

Research Paper

N6-methyladenosine RNA methylation regulators contribute to the progression of prostate cancer

Qunying Wu^{1*}, Xing Xie^{2*}, Yiming Huang^{3*}, Shanshan Meng², Youcheng Li⁴, Huifeng Wang¹✉ and Yanling Hu³✉

1. Department of Biochemistry and Molecular Biology, School of Pre-Clinical Medicine, Guangxi Medical University, Nanning, Guangxi, 530021, China.

2. Life Sciences Institute, Guangxi Medical University, Nanning, Guangxi, 530021, China.

3. Center of Genomic and Personalized Medicine, Guangxi Medical University, Nanning, Guangxi, 530021, China.

4. Department of Clinical Laboratory, Guigang City People's Hospital, The Eighth Affiliated Hospital of Guangxi Medical University, Guigang, Guangxi, 537100, China.

*These authors contributed equally to this work.

✉ Corresponding authors: E-mail: Yanling Hu, huyanling@gxmu.edu.cn; Huifeng Wang, wanghuifeng@gxmu.edu.cn.

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Abstract

Prostate cancer (PCa) is one of the most common epithelial malignant tumors and the fifth leading cause of cancer death in men. An increasing number of studies have demonstrated that N6-methyladenosine (m⁶A) plays a crucial role in tumorigenesis and tumor development. However, little is known about the role and levels of common m⁶A regulators and m⁶A levels in PCa. In this study, we analyzed the characteristic expression of m⁶A regulators in PCa and castration-resistant prostate cancer (CRPC). UALCAN and cBioPortal were used to estimate the clinical value and genetic alterations of m⁶A regulators, respectively. The correlation between m⁶A regulators and androgen receptor (AR) was assessed using Gene Expression Profiling Interactive Analysis (GEPIA) by Pearson correlation statistics. Total m⁶A levels were detected in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice and PCa cell lines. Results showed that the expression of methyltransferase-like 3 (METTL3) and YTH domain family members, namely, YTHDC2, YTHDF1, and YTHDF2 were generally upregulated in PCa, whereas those of fat mass and obesity-associated protein (FTO), AlkB homolog 5 (ALKBH5), and methyltransferase-like 14 (METTL14) were downregulated. The expression of METTL3, METTL14, Wilms' tumor 1-associating protein (WTAP), YTHDC2, YTHDF1, and YTHDF2 were remarkably higher in CRPC with lymph node metastasis than that in CRPC with bone metastasis, whereas ALKBH5, FTO, and YTHDF3 significantly decreased in CRPC with lymph node metastasis tissues. YTHDF1, YTHDF2, and YTHDC2 were positively correlated with the Gleason grades of PCa, and METTL14, FTO, and ALKBH5 were negatively associated with the Gleason classification. M⁶A regulators were positively correlated with AR. Patients with a genomic alteration of m⁶A were associated with poor disease-free survival (DFS). The total m⁶A levels in TRAMP mice increased dramatically compared with those in tumor-free mice, and m⁶A levels in LNCaP cell lines were higher than DU145 and PC3 cell lines. In summary, METTL3, METTL14, ALKBH5, FTO, YTHDC2, YTHDF1, and YTHDF2 were abnormally expressed in PCa and related to Gleason classification. Changes in m⁶A levels maybe contributed to the development and progression of PCa.

Key words: prostate cancer, N6-methyladenosine, castration-resistant prostate cancer, tumorigenesis and progression, androgen receptor

Introduction

More than 160 posttranscriptional modifications of cellular RNAs have been discovered [1], and N6-methyladenosine (m⁶A) is the most prevalent RNA modification of mRNA and non-coding RNA in eukaryotic cells [2]. M⁶A methylation regulators include methyltransferase complex, RNA

demethylases, and specific RNA-binding proteins, which are also known as “writers,” “erasers,” and “readers,” respectively [3]. Methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14), and Wilms' tumor 1-associating protein (WTAP) act as writers in mammalian cells [4, 5]. AlkB homolog 5

(ALKBH5) and fat mass and obesity-associated protein (FTO) are m⁶A demethylases that mediate the reversal of m⁶A methylation [6, 7]. The RNA modifications of m⁶A are recognized by “reader” proteins. These proteins are the YTH domain family members, including YTHDC1, YTHDC2, YTHDF1, YTHDF2, and YTHDF3 [8]. Growing evidence demonstrates that the aberrant expression of m⁶A RNA methylation is closely related to various cancers, such as hepatocellular carcinoma (HCC) [9], gastric cancer (GC) [10, 11], endometrial cancer [12], and glioblastoma [13]. Targeting FTO/m⁶A/MYC/CEBPA signaling exerts an anti-tumor effect in most leukemia samples [14]. The knockout of METTL14 substantially decreases the growth of Epstein–Barr virus-associated tumorigenesis in a xenograft model [15]. These studies indicate that m⁶A modification plays a critical role in cancer and maybe a potential target for cancer therapy.

Prostate cancer (PCa) is one of the most common epithelial malignant tumors in aging males worldwide and the fifth leading cause of cancer death in men [16, 17]. Androgen deprivation therapy (ADT) is the primary treatment for patients with advanced PCa, however, most patients eventually develop resistance to castration-resistant prostate cancer (CRPC) [18]. Despite the emergence of new therapeutic approaches, patients with metastatic PCa have a low quality of life and a low 5-year survival rate of less than 30% [19, 20]. The high morbidity and mortality of PCa necessitate more work to reveal its underlying mechanisms. Recent evidence suggests that m⁶A modification is closely connected to PCa. VIRMA and YTHDF3 are increased in stages III/IV than in stage II in PCa [21]. Li *et al.* [22] found that YTHDF2 is highly expressed in PCa and the downregulation of YTHDF2 considerably increases the level of m⁶A and inhibits the proliferation and migration of PCa cell lines. Cai *et al.* [23] reported that the silencing of METTL3 by shRNA inhibits cell proliferation, colony formation, and invasion of PCa cells and inhibits the growth of tumors *in vivo*. However, little is known about the role of different m⁶A regulators in the development and progression of PCa. In this study, we evaluated the expression of m⁶A regulators, the correlation between m⁶A regulators and clinical outcomes, and the genetic mutations of these regulators in PCa. We also analyzed the role of m⁶A levels in PCa by *in vivo* and *in vitro* experiments.

