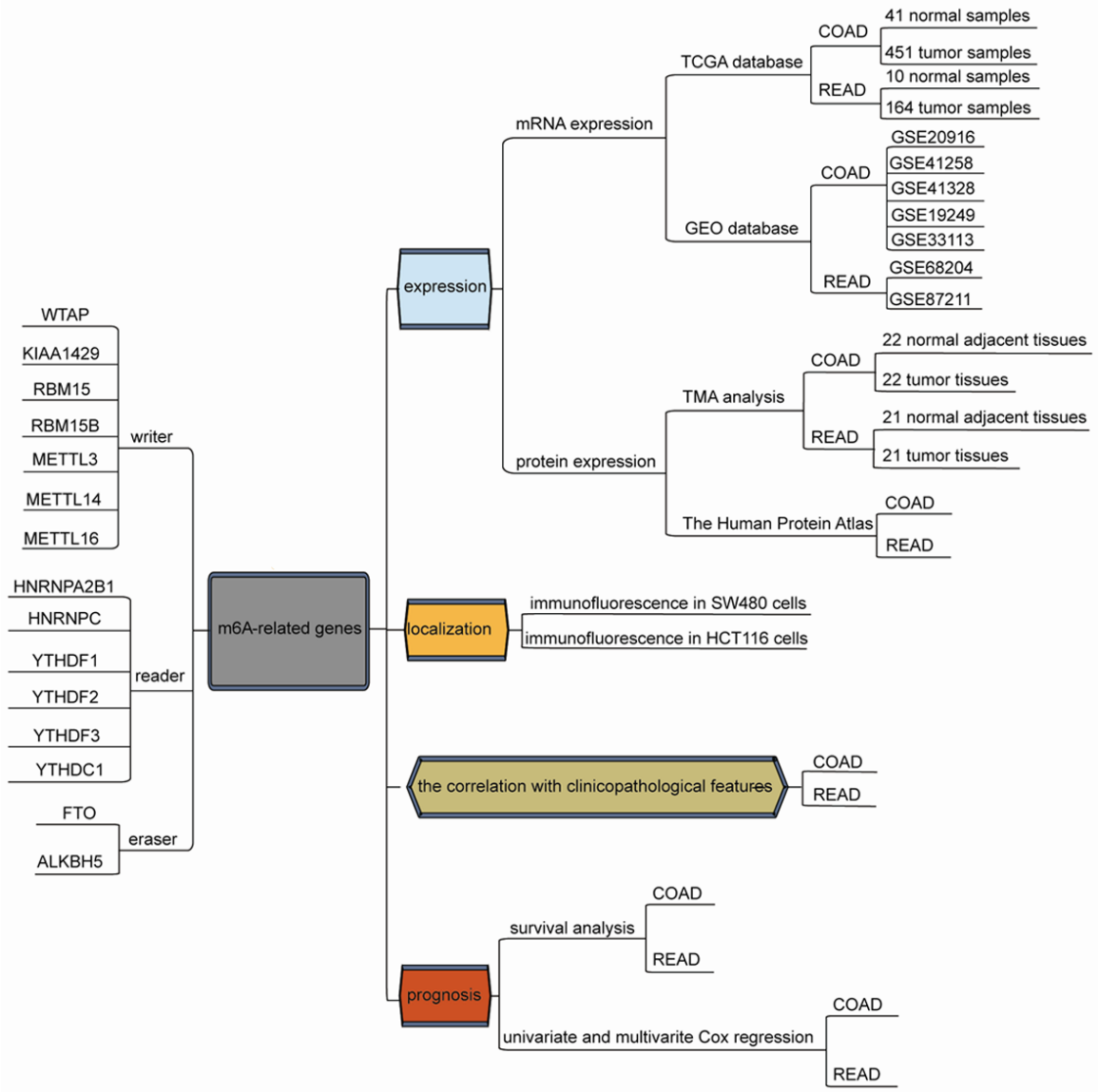


Figure 1. Study design and flow diagram. We focused on two types of colorectal cancer: COAD and READ. We first revealed the expression patterns of m^{6}A-related genes at the mRNA and protein levels based on the TCGA, GEO and the Human Protein Atlas databases and the TMA cohort. An immunofluorescence analysis was performed to determine the localization of the expression of m^{6}A-related genes in CRC cells. The correlation between m^{6}A-related gene expression and clinicopathological features was analyzed using χ^2 test. Furthermore, survival analysis and univariate and multivariate Cox regression analyses established that m^{6}A-related gene expression exerted a critical influence on the overall survival (OS) and recurrence-free survival (RFS) of CRC patients.

Cell lines and culture

The human colorectal cancer cell lines (HCT116 and SW480) used in this study were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 100 U/mL penicillin/streptomycin (Corning, New York, NY, USA) in a humidified incubator with an atmosphere of 5% CO_2 at 37°C. The cell lines were passed for less than 6 months in culture prior to the experiments.

Immunofluorescence

The cultured cells were inoculated into 24-well plates in DMEM with 10% FBS for 24 h, rinsed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized in 0.5% Triton

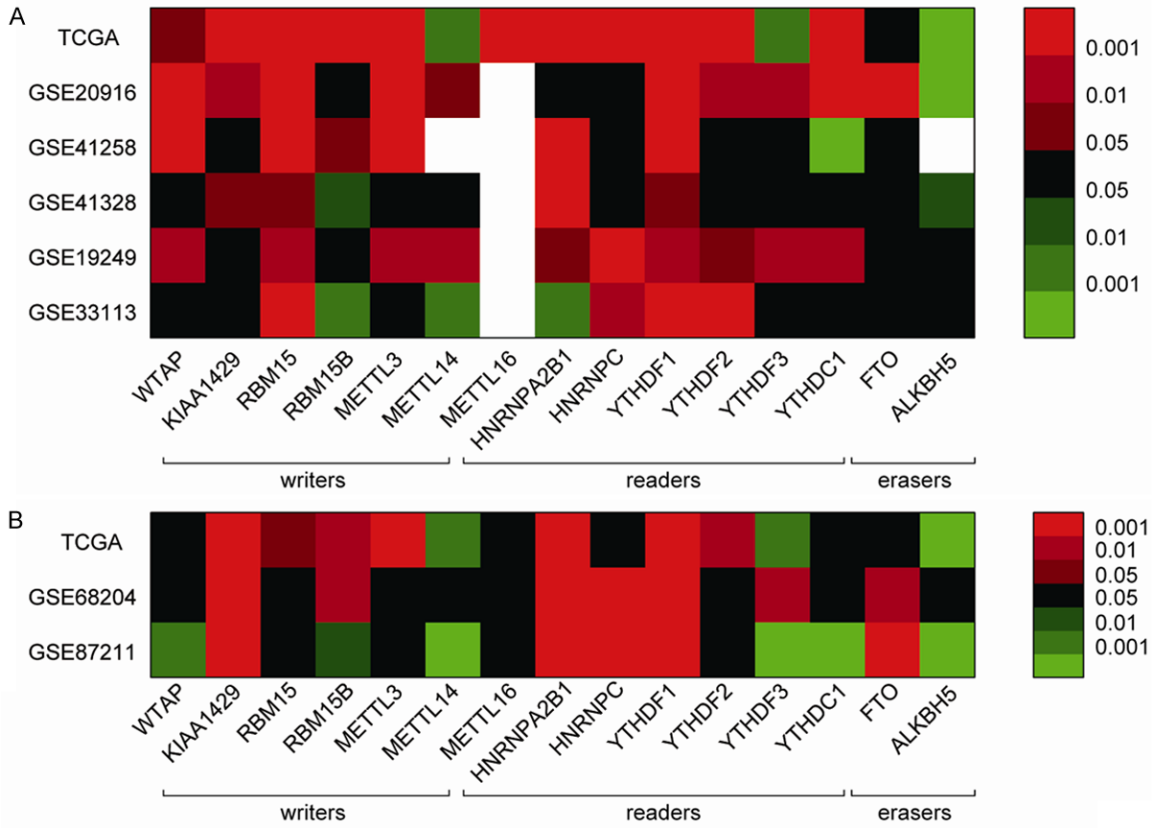


Figure 2. Heatmap showing the alterations in the mRNA expression of m^{6}A-related genes in the TCGA and GEO datasets. The red color indicates upregulated expression; the green color indicates downregulated expression; the black color indicates no significant changes, and the white color indicates that the related gene is absent in the datasets. The data were statistically analyzed by Student's t test (unpaired, two-tailed). A. mRNA expression patterns of m^{6}A-related genes in COAD. B. mRNA expression patterns of m^{6}A-related genes in READ.

X-100-PBS for 15 min. To block nonspecific binding sites, the cells were incubated with 1% bovine serum albumin (BSA) PBS. The cells were subsequently incubated with primary antibody (1:200 dilution) at 4°C overnight and then for 1 h with the appropriate secondary antibody (1:200 dilution). The nuclei were counterstained by mounting the cells in DAPI II (Abbott Molecular, Abbott Park, IL, USA). The immunofluorescent signals were then detected using a fluorescence microscope (Axio Observer A1). The characteristics of the antibodies used in our study are summarized in Table S2.

Statistical analysis

SPSS 23.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 7 (San Diego, CA, USA) were used for the statistical analyses. The TMA results were evaluated through the χ^2 test or Fisher's exact test. We analyzed the patients' survival time through Kaplan-Meier and log-rank tests. The best cut-off value for each gene and its survival curves were obtained using R Studio. The details are described in the Supplementary Methods. In addition, univariate and multivariate Cox regression analyses were performed to screen for independent factors that critically influenced the OS and RFS. Student's test was used for comparison between two groups. *P* (two-sided) values less than 0.05 were considered to indicate statistical significance.

Results

Expression pattern of m^{6}A "writers" in colorectal cancer

To explore the expression of m^{6}A-related proteins in human CRC, we first extracted and analyzed the expression of m^{6}A-related genes from the TCGA database at the mRNA level. In COAD,

all "writers" were substantially upregulated in tumor tissues compared with normal tissues, with the exception of METTL14, which was downregulated (**Figure 2A**). In contrast, the results showed that WTAP and METTL16 had no significant difference in READ tissues compared with normal tissues (**Figure 2B**). Furthermore, we used the GEO database to further validate the expression status of the m^{6}A "writers". **Figure 2** showed a hierarchical clustering heatmap of m^{6}A-related gene expression in COAD and READ, and the results presented a similar conclusion to that obtained from the analysis of the TCGA database.

Considering the difference between mRNA transcription and protein expression, the protein changes of the m^{6}A "writers" in CRC were further addressed by analyzing the TMA cohort consisting of 22 pairs of COAD and 21 pairs of READ tissue samples. The protein expression of all the "writers" was detected by IHC analysis (**Figure 3A** and **3B**). Subsequently, according to the staining intensity and the percentage of positive cells in the tissue sections, we categorized the patterns into high and low expression. In COAD, the protein expression of five (71.4%) "writers" (all except METTL14 and METTL16) was consistent with the gene expression levels. Specifically, METTL16 and METTL14 exhibited an obvious abundance and was weakly expressed at the mRNA level, respectively, but no significant difference was found at the protein level (**Figure 3C**). In READ, WTAP and METTL14 showed different expression patterns at the protein level, in contrast to the findings obtained at the mRNA level. WTAP was highly upregulated at the protein level but showed no significant difference at the mRNA level. Analogously with the findings in COAD, METTL14 was downregulated at the mRNA level but showed no significant changes at the protein level in READ (**Figure 3D**). Moreover, the IHC staining results and the patient data obtained from the Human Protein Atlas database also demonstrated the expression status of the m^{6}A "writers", as shown in **Figure 3E** and **3F**. The protein expression levels of most m^{6}A "writers" were in accordance with their transcriptional levels, but the database did not include any IHC information for METTL3.

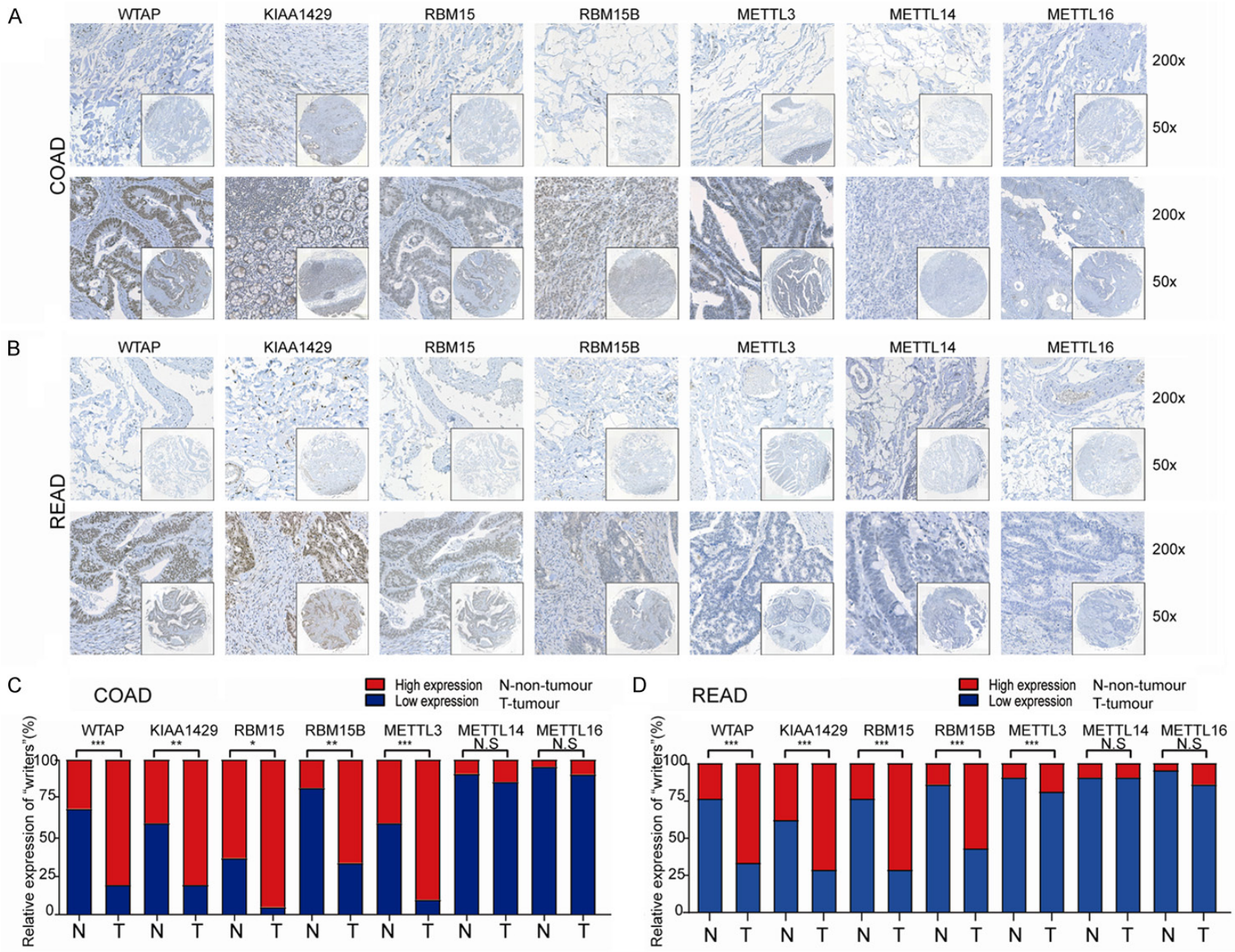
Expression status of m^{6}A "readers" in colorectal cancer

We analyzed the mRNA expression of the "readers" using the TCGA database. As shown in **Figure 2A**, YTHDF3 and the other "readers" was downregulated and overexpressed in COAD, respectively. However, no obvious discrepancies in HNRNPC and YTHDC1 expression were found in READ tissues compared with normal tissues (**Figure 2B**). Similar results were observed from the GEO database (**Figure 2A** and **2B**).

To further verify the expression patterns of m^{6}A "readers", we performed immunohistochemical analyses of COAD and READ TMAs (**Figure 4A** and **4B**). In COAD, all the "readers" showed significant changes in protein expression, and the direction of these changes was consistent with that found for the gene expression changes, with the exception of YTHDF3 and YTHDC1. Among these exceptions, the protein expression of YTHDF3 was contrary to its mRNA expression, and YTHDC1 showed an obvious abundance at the mRNA level but no significant change at the protein level, respectively (**Figure 4C**). The expression patterns found in READ were similar to those found in COAD via the TMA cohort (**Figure 4D**). YTHDF3 was also overexpressed at the protein level but downregulated at the mRNA level. However, HNRNPC was upregulated at the protein level but showed no apparent difference in expression at the mRNA level. In addition, the results from the Human Protein Atlas database demonstrated that the protein expression of most "readers" was consistent with their transcriptional level (**Figure 4E** and **4F**).

Expression of m^{6}A "erasers" in colorectal cancer

The m^{6}A "erasers" comprised FTO and ALKBH5, and an analysis of the TCGA database revealed that ALKBH5 showed obviously weaker mRNA expression in CRC than in normal tissue. No significant difference was found for the FTO gene in both COAD and READ. Similar results were found in the GEO database (**Figure 2A** and **2B**). The IHC analysis revealed that ALKBH5 was prominently overexpressed at the protein level but downregulated at the mRNA level and that the protein expression of FTO was concordant with its mRNA expression in both COAD and READ (**Figure 5A-D**). We found similar expression patterns in the Human Protein Atlas with respect to the transcriptional expression of m^{6}A "erasers" in CRC, with the exception of a deficiency of FTO in READ (**Figure 5E** and **5F**).


A
WTAP
KIAA1429
RBM15
RBM15B
METTL3
METTL14
METTL16
COAD
200x
50x
200x
50x
B
WTAP
KIAA1429
RBM15
RBM15B
METTL3
METTL14
METTL16
READ
200x
50x
200x
50x
C
COAD
High expression
Low expression
N-non-tumour
T-tumour
WTAP
KIAA1429
RBM15
RBM15B
METTL3
METTL14
METTL16
***
**
*
**
***
N.S
N.S
Relative expression of "writers"(%)
100
75
50
25
0
N
T
D
READ
High expression
Low expression
N-non-tumour
T-tumour
WTAP
KIAA1429
RBM15
RBM15B
METTL3
METTL14
METTL16
***
***
***
***
***
N.S
N.S
Relative expression of "writers"(%)
100
75
50
25
0
N
T

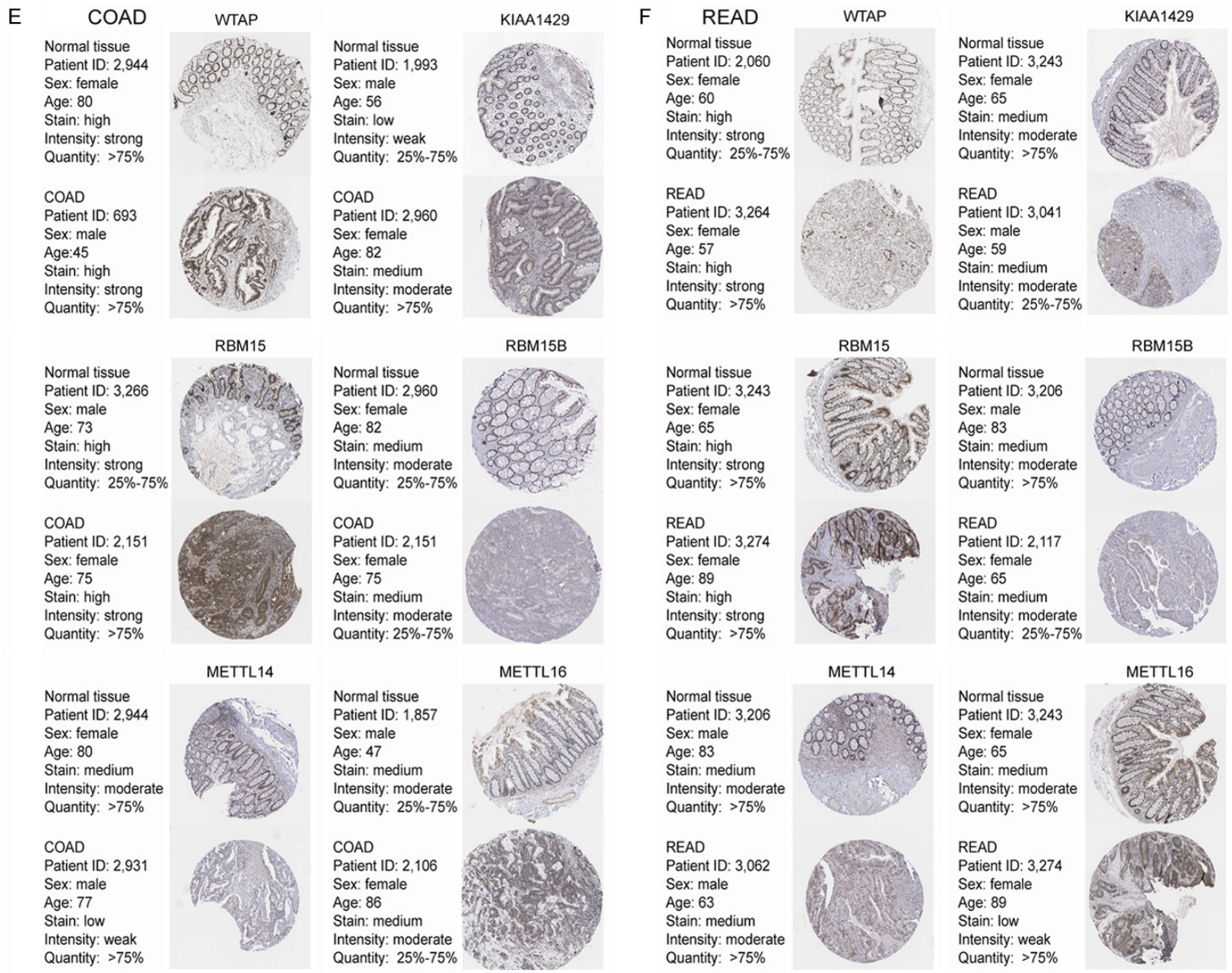
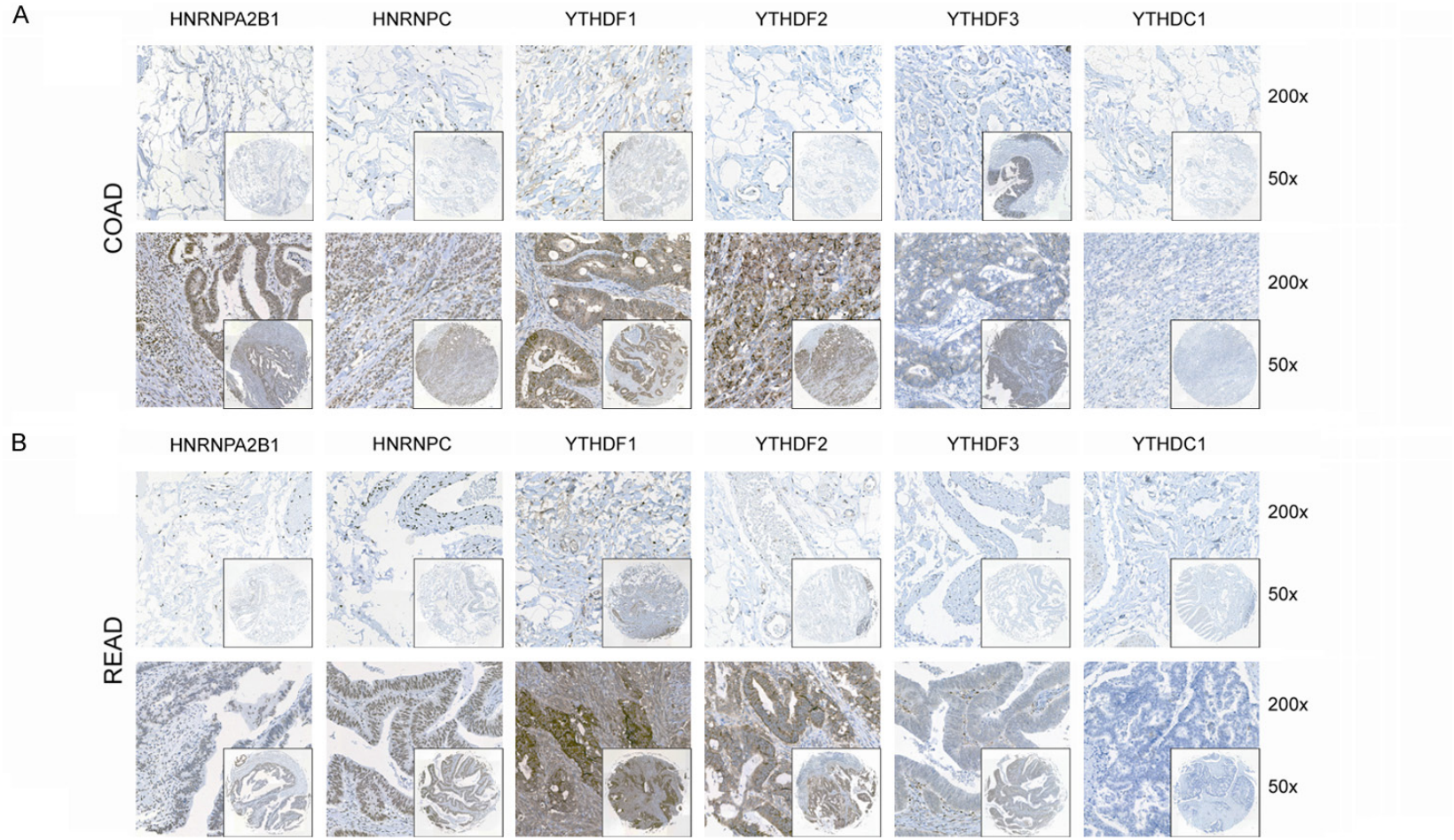
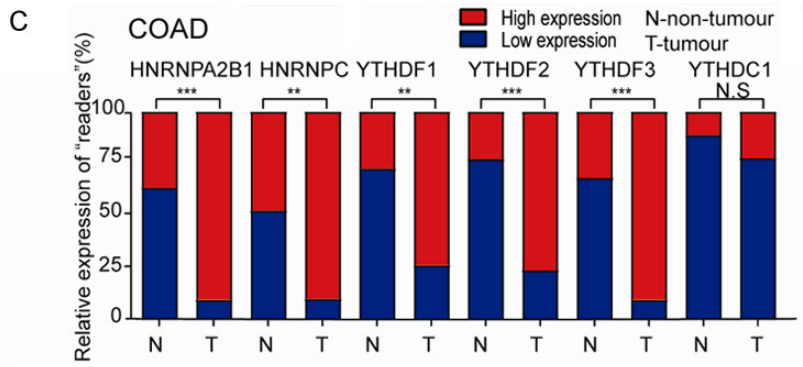
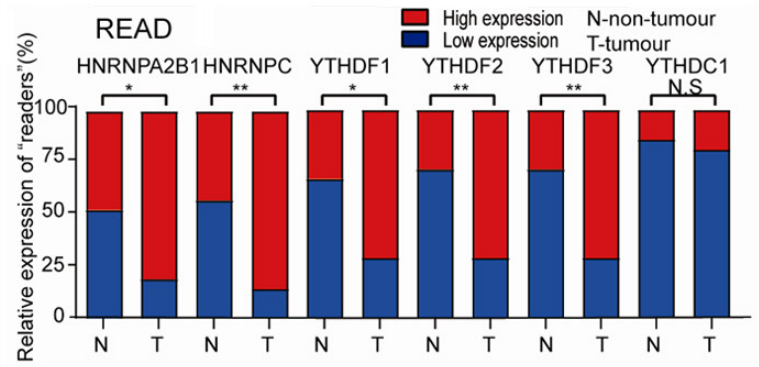
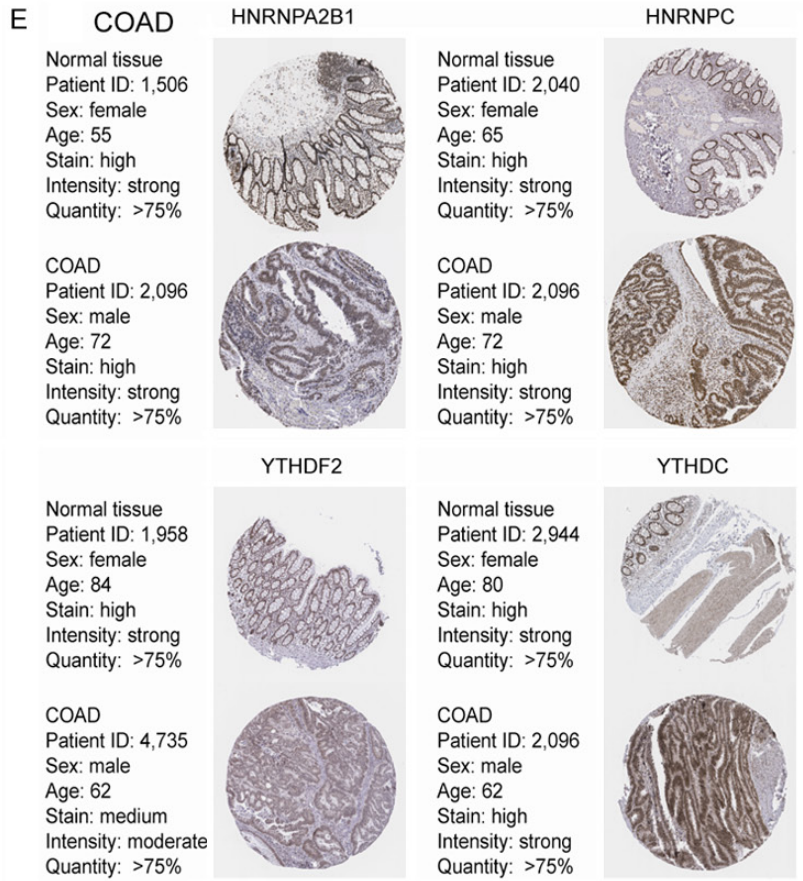