Materials and methods

Expression data sets

The Cancer Genome Atlas (TCGA)-Prostate

adenocarcinoma (TCGA-PRAD) cohort data and the gene expression of 495 patients with PCa (499 prostate tissues) and 52 normal samples were obtained from the National Cancer Institute Genomic Data Commons (<https://portal.gdc.cancer.gov/>). R version 3.6.3 software was utilized to normalize and process the expression data. OncoPrint database (<https://www.oncoPrint.org/>) is a powerful data-mining platform that contains 715 independent datasets and the data of 86,733 tumor tissues and adjacent normal tissues [24]. In this study, we analyzed the mRNA expression levels of m⁶A regulators in different types of cancer by OncoPrint data mining. The search parameters were as follows: type of analysis: cancer versus normal tissues; type of data: mRNA; *P* value: 0.05. The Human Cancer Metastasis Database (HCMDB, <http://hcmdb.i-sanger.com/index>) [25] is an online integrated database for storing and analyzing the large-scale expression data of cancer metastasis. We selected the data set of EXP00339 in HCMDB (dataset ID: GSE74685), which contains 89 CRPC samples that comprise 20 samples of bone metastasis and 69 of lymph node metastasis, to explore the expression of m⁶A regulators in CRPC with different metastasis sites.

M⁶A regulators and Gleason classification

UALCAN (<http://ualcan.path.uab.edu>) is an interactive online portal providing the gene expression and clinical analysis of tumor subsets, using TCGA level 3 RNA sequencing and clinical data from 31 cancer types [26]. UALCAN was implemented to analyze the relative expression of m⁶A regulators in different Gleason classifications to understand the clinical importance of m⁶A regulators in PCa.

M⁶A regulators and androgen receptor (AR)

The Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia.cancer-pku.cn/index.html>) [27] is a website for TCGA cancer and the Genotype-Tissue Expression profile analysis and interaction analysis. GEPIA can be used to perform the correlation analysis of gene pairs by Spearman, Pearson, or Kendall correlation statistics. The correlation between m⁶A regulators and AR was analyzed using GEPIA by Pearson correlation statistics.

Genetic alteration of m⁶A regulators in PCa

CBioPortal (<http://www.cbioportal.org/>) is an open-access website that explores, visualizes, and analyzes multidimensional cancer genomics data. We selected eight studies (Prostate Adenocarcinoma [Broad/Cornell, Nat Genet 2012], Prostate

Adenocarcinoma [Fred Hutchinson CRC, Nat Med 2016], Prostate Adenocarcinoma [MSKCC, PNAS 2014], Prostate Adenocarcinoma [TCGA, Firehose Legacy], Prostate Adenocarcinoma [CPC-GENE, Nature 2017], Prostate Adenocarcinoma [MSKCC, Cancer Cell 2010], Prostate Adenocarcinoma [MSKCC/DFCI, Nature Genetics 2018], and Prostate Cancer [DKFZ, Cancer Cell 2018]) to analyze the genetic alterations of m⁶A regulators in PCa. Disease-free survival (DFS) curve was drawn by the Kaplan-Meier method, and the difference in DFS was evaluated by log-rank test.

Cell lines and culture conditions

Three human PCa cell lines were used in this study: PC3, DU145, and LNCaP. PC3 and DU145 cell lines were obtained from the Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences. LNCaP cell line was acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The basal medium was RPMI-1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco), 1% penicillin and 1% streptomycin (Invitrogen). LNCaP cells were cultured in basic medium supplemented with 1.0 mM L-glutamine and 1.0 mM sodium pyruvate (Invitrogen). All cells were incubated in a humidified incubator at 37°C and 5% CO₂ (Thermo Scientific).

TRAMP mice

Hemizygous male transgenic adenocarcinoma of the mouse prostate (TRAMP) mice (8247 Ng/J) and wild-type C57BL/6 females were obtained from the Model Animal Research Center of Nanjing University (MARCNU, Nanjing, Jiangsu, China). The mice were reared and propagated by the MARCNU protocol. Male offspring were genotyped by polymerase chain reaction (PCR) according to protocol. All animal experiments were performed following institutional guidelines and approved by the Institutional Animal Care and Use Committee of Guangxi Medical University.

Detection of total m⁶A levels in TRAMP mice and PCa cell lines

Twelve TRAMP mice were randomly divided into four groups with three mice in each group. The prostate tissues of the mice at 8, 16, 26, and 36 weeks were collected for RNA extraction. Total RNA was extracted from the prostate tissues and PCa cell lines by TRIzol Reagent (Thermo Fisher Scientific). EpiQuik m⁶A RNA Methylation Quantification Kit (EpiGentek) was used to detect the total m⁶A RNA methylation levels.

Statistical analysis

Statistical data were analyzed using GraphPad Prism version 7.0 (GraphPad Software, USA). An independent t-test was used to evaluate the expression of m⁶A regulators between PCa tissues and normal tissues, and the results were presented as the mean ± SD. Differences were considered statistically significant if $P < 0.05$.

Results

M⁶A regulators in PCa were distinct from normal tissues

We extracted the mRNA expression matrix of 10 common m⁶A regulators from TCGA-PRAD to evaluate their expression in PCa. The results showed that METTL3, a chief component of m⁶A methyltransferase, was remarkably increased in PCa tissues than in normal controls ($P < 0.05$, Figure 1A, 1B). YTHDC2, YTHDF1, and YTHDF2 were also substantially higher in PCa tissues than in normal controls, whereas the expression of FTO, ALKBH5, and METTL14 decreased in PCa. Then, the correlation among m⁶A regulators was analyzed. Results indicated that METTL14 was positively correlated with YTHDC1, YTHDF2, and YTHDC2, and YTHDC1, YTHDC2, YTHDF2, and YTHDF1 were positively correlated with each other (Figure 1C).

We examined the mRNA levels of m⁶A regulators in different PCa datasets based on the Oncomine database to confirm the expression patterns of the m⁶A regulators in PCa. The results showed that the expression trends of the 10 common m⁶A regulators were consistent with the TCGA-PRAD (Figure 2). METTL3 was highly expressed in 10 PCa datasets, and FTO and ALKBH5 were downregulated in nine and six datasets, respectively.

Differential expression profiles of m⁶A regulators in CRPC

We detected the mRNA levels of m⁶A regulators in CRPC with different metastasis sites using HCMDDB to further examine their expression in PCa. The results showed that, except YTHDC1, the expression of m⁶A regulators was statistically significant at different CRPC metastasis sites ($P < 0.01$, Figure 3). The expression of METTL3, METTL14, WTAP, YTHDC2, YTHDF1, and YTHDF2 was remarkably higher in CRPC with lymph node metastasis than in CRPC with bone metastasis, whereas ALKBH5, FTO, and YTHDF3 were substantially decreased in CRPC with lymph node metastasis.

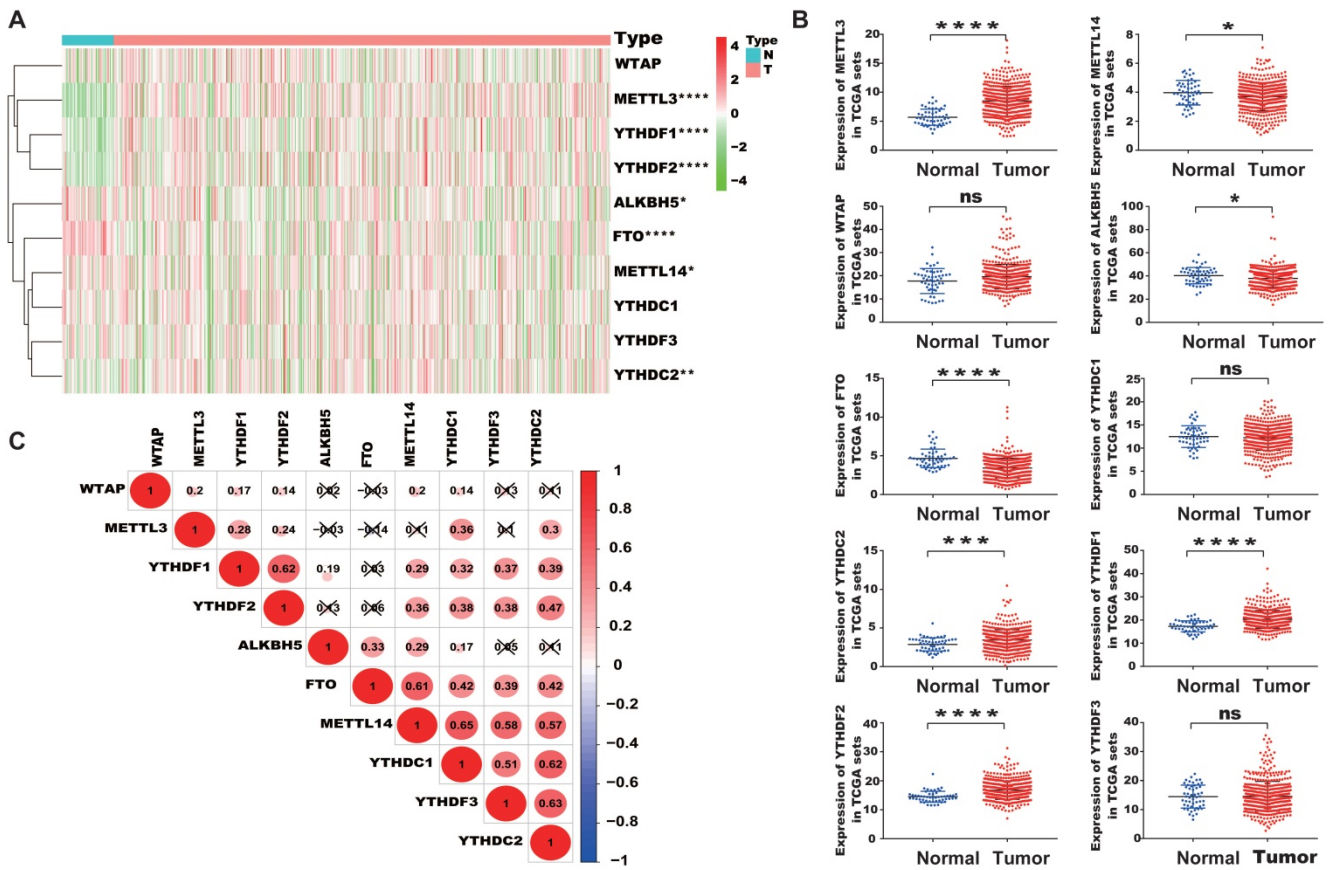


Figure 1. Characteristic expression of m⁶A regulators in PCa. (A) Heatmap of the expression of different m⁶A regulators between PCa tissues and normal tissue samples. (B) Scatter plots of the expression of m⁶A regulators in PCa. (C) Spearman correlation analysis of m⁶A regulators in PCa (Red and green colors in the plots symbolized relatively high and low expression, respectively).