E
COAD
WTAP
Normal tissue
Patient ID: 2,944
Sex: female
Age: 80
Stain: high
Intensity: strong
Quantity: >75%
COAD
Patient ID: 693
Sex: male
Age:45
Stain: high
Intensity: strong
Quantity: >75%
KIAA1429
Normal tissue
Patient ID: 1,993
Sex: male
Age: 56
Stain: low
Intensity: weak
Quantity: 25%-75%
COAD
Patient ID: 2,960
Sex: female
Age: 82
Stain: medium
Intensity: moderate
Quantity: >75%
RBM15
Normal tissue
Patient ID: 3,266
Sex: male
Age: 73
Stain: high
Intensity: strong
Quantity: 25%-75%
COAD
Patient ID: 2,151
Sex: female
Age: 75
Stain: high
Intensity: strong
Quantity: >75%
RBM15B
Normal tissue
Patient ID: 2,960
Sex: female
Age: 82
Stain: medium
Intensity: moderate
Quantity: 25%-75%
COAD
Patient ID: 2,151
Sex: female
Age: 75
Stain: medium
Intensity: moderate
Quantity: 25%-75%
METTL14
Normal tissue
Patient ID: 2,944
Sex: female
Age: 80
Stain: medium
Intensity: moderate
Quantity: >75%
COAD
Patient ID: 2,931
Sex: male
Age: 77
Stain: low
Intensity: weak
Quantity: >75%
METTL16
Normal tissue
Patient ID: 1,857
Sex: male
Age: 47
Stain: medium
Intensity: moderate
Quantity: 25%-75%
COAD
Patient ID: 2,106
Sex: female
Age: 86
Stain: medium
Intensity: moderate
Quantity: 25%-75%
F
READ
WTAP
Normal tissue
Patient ID: 2,060
Sex: female
Age: 60
Stain: high
Intensity: strong
Quantity: 25%-75%
READ
Patient ID: 3,264
Sex: female
Age: 57
Stain: high
Intensity: strong
Quantity: >75%
KIAA1429
Normal tissue
Patient ID: 3,243
Sex: female
Age: 65
Stain: medium
Intensity: moderate
Quantity: >75%
READ
Patient ID: 3,041
Sex: male
Age: 59
Stain: medium
Intensity: moderate
Quantity: 25%-75%
RBM15
Normal tissue
Patient ID: 3,243
Sex: female
Age: 65
Stain: high
Intensity: strong
Quantity: >75%
READ
Patient ID: 3,274
Sex: female
Age: 89
Stain: high
Intensity: strong
Quantity: >75%
RBM15B
Normal tissue
Patient ID: 3,206
Sex: male
Age: 83
Stain: medium
Intensity: moderate
Quantity: >75%
READ
Patient ID: 2,117
Sex: female
Age: 65
Stain: medium
Intensity: moderate
Quantity: 25%-75%
METTL14
Normal tissue
Patient ID: 3,206
Sex: male
Age: 83
Stain: medium
Intensity: moderate
Quantity: >75%
READ
Patient ID: 3,062
Sex: male
Age: 63
Stain: medium
Intensity: moderate
Quantity: >75%
METTL16
Normal tissue
Patient ID: 3,243
Sex: female
Age: 65
Stain: medium
Intensity: moderate
Quantity: >75%
READ
Patient ID: 3,274
Sex: female
Age: 89
Stain: low
Intensity: weak
Quantity: >75%

Figure 3. Protein expression patterns of m^{6}A-related "writers" in CRC and normal tissues. A. Representative IHC staining of m^{6}A-related "writers" in COAD in the TMA cohort. B. Representative IHC staining of m^{6}A-related "writers" in READ in the TMA cohort. C. Comparison of the relative expression of m^{6}A-related "writers" between COAD and normal tissues in the TMA cohort. D. Comparison of the relative expression of m^{6}A-related "writers" between READ and normal tissues in the TMA cohort. E. Information on the IHC staining of several m^{6}A-related "writers" in COAD in The Human Protein Atlas database. F. Information on the IHC staining of several m^{6}A-related "writers" in READ in The Human Protein Atlas database. (*P<0.05, **P<0.01, ***P<0.001, N.S: no significance).




C
COAD
High expression
Low expression
N-non-tumour
T-tumour
HNRNPA2B1 HNRNPC YTHDF1 YTHDF2 YTHDF3 YTHDC1
***
**
**
***
***
N.S
Relative expression of "readers"(%)
100
75
50
25
0
N T N T N T N T N T N T


D
READ
High expression
Low expression
N-non-tumour
T-tumour
HNRNPA2B1 HNRNPC YTHDF1 YTHDF2 YTHDF3 YTHDC1
*
**
*
**
**
N.S
Relative expression of "readers"(%)
100
75
50
25
0
N T N T N T N T N T N T


E
COAD
HNRNPA2B1
HNRNPC
Normal tissue
Patient ID: 1,506
Sex: female
Age: 55
Stain: high
Intensity: strong
Quantity: >75%
Normal tissue
Patient ID: 2,040
Sex: female
Age: 65
Stain: high
Intensity: strong
Quantity: >75%
COAD
Patient ID: 2,096
Sex: male
Age: 72
Stain: high
Intensity: strong
Quantity: >75%
COAD
Patient ID: 2,096
Sex: male
Age: 72
Stain: high
Intensity: strong
Quantity: >75%
YTHDF2
YTHDC
Normal tissue
Patient ID: 1,958
Sex: female
Age: 84
Stain: high
Intensity: strong
Quantity: >75%
Normal tissue
Patient ID: 2,944
Sex: female
Age: 80
Stain: high
Intensity: strong
Quantity: >75%
COAD
Patient ID: 4,735
Sex: male
Age: 62
Stain: medium
Intensity: moderate
Quantity: >75%
COAD
Patient ID: 2,096
Sex: male
Age: 62
Stain: high
Intensity: strong
Quantity: >75%

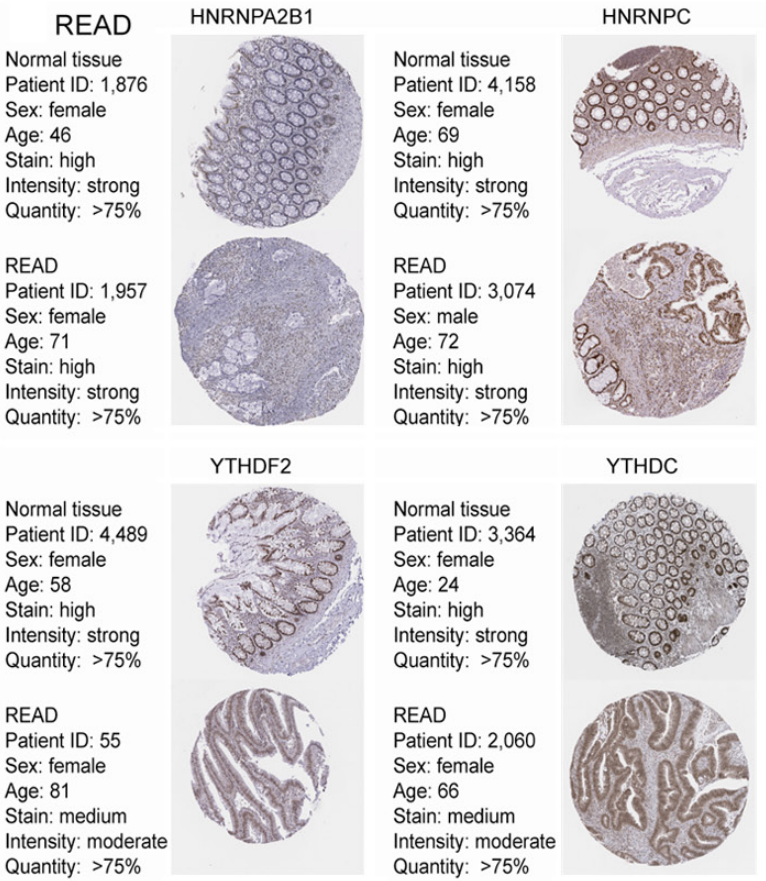

F
READ
HNRNPA2B1
HNRNPC
Normal tissue
Patient ID: 1,876
Sex: female
Age: 46
Stain: high
Intensity: strong
Quantity: >75%
Normal tissue
Patient ID: 4,158
Sex: female
Age: 69
Stain: high
Intensity: strong
Quantity: >75%
READ
Patient ID: 1,957
Sex: female
Age: 71
Stain: high
Intensity: strong
Quantity: >75%
READ
Patient ID: 3,074
Sex: male
Age: 72
Stain: high
Intensity: strong
Quantity: >75%
YTHDF2
YTHDC
Normal tissue
Patient ID: 4,489
Sex: female
Age: 58
Stain: high
Intensity: strong
Quantity: >75%
Normal tissue
Patient ID: 3,364
Sex: female
Age: 24
Stain: high
Intensity: strong
Quantity: >75%
READ
Patient ID: 55
Sex: female
Age: 81
Stain: medium
Intensity: moderate
Quantity: >75%
READ
Patient ID: 2,060
Sex: female
Age: 66
Stain: medium
Intensity: moderate
Quantity: >75%

Figure 4. Protein expression patterns of m^{6}A-related "readers" in CRC and normal tissues. A. Representative IHC staining of m^{6}A-related "readers" in COAD in the TMA cohort. B. Representative IHC staining of m^{6}A-related "readers" in READ in the TMA cohort. C. Comparison of the relative expression of m^{6}A-related "readers" between COAD and normal tissues in the TMA cohort. D. Comparison of the relative expression of m^{6}A-related "readers" on between READ and normal tissues in the TMA cohort. E. Information on the IHC staining of m^{6}A-related "readers" in COAD in The Human Protein Atlas database. F. Information on the IHC staining of m^{6}A-related "readers" in READ in The Human Protein Atlas database. (*P<0.05, **P<0.01, ***P<0.001, N.S: no significance).