Analysis Type of Cancer	Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal	
	METTL3	METTL14	WTAP	FTO	ALKBH5	YTHDC1	YTHDC2	YTHDF1	YTHDF2	YTHDF3	METTL3	METTL14	WTAP	FTO	ALKBH5	YTHDC1	YTHDC2	YTHDF1	YTHDF2	YTHDF3
Bladder Cancer	4	1	2	2	3	9	2	1		4	3	5	1	5	4		3	1	3	4
Brain and CNS Cancer	11	2	9	5	18	13	6	13	5	9	14	9	9	6	9		6	7	19	4
Breast Cancer	3	20	8	9	16	27	2	25	11	13	12	30	11	16	24	1	25	7	11	11
Cervical Cancer	4		1	3	2	6	1	1	2	5	2	5	1	4	4		5		4	
Colorectal Cancer	16	13	7	18	14	15	14	2	10	22	14	21	13	18	16		16	14	13	9
Esophageal Cancer	3	2		2	3	6	4		1	2		7	1	5	2		1	2	4	1
Gastric Cancer	6	4	6	10	6	9	4	4	3	12	4	11	4	6	14		4	6	19	
Head and Neck Cancer	11	7	4	3	14	8	4	4	7	6	5	12	6	7	4		11	1	15	1
Kidney Cancer	6	7	1	3	9	5	13		5		6	8	7	5	3		5	10	9	
Leukemia	9	6	7	2	15	9	15	2	12	6	10	10	5	9	4		10	2	11	14
Liver Cancer	7			3	4	8		6	1	5	7	9	6	1	3		5	5	6	1
Lung Cancer	6	5		6	4	15	1	17	4	8	6	14	2	14	8		4	3	7	5
Lymphoma	6	15	7	6	9	19	11	3	12	9	8	28	6	20		3	2	12	4	16
Melanoma			2	1	4	2	2		1		4	1	3	1		1	1	1	1	2
Myeloma	2	2	2	2	2	1	2	2	1	3	4	1	4	1		1	3	1	1	1
Other Cancer	12	7	3	5	19	2	10	8	6	15	10	10	9	12	3		15	3	15	3
Ovarian Cancer	6	3		3	3	11		9		2	12	5	7	3			6	2	2	4
Pancreatic Cancer	4		1		2	5	4		3	4	1	3	3		4	1	3	1	3	3
Prostate Cancer	10		1	2	6	5		9		6	3	6	3	2	3	1	4	1	8	1
Sarcoma	9	1	1		5	8	1	8	3		7	6	13	2	2		10		9	
Significant Unique Analysis	134	93	60	84	152	185	96	116	86	132	116	211	109	139	111	7	138	79	163	78
Total Unique Analyses	374		264		445		418		312		431		425		180		389		392	

Figure 2. Transcriptional levels of m⁶A regulators in PCa (OncoPrint). Arabic numbers in the figure showed the numbers of datasets in which m⁶A regulator expression was significantly upregulated (red) or downregulated (blue) in PCa tissues compared with adjacent normal tissues.

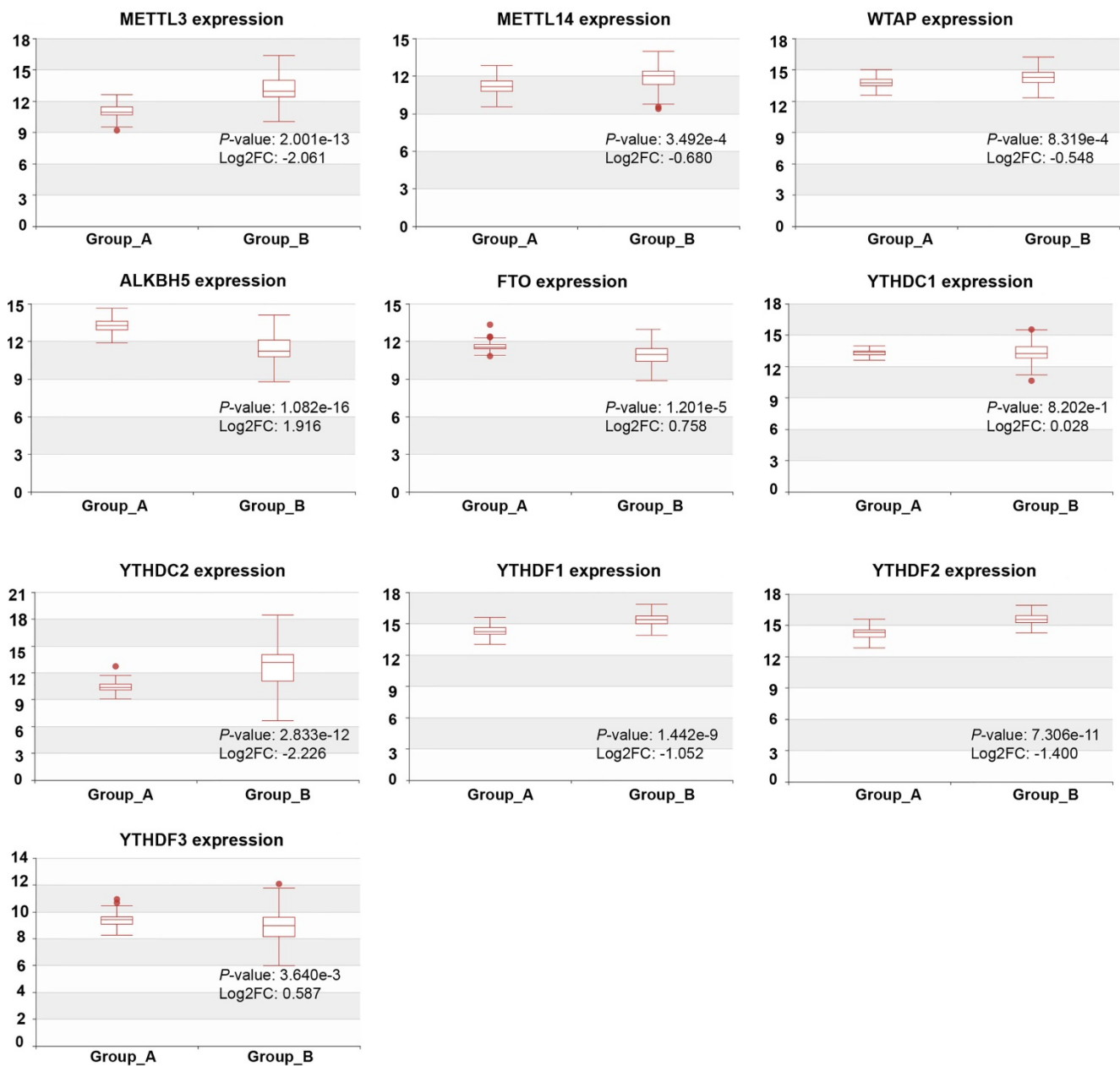


Figure 3. Expression of m⁶A regulators in CRPC with different metastasis sites. Group_A, metastasis tumor of CRPC with bone metastasis; Group_B, metastasis tumor of CRPC with lymph node metastasis experiment.

Correlation between the expression of m⁶A regulators and Gleason classification

The correlation between the expression of m⁶A regulators in PCa and Gleason classification was assessed using the UALCAN database. The results were shown in Figure 4. YTHDF1, YTHDF2, and YTHDC2 were positively correlated with Gleason grade, and the mRNA level of METTL3 was higher in patients with different Gleason grades of PCa than in normal samples. METTL14, FTO, and ALKBH5 were negatively associated with the Gleason classification of PCa. These results suggested that the mRNA expression of m⁶A regulators was related to the

clinical features of patients with PCa.

Correlation of m⁶A regulators and AR

AR is a primary oncogene driver of PCa and plays a key role in the development and progression of PCa [28, 29]. The progress of CRPC still depends on continuous AR signals [30]. The relationship of the expression of m⁶A regulators and AR was assessed by GEPIA, and the results were presented in the scatter diagrams in Figure 5. Surprisingly, all the m⁶A regulators were positively correlated with AR ($P < 0.05$). METTL14, FTO, YTHDC1, YTHDC2, YTHDF1, YTHDF2, and YTHDF3 were remarkably related to AR ($P < 0.0001$, $R > 0.6$).

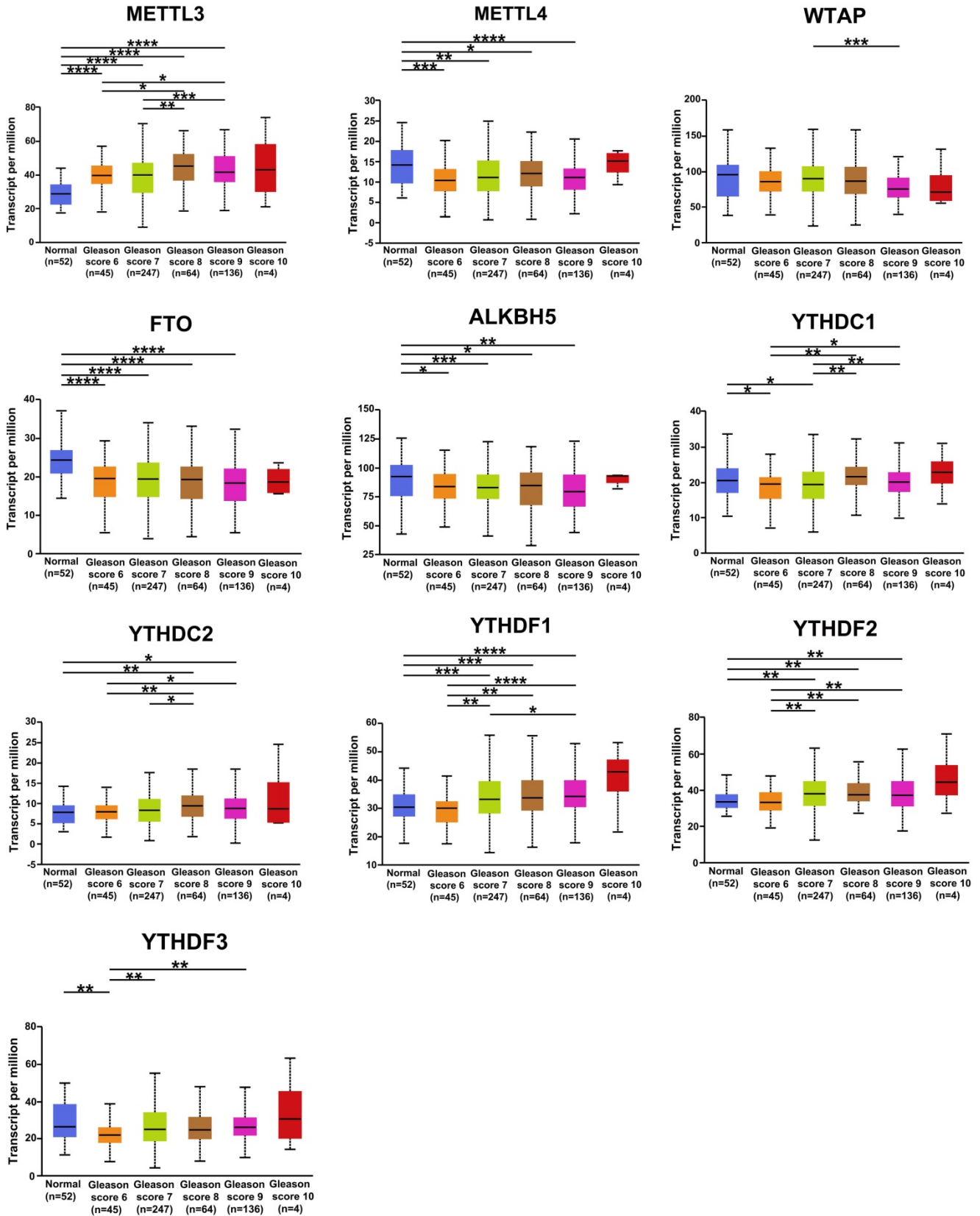


Figure 4. Relative expression of m⁶A regulators in normal and PCa tissues with different Gleason classifications. * P < 0.05, ** P < 0.01, ***P < 0.001.