Localization of the expression of m^{6}A-related genes in colorectal cells

Although the expression patterns of m^{6}A-related genes in CRC at the mRNA and protein levels have been studied, information on the localization of m^{6}A-related genes in CRC cells remains to be elucidated. Therefore, an IF analysis was performed to further identify the subcellular distribution of m^{6}A-related proteins in CRC cells. As shown in **Figure 6**, most of the m^{6}A-related genes were mainly expressed in the nucleus and cytoplasm. Specifically, some proteins (KIAA1429, RBM15, RBM15B, HNRNPA2B1, YTHDC1 and ALKBH5) showed strong nuclear staining as well as weak cytoplasmic staining. YTHDF1 and YTHDF2 were detected only in the cytoplasm, where as HNRNPC signals were found in the nucleus.

Relationship between m^{6}A-related genes and clinicopathological features in CRC

We further analyzed the correlation between the expression of m^{6}A-related genes and clinicopathological characteristics in COAD and READ to explore the clinical significance of m^{6}A-related gene expression. As shown in **Figure 7**, in COAD, the KRAS mutation was associated with YTHDF1 expression (P=0.035) (**Figure 7A**), and the BRAF mutation could affect the expression of METTL3 (P=0.033), YTHDF1 (P<0.0001) and ALKBH5 (P=0.011) (**Figure 7B**). Moreover, the expression of KIAA1429 (P=0.036), RBM15B (P=0.003), YTHDF1 (P=0.022) and ALKBH5 (P=0.022) was correlated with age (**Figure 7C**). In addition, gender was related to WTAP (P=0.046), METTL16 (P=0.005), HNRNPC (P=0.018) and YTHDF1 (P=0.035) expression (**Figure 7D**). Race was found to be associated with WTAP (P=0.019) expression (**Figure 7E**), and the TNM stage was verified to have correlation with YTHDC1 (P=0.011) expression (**Figure 7F**). However, some differences were found in READ. The KRAS mutation was associated with YTHDF1 (P=0.005) expression (**Figure 8A**), and a significant relationship was found between BRAF mutation and the expression of RBM15 (P=0.025), METTL3 (P=0.03), METTL14 (P=0.007), YTHDF2 (P=0.043), YTHDF3 (P=0.018) and YTHDC1 (P=0.002) (**Figure 8B**). Moreover, age was found to be related to KIAA1429 (P=0.017), RBM15 (P=0.015) and METTL16 (P<0.0001) expression (**Figure 8C**), and no obvious association was found for gender, race and TNM stage with m^{6}A-related gene expression (Figure S1).

Survival analysis of m^{6}A-related proteins in colorectal cancer

To evaluate the prognostic roles of m^{6}A-related proteins in CRC progression, we first classified COAD and READ patients into two groups (high-expression group and low-expression group) according to the optimal cut-off value. The correlation of m^{6}A-related gene expression with corresponding clinical follow-up information was determined through Kaplan-Meier analysis and a log-rank test. We first investigated whether the expression levels of m^{6}A-related genes were correlated with the outcome of CRC patients. In COAD patients, low FTO expression predicted poor OS (**Figure 9A**), and patients with high METTL3 expression was associated with a shorter RFS compared with those with low METTL3 expression (**Figure 9B**). Moreover, as shown in **Figure 9C-E**, in addition to high ALKBH5 expression, low METTL14 and METTL16 expression in READ tissues were clearly associated with worse OS. However, no significant difference in METTL16 and FTO mRNA expression was found between tumor and normal tissues. Additionally, m^{6}A-related genes did not predict RFS in READ (Figure S2). Further details were presented in Figures S3, S4, S5.

The univariate Cox regression analysis was performed to identify risk factors related to patient prognosis. Univariable analyzes of COAD revealed that the TNM (tumor, node, and metastasis) stage and high METTL3 expression were significant prognostic factors for RFS (**Figure**

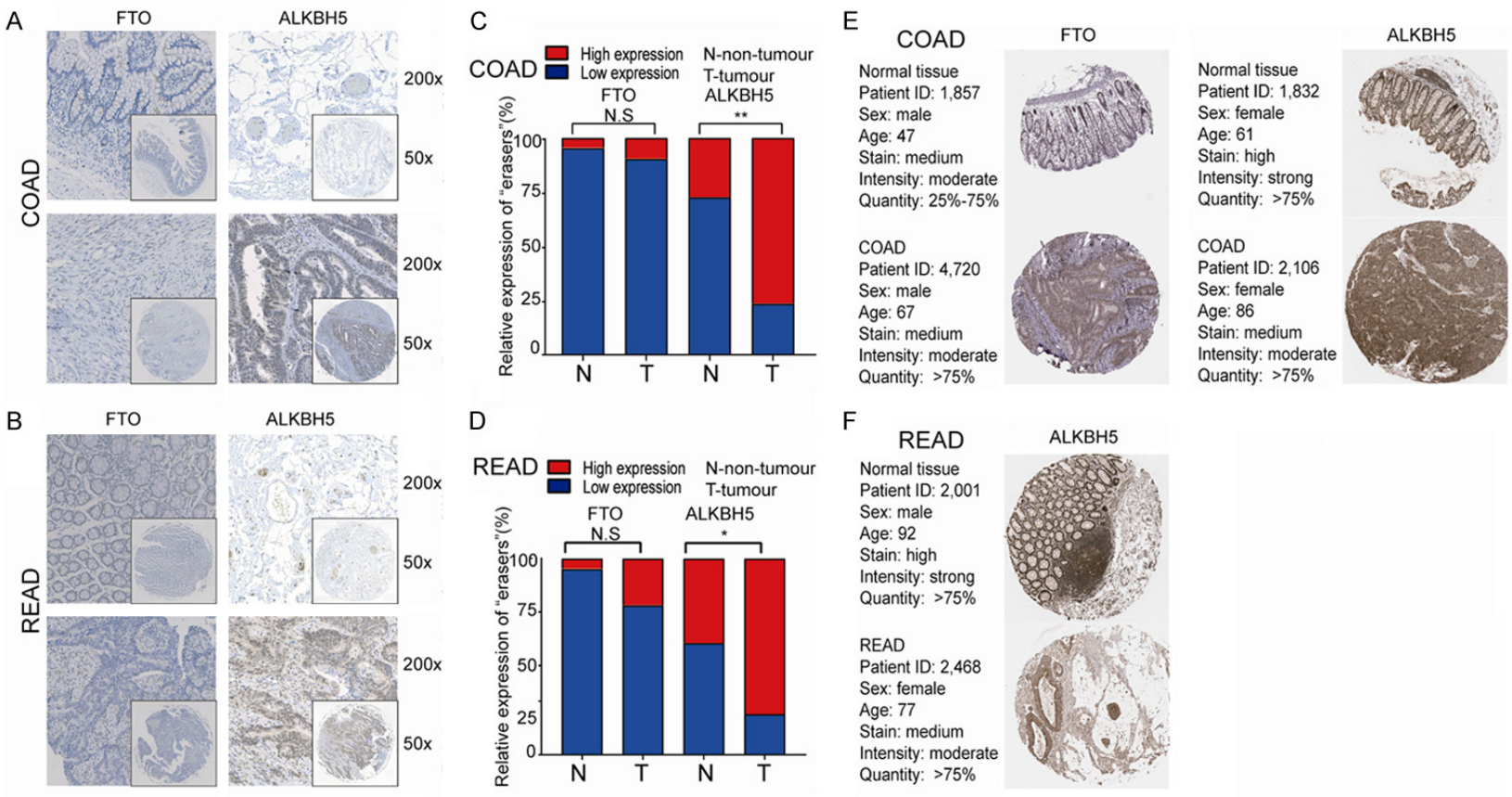


Figure 5. Protein expression patterns of m6A-related "erasers" in CRC and normal tissues. A. Representative IHC staining of m6A-related "erasers" in COAD in the TMA cohort. B. Representative IHC staining of m6A-related "erasers" in READ in the TMA cohort. C. Comparison of the relative expression of m6A-related "erasers" between COAD and normal tissues in the TMA cohort. D. Comparison of the relative expression of m6A-related "erasers" between READ and normal tissues in the TMA cohort. E. Information on the IHC staining of m6A-related "erasers" in COAD in The Human Protein Atlas database. F. Information on the IHC staining of several m6A-related "erasers" in READ in The Human Protein Atlas database. (*P<0.05, **P<0.01, ***P<0.001, N.S: no significance).

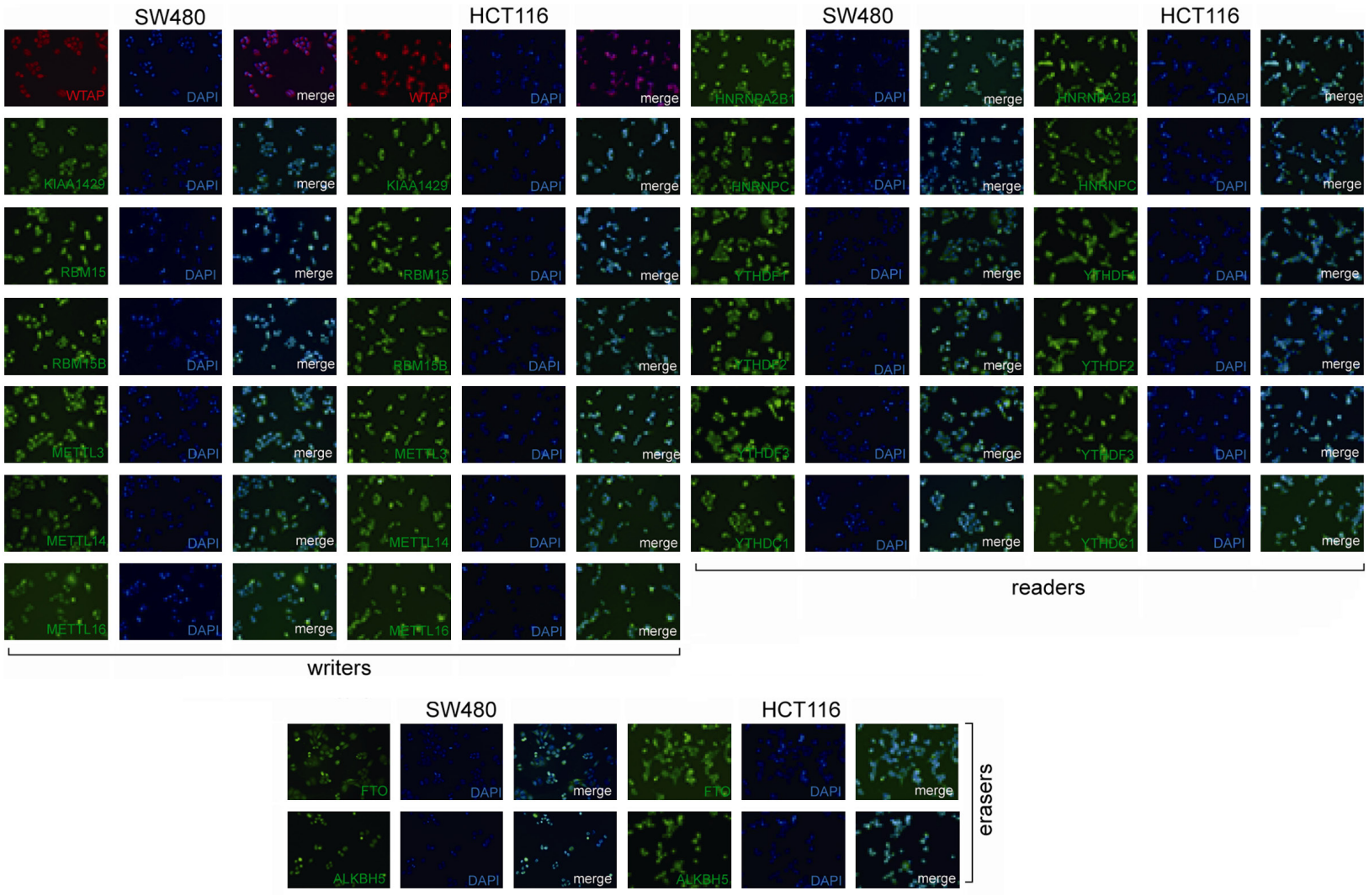
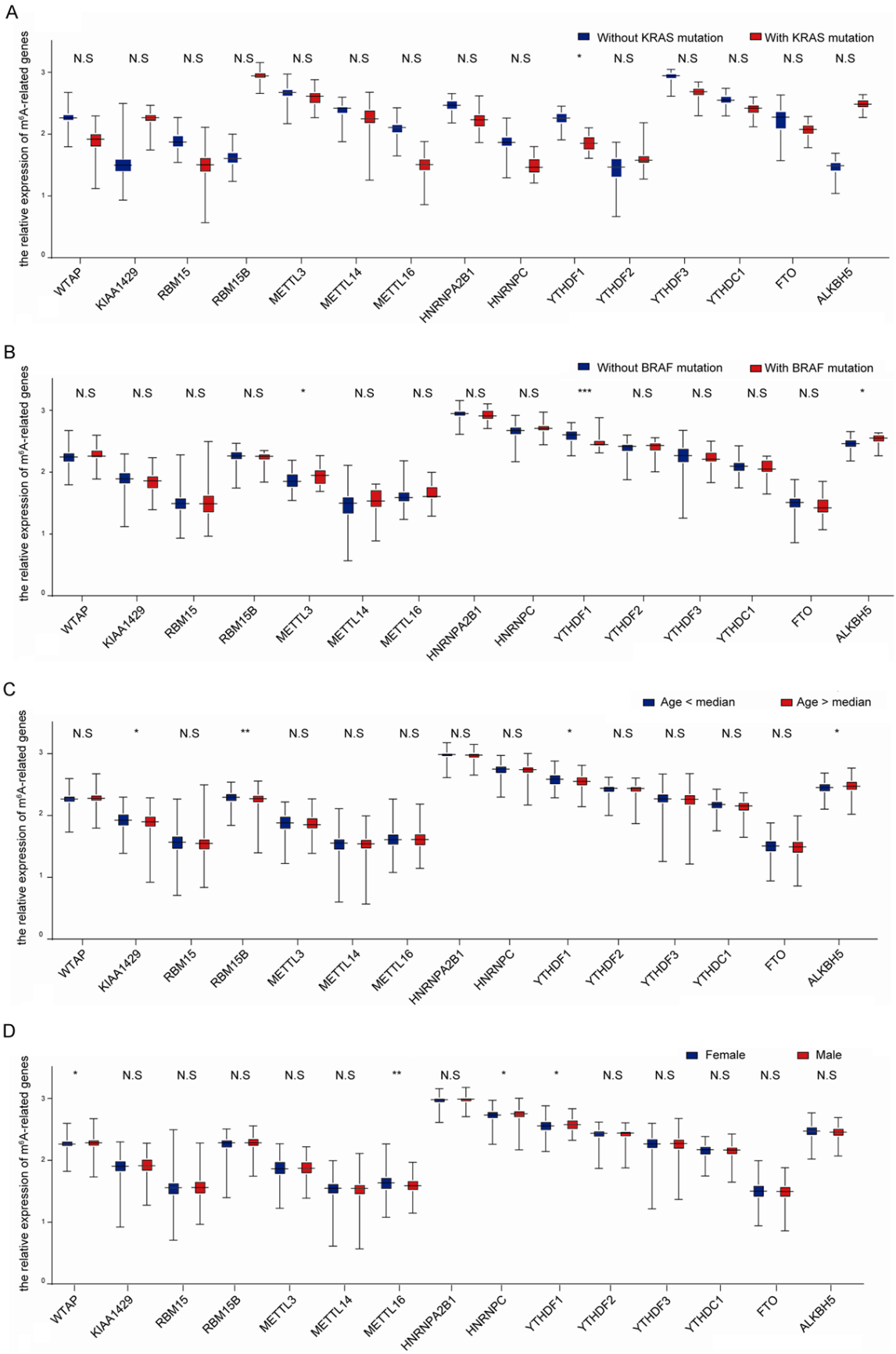