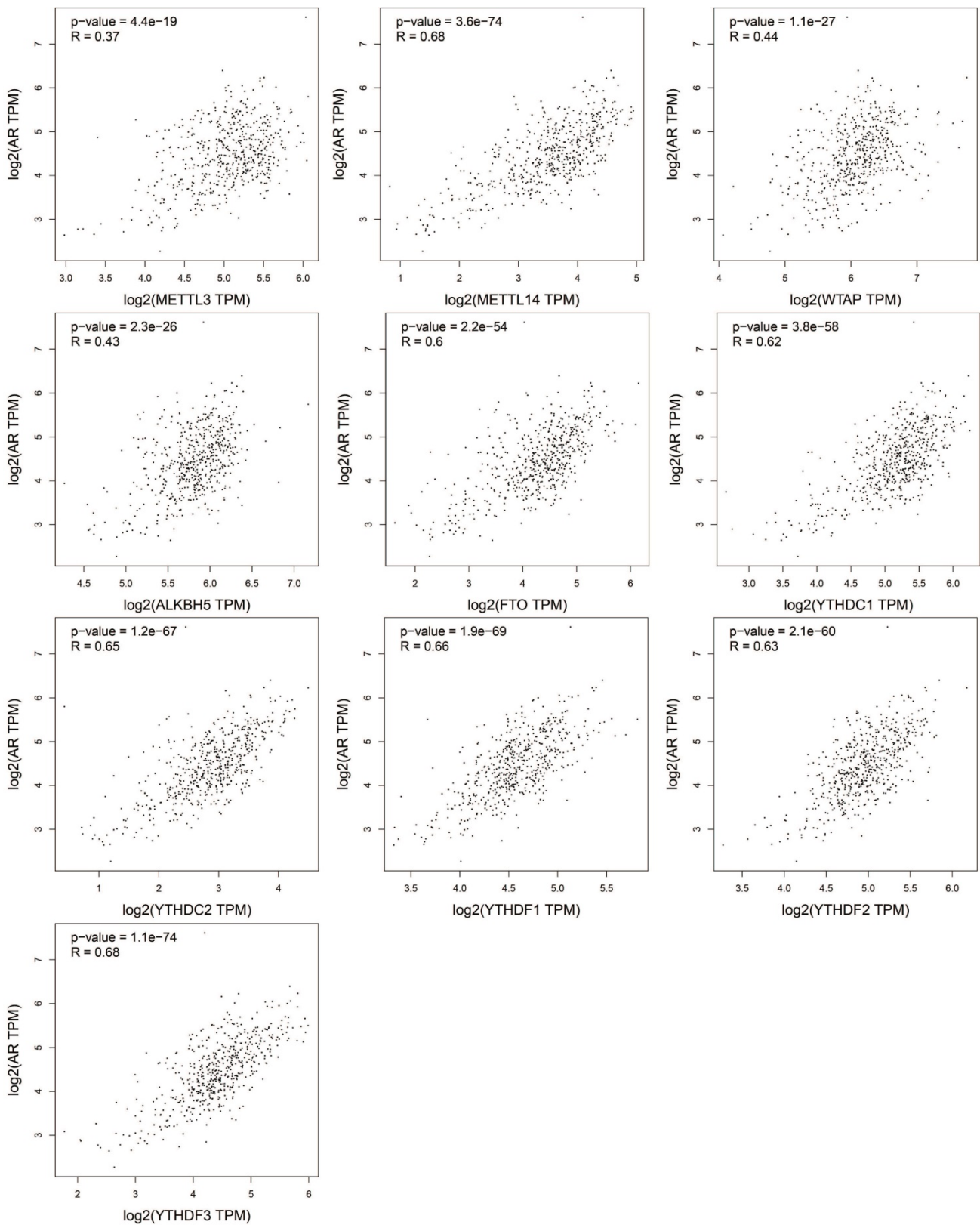


Figure 5. Correlation between the mRNA expression of m⁶A regulators and AR in PCa.

Genetic mutations of m⁶A regulators and their associations with the DFS of patients with PCa

The genetic variations of the m⁶A regulators

were analyzed using the cBioPortal database. We found 343 (12%) alterations from 2797 queried patients, including mutation, multiple alterations, amplification, and deep deletions. Deep deletion was

the most common type of genomic alteration among these variations (Figure 6A). The DFS curves indicated that patients with a genomic alteration of m⁶A had remarkably worse DFS (Figure 6B). The alterations frequencies of YTHDC2, YTHDF3, METTL14, WTAP, YTHDC1, YTHDF2, FTO, YTHDF1, ALKBH5, and METTL3 were 3%, 3%, 2.2%, 1.8%, 1.6%, 0.9%, 0.8%, 0.6%, 0.5%, and 0.4%, respectively (Figure 6C).

Expression of total m⁶A levels in TRAMP mice and PCa cell lines

TRAMP mice can spontaneously develop PCa and their pathological progression is similar to that of human PCa [31]. We determined the total m⁶A expression of TRAMP mice at different stages of PCa to characterize the specific m⁶A level profile. Results showed that the m⁶A level of TRAMP mice increased

significantly compared with that of tumor-free mice ($P < 0.01$; Figure 7A). We also found that m⁶A expression level was significantly upregulated with the increase in cancer progression ($P < 0.05$; Figure 7B). To evaluate the functional relevance of m⁶A between PCa and CRPC, we observed the m⁶A expression levels of AR-independent (DU145 and PC3) and AR-dependent PCa cell lines (LNCaP). The results showed that the m⁶A level of LNCaP cells increased significantly compared with those of DU145 and PC3 cells (Figure 7C).

Discussion

M⁶A modification is involved in tumorigenesis, metastasis, and drug resistance [32]. Wang *et al.* [10] found that increased expression of METTL3 in GC promotes tumor angiogenesis, supporting that

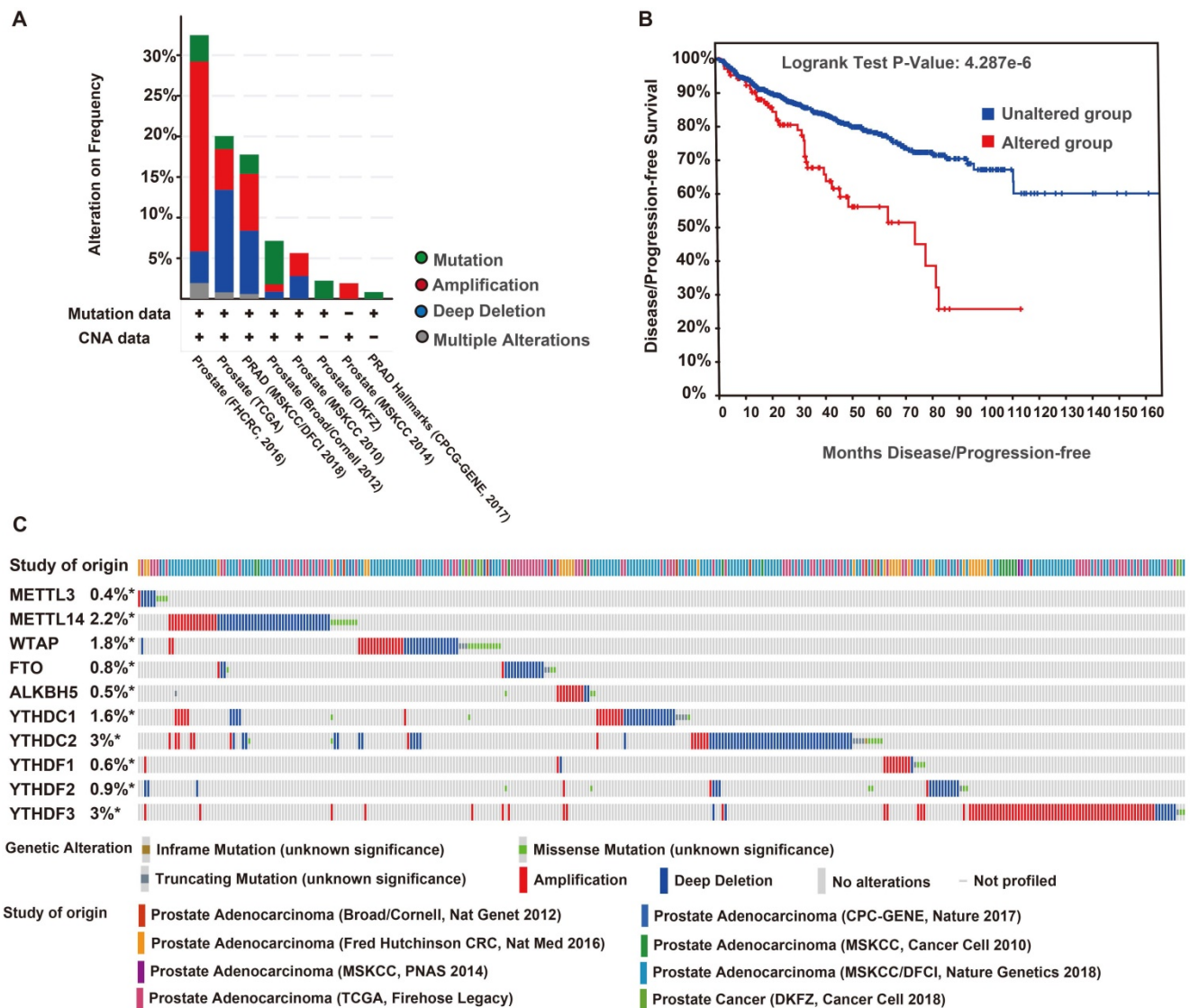


Figure 6. Genomic alteration and prognosis of m⁶A regulators in eight PCa studies (cBioportal). (A) OncoPrint of 10 m⁶A regulators in PCa. (B) DFS/progression-free survival (PFS) of patients with PCa who had altered (red) and unaltered (blue) mRNA expression of the m⁶A regulators. (C) Genetic alteration frequencies of the 10 m⁶A regulators in PCa.

METTL3 is a promising prognostic biomarker and therapeutic target for GC. It was reported that the downregulation of METTL14 is related to poor prognosis in patients with colorectal cancer (CRC), and the knockdown of METTL14 promotes the proliferation and invasion of CRC cells [33]. Low expression of ALKBH5 was found to be closely linked to the occurrence and development of non-small cell lung cancer (NSCLC) [34]. Elevated expression of some subtypes of FTO has a critical oncogenic role in acute myeloid leukemia (AML) [35]. The upregulation of FTO promotes the development of melanoma and its resistance to PD-1 [2]. However, little is known about the role of multiple m⁶A regulators in PCa [23].