Figure 6. Subcellular localization of m^{6}A-related genes in SW480 and HCT116 cell lines. The cells were fixed and reacted with the corresponding antibodies. The secondary antibodies were anti-rabbit IgG-conjugated to fluorescein isothiocyanate and anti-mouse IgG-conjugated to rhodamine red. The nucleus was stained with DAPI (blue). The images were captured with a fluorescence microscope.


A
Without KRAS mutation
With KRAS mutation
the relative expression of m6A-related genes
N.S N.S N.S N.S N.S N.S N.S N.S N.S * N.S N.S N.S N.S N.S
WTAP KIAA1429 RBM15 RBM15B METTL3 METTL14 METTL16 HNRNPA2B1 HNRNPC YTHDF1 YTHDF2 YTHDF3 YTHDC1 FTO ALKBH5
B
Without BRAF mutation
With BRAF mutation
the relative expression of m6A-related genes
N.S N.S N.S N.S * N.S N.S N.S N.S *** N.S N.S N.S N.S *
WTAP KIAA1429 RBM15 RBM15B METTL3 METTL14 METTL16 HNRNPA2B1 HNRNPC YTHDF1 YTHDF2 YTHDF3 YTHDC1 FTO ALKBH5
C
Age < median
Age > median
the relative expression of m6A-related genes
N.S * N.S ** N.S N.S N.S N.S N.S * N.S N.S N.S N.S *
WTAP KIAA1429 RBM15 RBM15B METTL3 METTL14 METTL16 HNRNPA2B1 HNRNPC YTHDF1 YTHDF2 YTHDF3 YTHDC1 FTO ALKBH5
D
Female
Male
the relative expression of m6A-related genes
* N.S N.S N.S N.S N.S ** N.S * * N.S N.S N.S N.S N.S
WTAP KIAA1429 RBM15 RBM15B METTL3 METTL14 METTL16 HNRNPA2B1 HNRNPC YTHDF1 YTHDF2 YTHDF3 YTHDC1 FTO ALKBH5

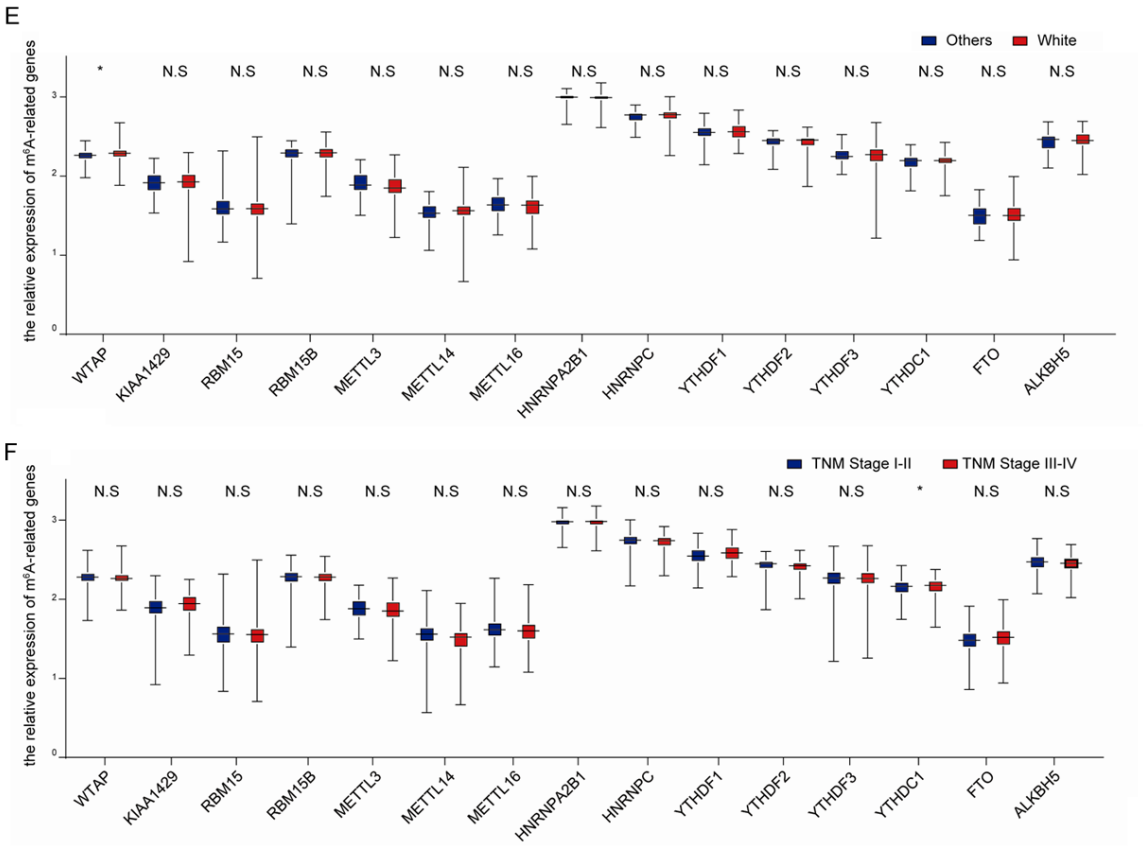


Figure 7. Relationship between m^{6}A-related gene expression and molecular/clinicopathological features in COAD. A. YTHDF1 expression was associated with the KRAS mutation. B. The expression of METTL3, YTHDF1 and ALKBH5 was related to the BRAF mutation. C. A significant correlation was found between age and the expression of KIAA1429, RBM15B, YTHDF1 and ALKBH5. D. Gender was related to WTAP, METTL16, HNRNPC and YTHDF1 expression. E. WTAP expression was important to race. F. The TNM stage was correlated with YTHDC1 expression.

9F) and that the TNM stage and age were prognostic factors for OS (**Figure 9G**). For READ patients, age, the TNM stage and the expression of METTL14, METTL16 and ALKBH5 were found to have a critical influence on OS (**Figure 9H**), and the TNM stage was the only prognostic factor for RFS (**Figure 9I**). The details are shown in Tables S3 and S4. Furthermore, the multivariate Cox regression analysis revealed that the TNM stage was an independent risk factor for OS (P<0.0001) and RFS (P<0.0001) in COAD (**Tables 1** and **2**). Age was also found to be a factor affecting RFS (P=0.001) (**Table 2**). In READ, the expression of METTL14 (P=0.004) and ALKBH5 (P<0.0001) and the TNM stage (P<0.025) were verified to be independent factors of OS (**Table 3**).

Discussion

M^{6}A was initially reported by Ronald Desrosiers in 1974 [17], but the precise mechanism and regulatory function of the m^{6}A modification remained largely unknown until recently [18]. Many studies have revealed that the m^{6}A modification affects almost every aspect of RNA metabolism, including RNA expression, splicing, nuclear export, translation, decay and RNA-protein interactions (**Figure 10**) [19-21]. Studies conducted in recent years have demonstrated that m^{6}A can regulate multiple spatial and temporalphysiological processes, including gametogenesis, sex determination, embryogenesis, cell fate determination, circadian rhythms, heat shock responses, DNA damage response, pluripotency, reprogramming and neuronal functions [5, 22]. Furthermore, emerging evidence has revealed that m^{6}A plays crucial roles in human diseases. For example, the m^{6}A modification might lead to obesity [23], type 2 diabetes mellitus [24], and infertility [25], among other diseases. Although the acknowledgement of m^{6}A methylation remains at controversial, advanced methods, such as high-through-

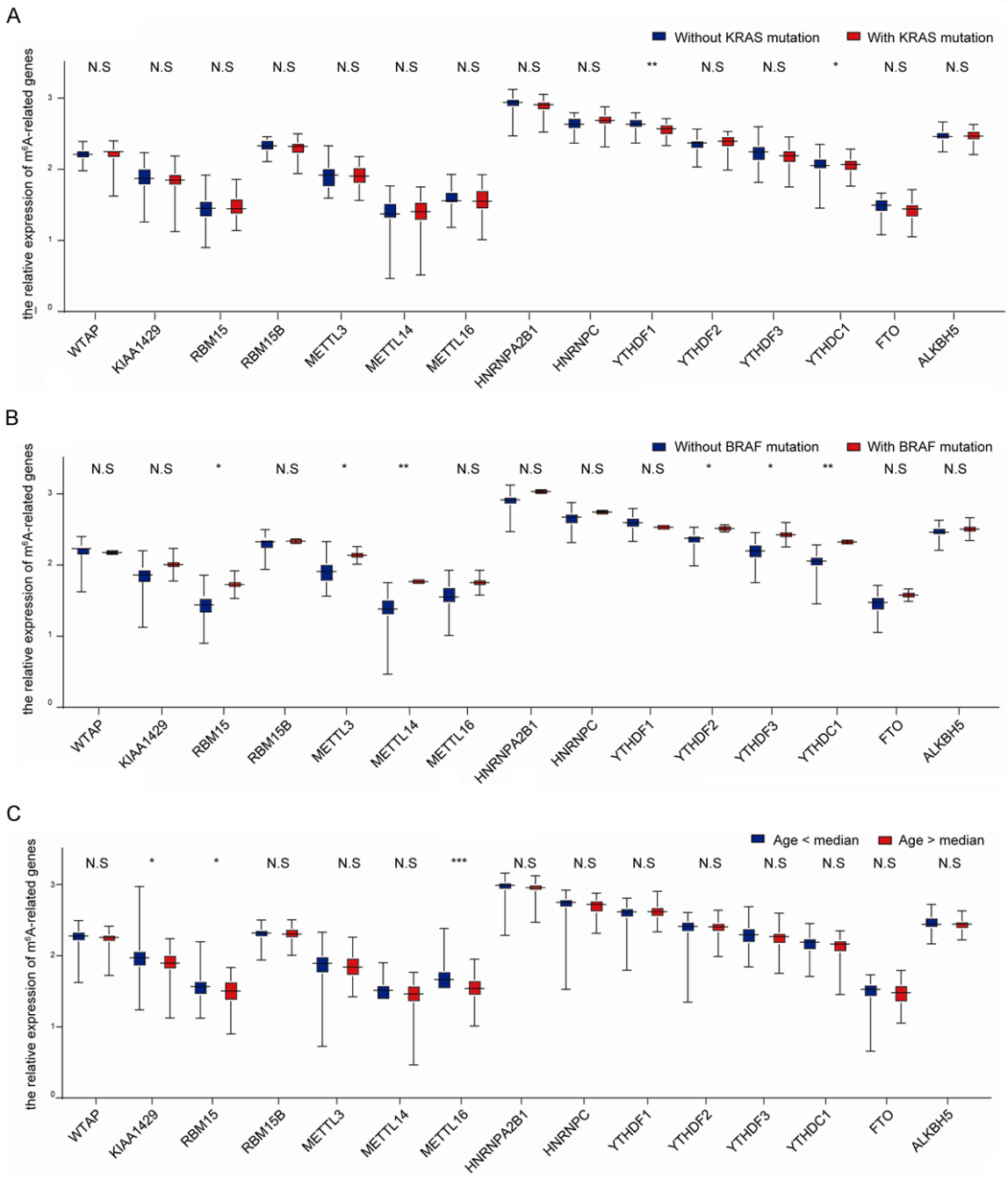


Figure 8. Relationship between m^{6}A-related gene expression and molecular/clinicopathological features in READ. A. The KRAS mutation was associated with YTHDF1 expression. B. An obvious linkage between the BRAF mutation and the expression of RBM15, METTL3, METTL14, YTHDF2, YTHDC1 and YTHDF3 was found. C. Age could affect the expression of KIAA1429, RBM15 and METTL16.

put sequencing, have enabled researchers to explore the implication of m^{6}A in human diseases, particularly cancer. An increasing number of studies have indicated that m^{6}A plays an essential role in the initiation and progression of tumors. Additionally, aberrant m^{6}A RNA methylation is closely associated with cancer, but the specific regulatory role of m^{6}A in tumorigenesis and cancer progression needs to be fully elucidated. In this manuscript, we provided an overall summary of the roles of m^{6}A in the regulation of CRC.

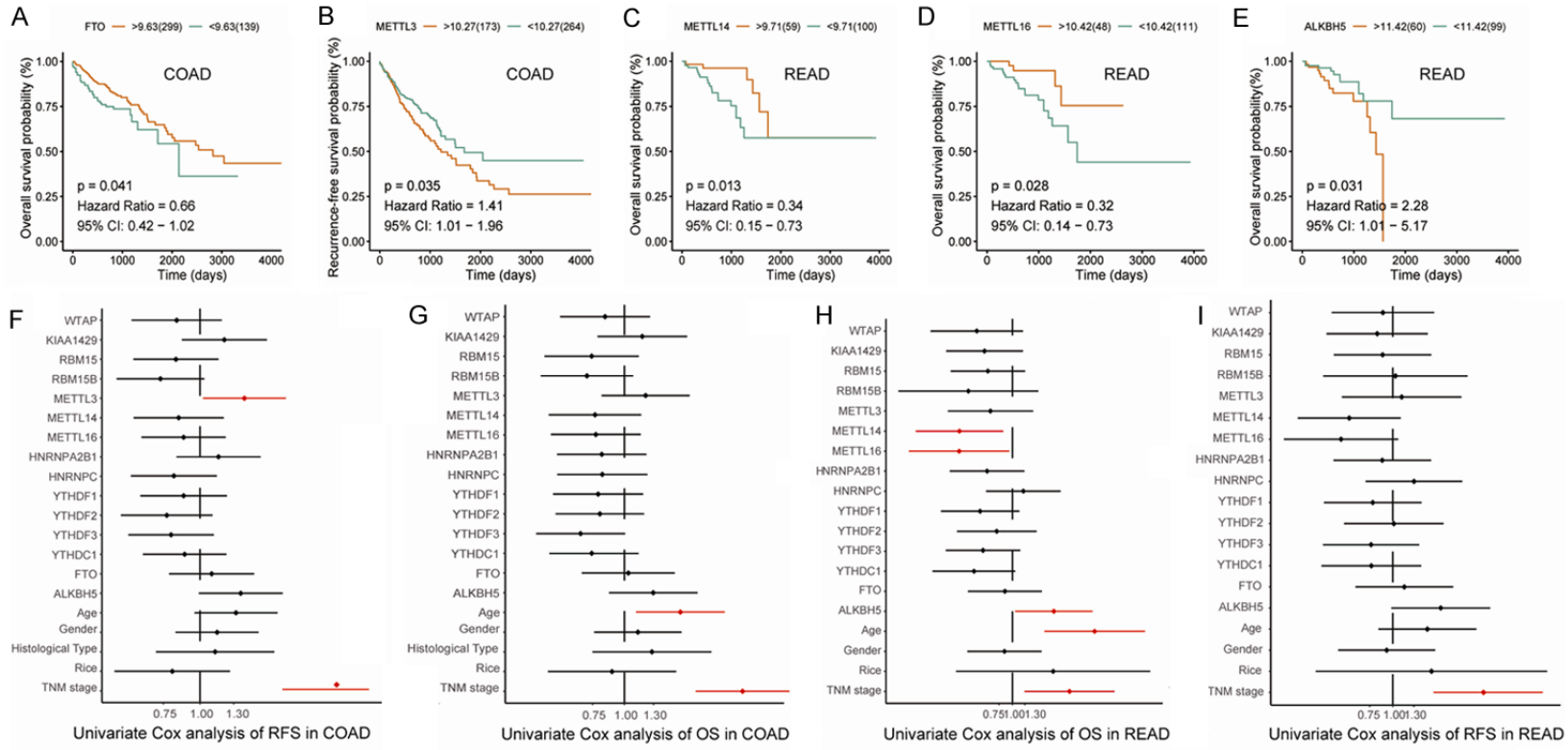


Figure 9. Kaplan-Meier curves and univariate and multivariate Cox regression analyses of the TCGA database. A. Low FTO expression predicted poor OS in COAD patients. B. High METTL3 expression predicted a shorter RFS in COAD patients. C. Low METTL14 expression predicted poor OS in READ patients. D. Low METTL16 expression predicted poor OS in READ patients. E. READ patients with high ALKBH5 expression had a shorter OS compared with those with low ALKBH5 expression. F. Univariate Cox regression analysis of the RFS of COAD patients. G. Univariate Cox regression analysis of the OS of COAD patients. H. Univariate Cox regression analysis of the OS of READ patients. I. Univariate Cox regression analysis of the RFS of READ patients.

Table 1. Multivariate Cox regression analysis of OS in COAD

Parameters	HR	95% CI	*P*-value
Age	2.055	1.353-3.121	0.001**
TNM stage	3.314	2.155-5.097	<0.0001***

**P*<0.01, ***P*<0.001.

Table 2. Multivariate Cox regression analysis of RFS in COAD

Parameters	HR	95% CI	*P*-value
METTL3	1.356	0.975-1.886	0.07
TNM stage	2.676	1.905-3.758	<0.0001***

***P*<0.001.

Table 3. Multivariate Cox regression analysis of OS in READ

Parameters	HR	95% CI	*P*-value
METTL14	0.133	0.034-0.524	0.004**
ALKBH5	6.013	2.244-16.111	<0.0001***
METTL16	0.303	0.048-1.905	0.203
Age	0.353	0.081-1.530	0.164
TNM stage	3.298	1.165-9.336	0.025*

**P*<0.05, ***P*<0.01, ****P*<0.001.

Analyses of the TCGA and GEO databases revealed that most of the m6A-related genes were dysregulated in CRC. The results revealed that higher expression of KIAA1429, RBM15B, RBM15, HNRNPA2B1, YTHDF1, YTHDF2 and METTL3 and weaker expression of ALKBH5, YTHDF3 and METTL14 in COAD and READ. In addition, we performed an IHC analysis to further substantiate the m6A-related gene expression patterns at the protein level based on the Human Protein Atlas and TMA cohort. The results were almost consistent and revealed that m6A-related genes were dysregulated in CRC tissues, which indicated that most of these genes might play an oncogenic role in CRC. Similar results were previously reported. Specifically, Chen *et al* found that METTL3 was upregulated in liver cancer and promoted liver cancer progression through the YTHDF2-dependent post-transcriptional silencing of SOCS2 [26]. Joao Lobo reported that RBM15B was highly expressed in urological tumors, such as prostate cancer, testicular germ cell tumors and papillary renal cell carcinoma [27]. In addition, METTL14 exhibited low expression in hepatocellular carcinoma [28], glioblastoma [29] and breast adenocarcinoma [30]. And ALKBH5 reportedly downregulated the motility of pancreatic cancer by demethylating the long non-coding RNA (lncRNA) KCNK15-AS1 [31]. Nishizawa Y revealed that high YTHDF1 expression was associated with poor prognosis and that its overexpression was driven by c-MYC in CRC [32]. We also demonstrated a significant relationship between the expression of many m6A-related genes and molecular/clinicopathological features in COAD, such as the KRAS and BRAF mutations, age, gender, race and TNM stage. However, we did not find a correlation between m6A-related gene expression and gender, race and TNM stage in READ. In addition, Kowk *et al* demonstrated that genetic alterations in m6A regulators could predict poorer survival and were associated with TP53 mutations in acute myeloid leukemia [33]. This study first verified the clinicopathological features related to the regulation of m6A-related genes and provided novel insights for further study, and the results demonstrate that m6A-related-genes might play a vital role in CRC.

Abundant studies have reported that the dysregulation of m6A-related genes is related to poor prognosis. For example, Liu *et al* verified that the m6A demethylase FTO facilitated tumor progression in lung squamous cell carcinoma by regulating the expression of MZF1 [34]. Chen *et al* revealed that bladder cancer patients with positive WTAP expression had a higher postoperative recurrence risk than those with negative WTAP expression [35]. In addition, ALKBH5 was reportedly a novel prognostic biomarker that predicted the prognosis of pancreatic cancer [36]. Ma *et al* found that hepatocellular carcinoma patients with reduced METTL14 expression experienced more frequent recurrence and poorer survival [28]. Consistent with these findings, using TCGA data, we found that high METTL3 expression and decreased regulation of METTL14, METTL16, FTO and ALKBH5 were positively correlated with poor prognosis in CRC patients. Additionally, univariate and multivariate analyses showed that age, the TNM stage and the expression of METTL14 and ALKBH5 were independent prognostic factors in CRC.

Conclusions

M6A-related genes were dysregulated in CRC, and their expression was associated with CRC progression and poor prognosis. The results of this study showed the value of m6A-related

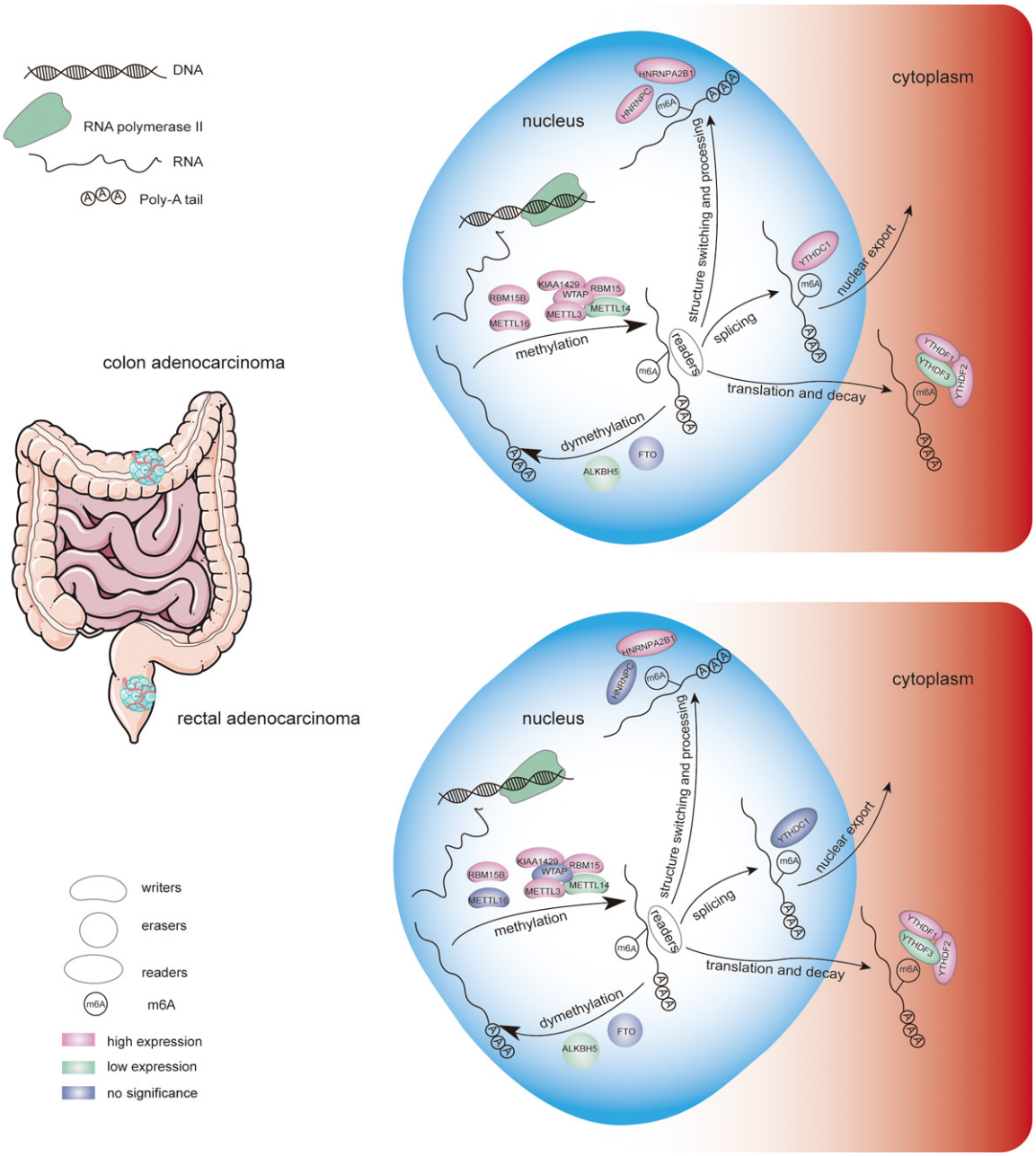


Figure 10. Mechanism of m^{6}A modification in CRC. M^{6}A RNA methylation is a dynamic and reversible process that affects almost every aspect of RNA metabolism and involves adenosine methyltransferases, demethylases, and RNA-binding proteins.

genes as clinical biomarkers in CRC and emphasized their potential as prognostic biomarkers in CRC patients.

Acknowledgements

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All the patients provided written informed consent, and the project was performed in accordance with the Helsinki Declaration of 1975. The clinical information of the patients utilized for this study is maintained in the databases of the First Affiliated Hospital of Zhengzhou University.