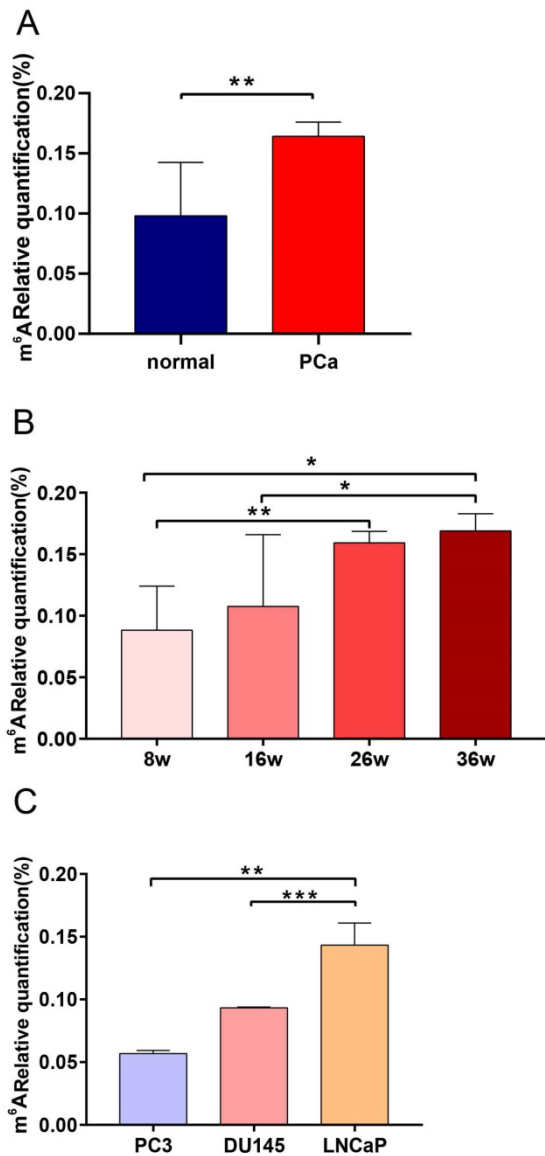


Figure 7. Expression levels of the m⁶A in TRAMP mice and PCa cell lines. (A) Expression of normal prostate tissues and PCa tissues in TRAMP mice. (B) Expression of m⁶A at different stages of TRAMP mice. (C) Expression of m⁶A in AR-independent and AR-dependent PCa cell lines.

In this study, the expression, clinical value, and mutation of 10 m⁶A regulators in PCa, as well as their correlation with AR, were analyzed. Total m⁶A expression levels were determined in PCa cell lines and TRAMP mice. The results of our study showed that FTO and ALKBH5 had low- expression in PCa and were also significantly decreased in CRPC with bone metastasis tissues than in CRPC with lymph node metastasis. METTL3, YTHDC2, YTHDF1, and YTHDF2 had a remarkably high expression in PCa and CRPC with lymph node metastasis. METTL14 decreased in PCa and CRPC with bone metastasis tissues. The Gleason grading system is an effective predictor of the prognosis of patients with PCa because it can evaluate the differentiation and malignancy of PCa [36]. We assessed the relationship of m⁶A regulators and their Gleason classifications. We found that METTL3, YTHDF1, YTHDF2, and YTHDC2 were generally upregulated with different Gleason grades, whereas METTL14, FTO, and ALKBH5 had low expression in patients with different Gleason classifications of PCa. We also found that patients with a genomic alteration of m⁶A were associated with poor DFS. These findings suggest that METTL3, METTL14, ALKBH5, FTO, YTHDC2, YTHDF1, and YTHDF2 were abnormally expressed in PCa and related to Gleason classification, and these m⁶A modulators were the potential molecular targets for the diagnosis and treatment for PCa.

TRAMP mice developed varying degrees of prostate hyperplasia at 6-12 weeks, progressed to severe hyperplasia and adenocarcinoma at 18 weeks, and presented primary tumors at 24-30 weeks [37]. M⁶A methylation level contributes to the progression of human cancer [38, 39]. We hypothesized that changes in m⁶A level promoted the development of PCa, thus, we detected the m⁶A levels in TRAMP mice and PCa cell lines. We found that the m⁶A level of TRAMP mice increased with ages. These results indicated that changes in m⁶A levels were possibly involved in the occurrence and development of PCa.

Our study had some limitations. First, although we found that m⁶A regulators were abnormally expressed in PCa, the potential diagnostic and therapeutic effects were not evaluated, hence, further research is necessary to explore whether m⁶A regulators could be used as diagnostic markers or therapeutic targets in PCa. Second, we did not elucidate the molecular mechanisms of m⁶A regulators in PCa. The detailed mechanism of m⁶A regulators in PCa warrants further investigation.

In conclusion, we analyzed the expression of m⁶A regulators, their relationship with Gleason grades and AR, and their genomic variations in PCa,

and detected m⁶A levels in TRAMP mice and PCa cell lines. The results indicated that the abnormal expression of m⁶A regulators and abnormal m⁶A levels contribute to the development of PCa. This study provides a further understanding of epigenetic RNA modification in PCa.

Abbreviations

PCa: Prostate cancer; m⁶A: N⁶-methyladenosine; CRPC: castration-resistant prostate cancer; AR: androgen receptor; DFS: disease-free survival; TRAMP: transgenic adenocarcinoma of the mouse prostate; METTL3: Methyltransferase-like-3; METTL14: Methyltransferase-like-14; WTAP: Wilms tumor 1 associated protein; ALKBH5: AlkB homolog 5; FTO: fat mass and obesity-associated protein; HCC: hepatocellular carcinoma; GC: gastric cancer; ADT: Androgen deprivation therapy; NCI-GDC: National Cancer Institute Genomic Data Commons; HCMDB: Human Cancer Metastasis Database; GEPIA: Gene Expression Profiling Interactive Analysis; NSCLC: non-small cell lung cancer; AML: acute myeloid leukemia; OV: ovarian cancer.

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Authors' Contributions

Yanling Hu, Qunying Wu, and Huifeng Wang designed the study and wrote the article. Xing Xie, Yiming Huang, and Huifeng Wang performed the experiments *in vitro* and *in vivo*. Qunying Wu, Huifeng Wang, Xing Xie, Yiming Huang interpreted the results and revised the manuscript. Shanshan Meng and Youcheng Li helped to interpret the data. All authors have read and approved the final manuscript.

Competing Interests

The authors have declared that no competing interest exists.

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