Disclosure of conflict of interest

None.

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Table S1. Characteristics of GEO databases used in this study

Cohort ID	Platform	Number of samples		Publication year	Country
		Nontumor	Tumor		
GSE20916	Affymetrix	79	66	Poland	2010
GSE41258	Affymetrix	103	255	Israel	2012
GSE41328	Affymetrix	10	10	USA	2012
GSE19249	Affymetrix	4	12	USA	2010
GSE33113	Affymetrix	6	90	Netherlands	2011
GSE68204	Agilent	21	59	Italy	2016
GSE87211	Agilent	160	203	USA	2017
Total		383	695		

Abbreviations: TNM = tumor node metastasis; GEO = Gene Expression Omnibus.

Table S2. Information on antibodies used in this study

Group	Antibody	IHC	IF	Specificity	Company
Writers	WTAP	1:500	1:200	Mouse Monoclonal	Proteintech Group, China
	KIAA1429	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China
	RBM15	1:500	1:200	Rabbit Polyclonal	Proteintech Group, China
	RBM15B	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China
	METTL3	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China
	METTL14	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China
	METTL16	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China
Erasers	FTO	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China
	ALKBH5	1:500	1:200	Rabbit Polyclonal	Proteintech Group, China
Readers	HNRNPA2B1	1:500	1:200	Rabbit Polyclonal	Proteintech Group, China
	HNRNPC	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China
	YTHDF1	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China
	YTHDF2	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China
	YTHDF3	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China
	YTHDC1	1:200	1:200	Rabbit Polyclonal	Proteintech Group, China

Abbreviations: IF = immunofluorescence; IHC = immunohistochemistry.

Supplementary Methods

The survival curves and the optimal cut-off values for each gene were obtained using R Studio. The details are as follows:

```
rm (list = ls ())

load (file = “combindmerge.rData”)

load (file = “merge.rData”)

load (file = “OSmerge.rData”)

load (file = “PFSmerge.rData”)

library (survival)

library (survminer)

library (ggplot2)

OS <-as.data.frame (combindmerge [,c (“OS”, “OStime”)])

OS <-cbind (OS, merge)

 OS <-na.omit (OS)

names (OS) [1] <- “fustat”

names (OS) [2] <- “futime”

save (OS, file = ‘OSmerge.rData’)

PFS <-as.data.frame (combindmerge [,c (“PFS”, “PFStime”)])

PFS <-cbind (PFS, merge)

PFS <-na.omit (PFS)

 names (PFS) [1] <- “fustat”

names (PFS) [2] <- “futime”

save (PFS, file = ‘PFSmerge.rData’)

a <-OS [,1:2]

b <-OS [,c (“genename”)]

svdata <-cbind (a,b)

replacecolumnName <- function (Matrix, oldname, newname){

   index = which (colnames (Matrix) == oldname)

   colnames (Matrix) [index] = newname

   return (Matrix)

}
```

```
svdata <-replacecolumnName (svdata, “gene name”, “new name”)
library (survival)
library (survminer)
res.cut <- surv_cutpoint (svdata, time = “futime”,
                                  event = “fustat”,
                                  variables = names (svdata) [3:ncol (svdata)],
                                 minprop = 0.3)
res.cat <- surv_categorize (res.cut)
my.surv <- Surv (res.cat$futime, res.cat$fustat)
pl <-list ()
for (i in colnames (res.cat) [3:ncol (svdata)]) {
  group <- res.cat [,i]
  survival_dat <- data.frame (group = group)
  fit <- survfit (my.surv ~ group)
```

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Expression patterns and prognostic value of m6A related genes in colorectal cancer

Authors:
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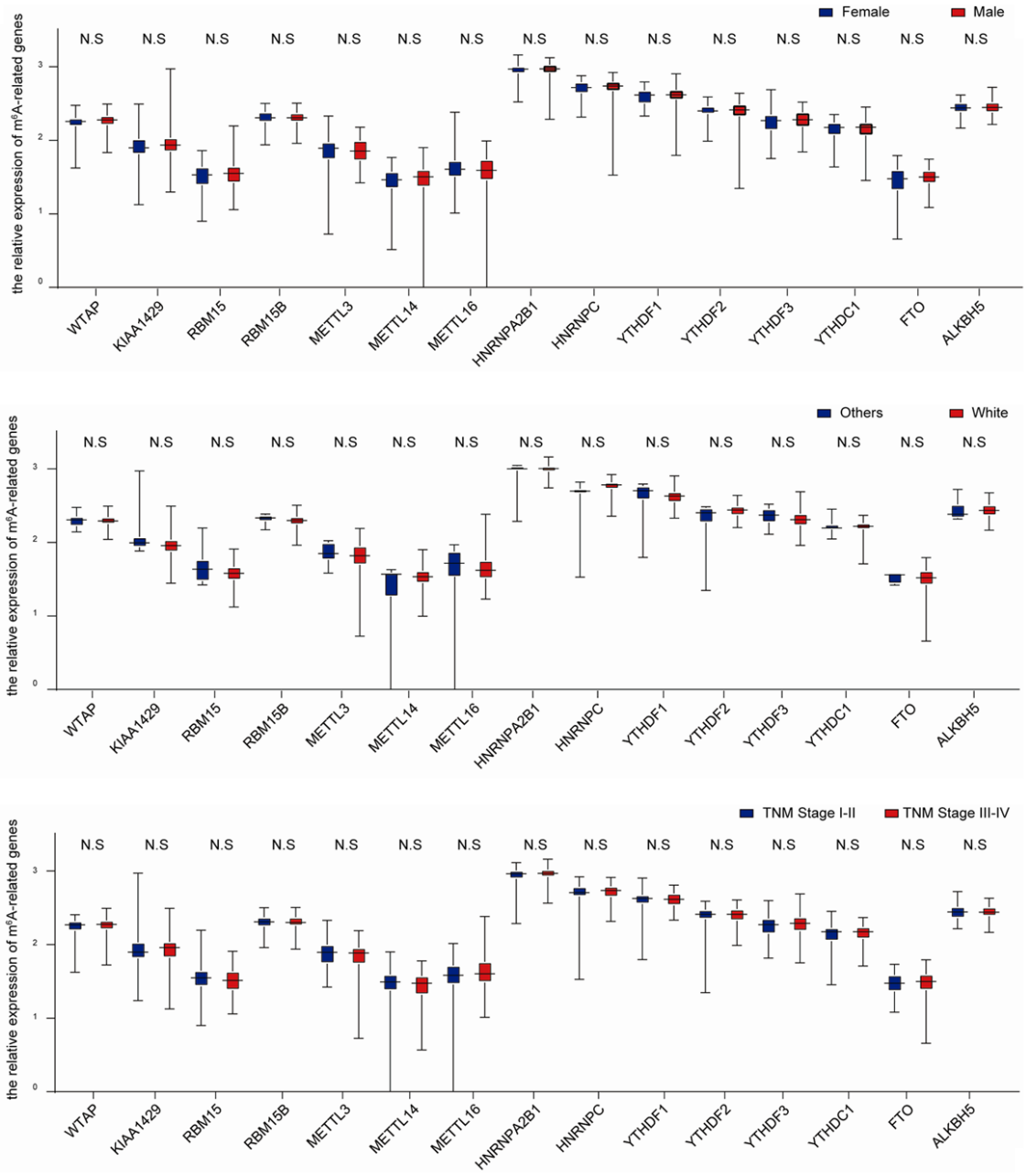

Figure S1. No correlation was found between m^{6}A-related gene expression and gender, race and TNM stage in READ.

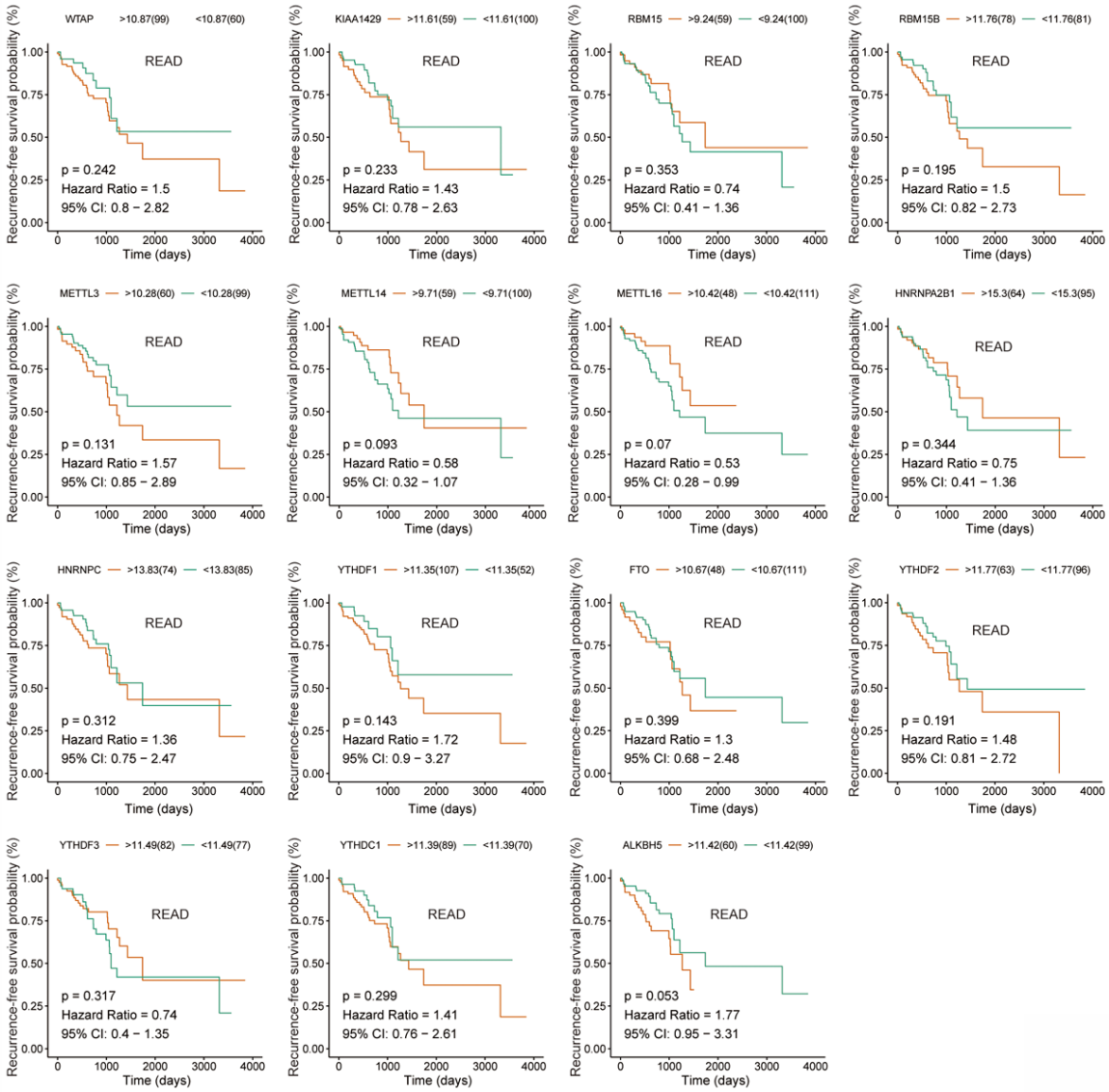

Figure S2. Kaplan-Meier survival curves showing the stratification of RFS of READ patients in the TCGA dataset based on m^{6}A-related gene expression (red: high expression; green: low expression).

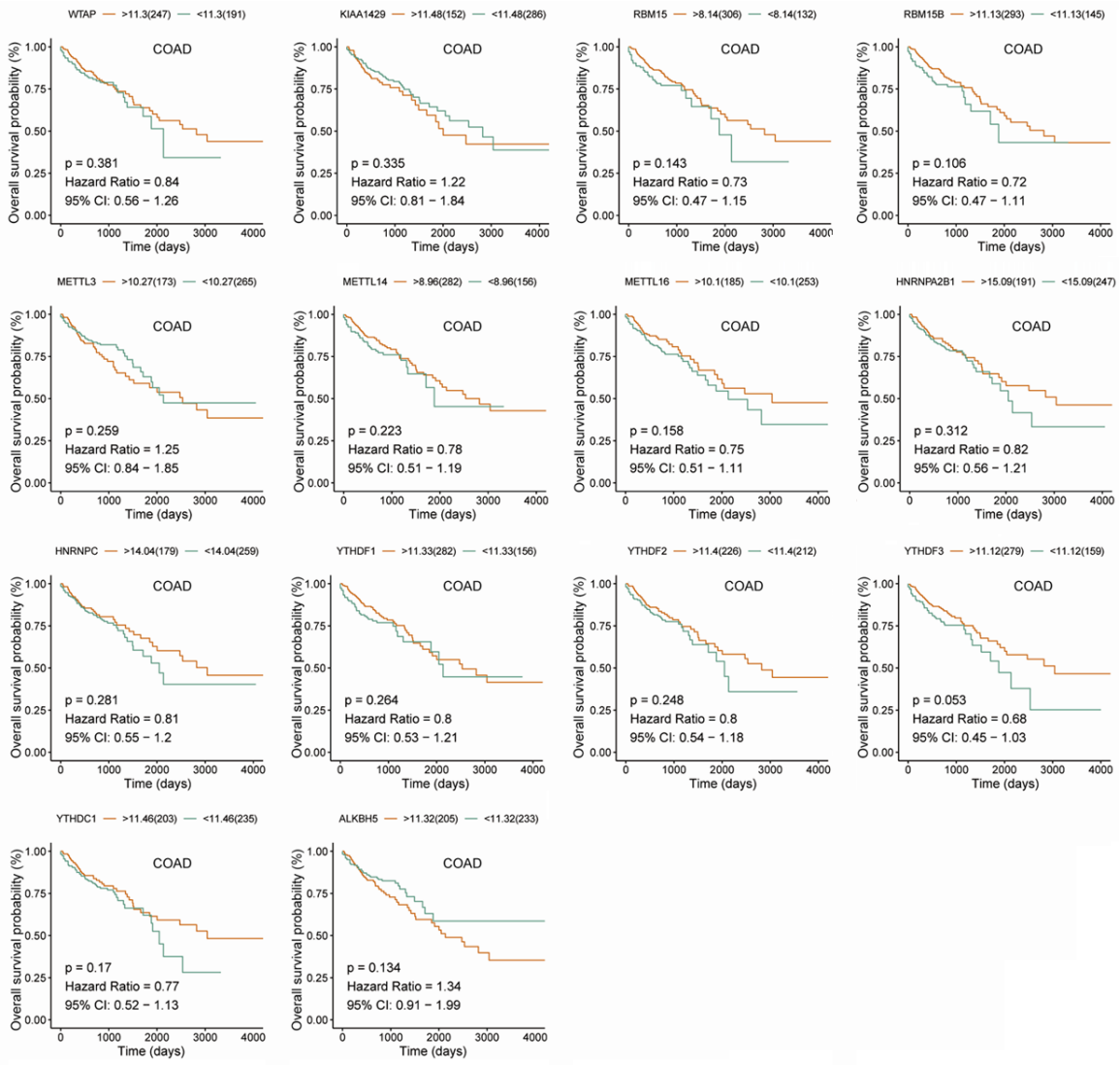

Figure S3. Kaplan-Meier survival curves showing the stratification of OS of COAD patients in the TCGA dataset based on m^{6}A-related gene expression (red: high expression; green: low expression).

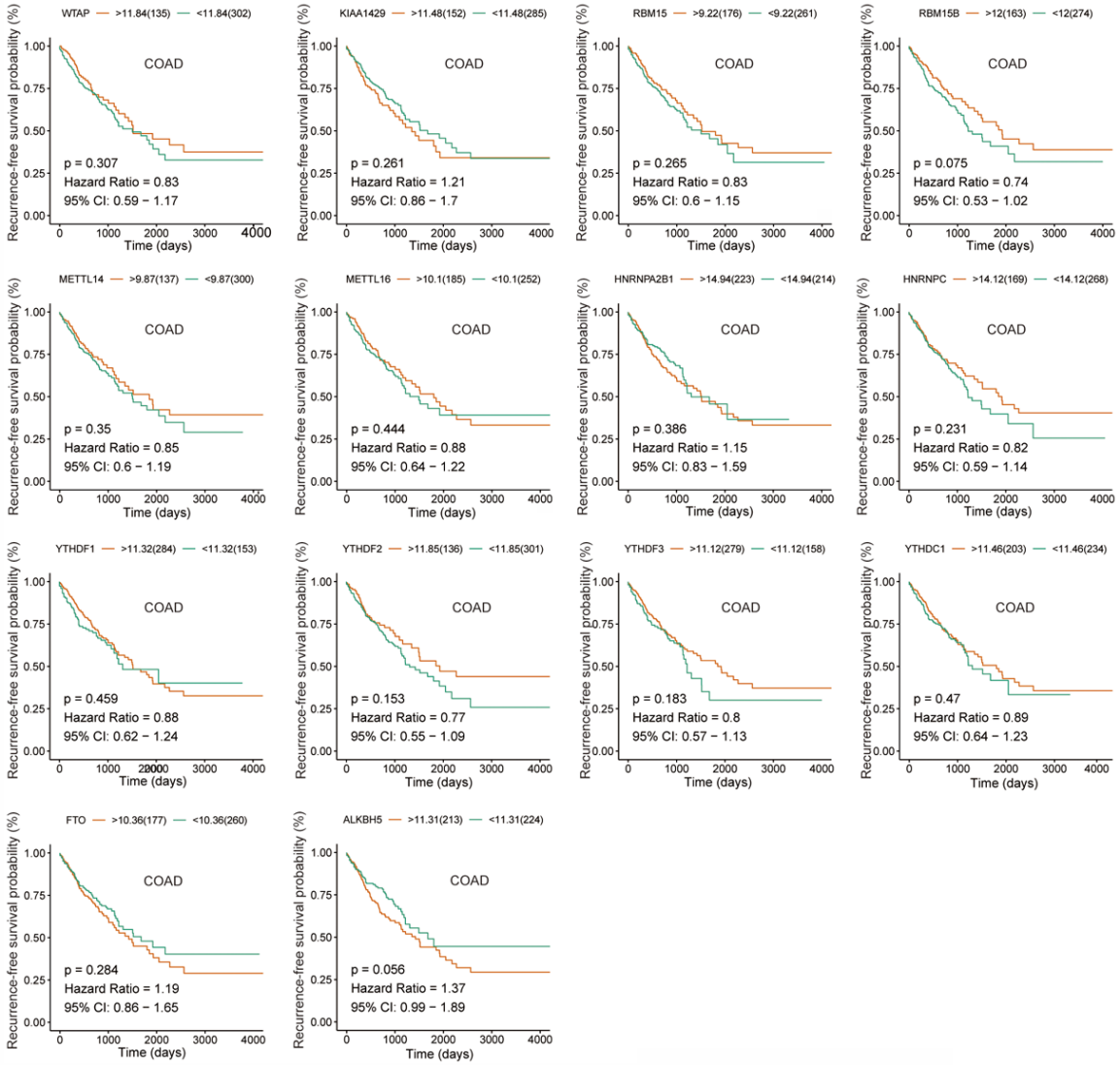

Figure S4. Kaplan-Meier survival curves showing the stratification of RFS of COAD patients in the TCGA dataset based on m^{6}A-related gene expression (red: high expression; green: low expression).

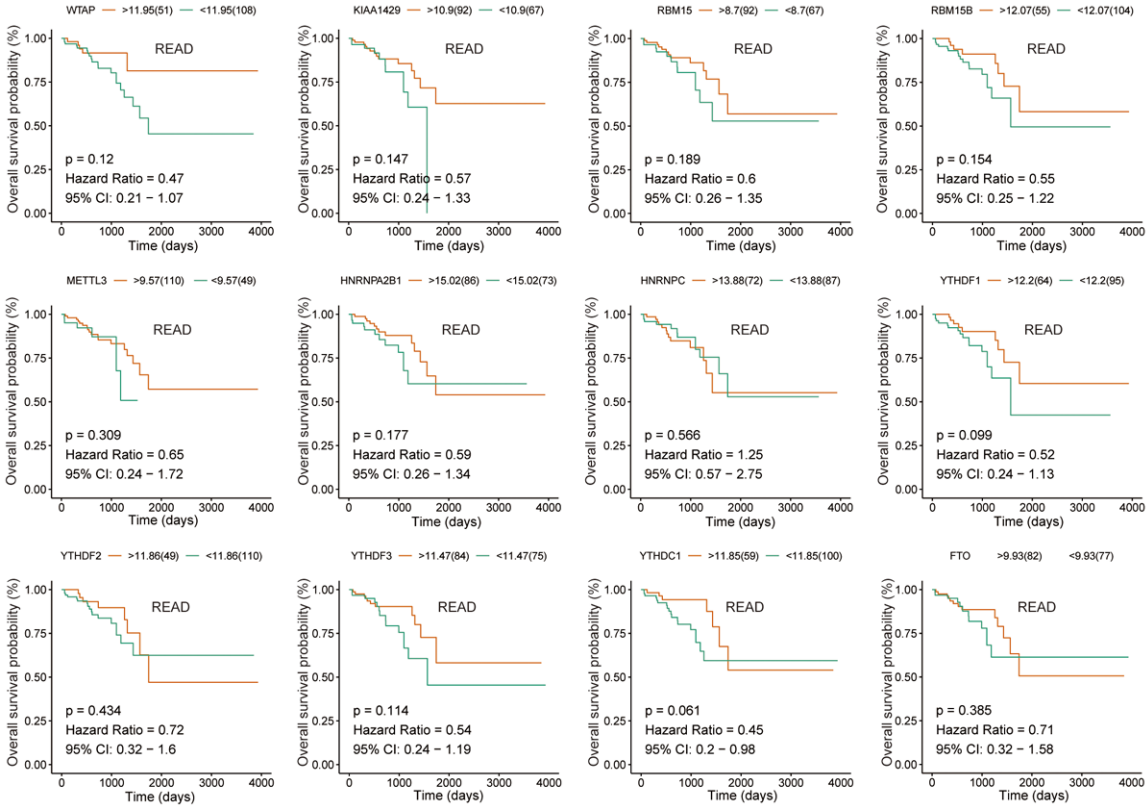

Figure S5. Kaplan-Meier survival curves showing the stratification of OS of READ patients in the TCGA dataset based on m^{6}A-related gene expression (red: high expression; green: low expression).

Table S3. Univariate Cox regression analysis of OS and RFS in COAD patients

Parameters	OS			RFS		
	HR	95% (CI)	*P* value	HR	95% (CI)	*P* value
WTAP	0.839	0.560-1.258	0.396	0.832	0.585-1.184	0.306
KIAAA1429	1.174	0.784-1.757	0.436	1.209	0.867-1.686	0.263
RBM15	0.744	0.486-1.137	0.172	0.827	0.592-1.154	0.264
RBM15B	0.713	0.470-1.081	0.111	0.733	0.520-1.033	0.076
METTL3	1.210	0.815-1.797	0.345	1.415	1.023-1.958	0.036*
METTL14	0.767	0.506-1.163	0.211	0.845	0.593-1.203	0.350
METTL16	0.772	0.515-1.157	0.210	0.879	0.632-1.222	0.442
HNRNPA2B1	0.815	0.545-1.219	0.319	1.155	0.831-1.605	0.390
HNRNPC	0.818	0.545-1.228	0.334	0.814	0.581-1.140	0.231
YTHDF1	0.788	0.526-1.182	0.250	0.879	0.626-1.233	0.455
YTHDF2	0.801	0.538-1.193	0.275	0.770	0.538-1.102	0.153
YTHDF3	0.673	0.451-1.005	0.053	0.796	0.570-1.113	0.183
YTHDC1	0.759	0.508-1.135	0.179	0.886	0.639-1.229	0.467
FTO	1.035	0.681-1.573	0.871	1.095	0.785-1.526	0.594
ALKBH5	1.296	0.870-1.930	0.202	1.375	0.990-1.910	0.057
Age	1.656	1.109-2.472	0.014*	1.325	0.957-1.834	0.090
Gender	1.129	0.762-1.672	0.545	1.143	0.826-1.581	0.420
Histological type	1.281	0.749-2.190	0.366	1.125	0.708-1.787	0.619
Rice	0.893	0.501-1.592	0.702	0.804	0.512-1.264	0.345
TNM stage	2.902	1.902-4.428	0.000***	2.679	1.908-3.761	0.000***

Abbreviations: TNM = tumor node metastasis; OS = overall survival; RFS = recurrence-free survival; COAD = colon adenocarcinoma; CI = confidence interval, *P<0.05, ***P<0.001.

Table S4. Univariate Cox regression analysis of OS and RFS in READ patients

Parameters	OS			RFS		
	HR	95% (CI)	*P* value	HR	95% (CI)	*P* value
WTAP	0.466	0.175-1.246	0.128	0.883	0.466-1.672	0.702
KIAAA1429	0.550	0.243-1.243	0.151	0.823	0.437-1.547	0.545
RBM15	0.591	0.268-1.302	0.192	0.880	0.480-1.615	0.681
RBM15B	0.390	0.088-1.730	0.215	1.033	0.420-2.540	0.944
METTL3	0.624	0.252-1.546	0.309	1.117	0.530-2.354	0.770
METTL14	0.323	0.127-0.819	0.017*	0.580	0.306-1.101	0.096
METTL16	0.321	0.110-0.935	0.037*	0.524	0.257-1.067	0.075
HNRNPA2B1	0.581	0.263-1.284	0.180	0.877	0.478-1.610	0.671
HNRNPC	1.258	0.570-2.779	0.570	1.303	0.713-2.378	0.389
YTHDF1	0.501	0.218-1.152	0.104	0.779	0.424-1.431	0.420
YTHDF2	0.714	0.306-1.663	0.435	1.013	0.544-1.883	0.969
YTHDF3	0.533	0.242-1.176	0.119	0.762	0.418-1.388	0.374
YTHDC1	0.440	0.182-1.062	0.068	0.765	0.411-1.422	0.397
FTO	0.849	0.387-1.866	0.684	1.154	0.628-2.122	0.644
ALKBH5	2.411	1.056-5.506	0.037*	1.819	0.983-3.367	0.057
Age	5.750	1.967-16.806	0.001**	1.538	0.836-2.831	0.167
Gender	0.842	0.383-1.851	0.669	0.926	0.506-1.695	0.804
Rice	2.374	0.301-18.710	0.412	1.620	0.382-6.863	0.512
TNM stage	3.364	1.289-8.776	0.013*	3.285	1.661-6.497	0.001***

Abbreviations: TNM = tumor node metastasis; OS = overall survival; RFS = recurrence-free survival; READ = rectal adenocarcinoma; CI = confidence interval, *P<0.05, **P<0.01, ***P<0.001.