Prognostic Value and Possible Mechanism of m6A Methyltransferase METTL3 in Prostate Cancer

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Research Article

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Abstract

Background: The N6-methyladenosine (m6A) methyltransferase METTL3 has been reported to be closely related to prostate cancer (PCa). Thus, we aimed to explore its prognostic value and possible mechanism in PCa.

Methods: The METTL3 gene and protein expression status in between the PCa and normal tissues as well as the correlation between METTL3 and Gleason score (GS) were evaluated using The Cancer Genome Atlas Project (TCGA) dataset and the tissue microarray (TMA) dataset. The prognostic value of *METTL3* was evaluated using 490 PCa patients from TCGA cohort followed by the verification using 515 PCa patients from the dataset integrated by TCGA cohort and International Cancer Genome Consortium (ICGC) cohort. The machine learning methods were used for the possible mechanism of METTL3 which was closely related to the prognosis of PCa. A nomogram was constructed to provide a quantitative approach to predict the prognosis of PCa.

Results: The gene and protein expression levels of METTL3 in the PCa tissues were significantly higher than those in the normal tissues, and the gene and protein expression levels of METTL3 in the high (GS>7) risk PCa tissues were significantly higher than those in the low-moderate risk PCa (GS≤7) tissues. The PCa patients with high expression level of *METTL3* had higher risk for the progression-free survival (PFS) events and poorer short-term and long-term PFS than those with low expression level of *METTL3*. Through using machine learning, a METTL3 related risk model (M-RM) consisted of seven m6A methyltransferase METTL3 target genes was obtained. The high M-RM score was revealed to be significantly related to the poor PFS of PCa as well as the high activity of the KEGG pathways closely related to the process of cell cycle.

Conclusion: *METTL3* has the potential to be the prognostic predictor of PCa. Its coded protein may affect the prognosis of PCa mainly through regulating the expression of their target genes closely related to the process of cell cycle. These outcomes will be benefit to improve the prognosis of patients, as well as reduce the mortality of PCa.

Introduction

Prostate cancer (PCa) is the most common malignancy for male, especially in old age, as its incidence is second to lung cancer among men in western countries [1]. In our country, PCa has become the 6th incidence and 7th death of male malignant tumors. With the development of medicine, some curable therapeutic methods such as radical prostatectomy (RP) are proposed and widely used in clinical practice. However, a high recurrence rate still exists [2]. Salvage treatment at the early stage of recurrence is beneficial to prolong survival and even can cure tumor [3]. Therefore, recurrence prediction is of great significance to reduce mortality and improve prognosis of the patients with PCa.

Serum prostate-specific antigen (PSA), Gleason score (GS) and pathological TNM (pTNM) staging are the common methods used to evaluate the recurrence and prognosis of PCa patients. However, there are still

some shortcomings. The rising serum PSA level after curable treatment is unreliable to predict the prognosis of PCa patients, because it does not mean that the patients with rising serum PSA levels are all at a high risk of death from PCa [4]; both GS and pTNM staging are limited by subjective assessment, distant micro-metastasis, and the variations among patients with the same tumor stage or GS [4]. These above have provided the motivation and goal for further exploring a more credible prognostic predictor for PCa patients.

Recently, METTL3 (methyltransferase like 3), a critical N6-methyladenosine (m6A) methyltransferase, has gained increasing attentions. It can maintain the homeostasis of m6A methylation by methylating its target mRNAs, thus participating in diverse pathological processes [5–7]. It is reported that the up-regulated METTL3 was closely related to the progression and poor prognosis of many kinds of cancers, such as gastric cancer [8], bladder cancer [9], oral squamous cell carcinoma [10], lung adenocarcinoma [11], ect. In case of PCa, Ma et al. and Li et al. proposed that the up-regulation of *METTL3* was significantly related to the poor over survival (OS) of PCa by using The Cancer Genome Atlas Project (TCGA) cohort [12–13]. However, due to the few OS events in TCGA PRAD cohort (only ten OS events out five hundred cases), OS is not recommended for PCa survival studies by using TCGA cohort [14]. In addition, how METTL3 affects the prognosis of PCa remains largely uncertain.

Therefore, in this study, we used progression-free survival (PFS) event, the recommended clinical outcome endpoint of PCa survival studies by using TCGA database [14], as a substitute for OS to evaluate the prognostic value of *METTL3* for the PCa patients, which is verified subsequently by using the dataset integrated by TCGA cohort and International Cancer Genome Consortium (ICGC) cohort. Then, a series of machine learning methods such as gene co-expression analysis, single-sample gene set enrichment analysis (ssGSEA), etc. were performed to uncover the possible mechanism of METTL3 closely related to the prognosis of PCa. Finally, the nomogram of *METTL3* combined with other clinical risk factors was constructed, which provided a quantitative approach to predict the prognosis of PCa. The detail strategy is showed in Figure 1.

Materials And Methods

Gene expression dataset of the prostate tissues

TCGA gene expression data of the prostate tissues were downloaded from TCGA database (<https://cancergenome.nih.gov/>) and its clinical data for PFS analysis was downloaded from TCGA Pan-Cancer Clinical Data Resource (TCGA-CDR) [14]. ICGC gene expression data of PCa tissues and its clinical data for PFS analysis were downloaded from ICGC database (<https://dcc.icgc.org>). The patients with a PFS event were defined as those who had a new tumor event after RP, whether it was a progression of the disease, local recurrence, distant metastasis, new primary tumors at all sites, or died of cancer without a new tumor event, including cases with a new tumor event whose type was N/A [14]. The clinical features were shown in Table 1. RNA modification data was obtained from the RNA modification database by using RMBase (<http://rna.sysu.edu.cn>) [15].

Table 1

Clinical features of the PCa patients

Clinical features	TCGA cohort	TCGA + ICGC cohort
Age (Mean +/- SE)	60.99 +/- 0.309	61.12 +/- 6.828
GS (6/7/8/9/10 patients)	45/244/63/135/3	Unknown
pT (T2/T3-T4 patients)	377/109	402/109
pN (N0/N1 patients)	340/77	365/77
Distant metastasis (patients)	6	8
Death (patients)	4	5
Death from PCa (patients)	2	Unknown
Patients with PFS event (patients)	89	99
Prior treatment (patients)	Unknown	Unknown
SE: standard error		

Immunohistochemical (IHC) analysis

The PCa TMA was purchased from Xinchao Biotech, Shanghai, China. It contained three normal prostate tissues, eight adjacent tissues of PCa, and forty-nine PCa tissues. All paraffin tissue sections obtained from the TMA were dewaxed and rehydrated. After Antigen retrieval, blocking with bovine serum albumin (Sango Biotech, Shanghai, China), the slides were incubated with anti-METTL3 (Abcam, Cambridge, Massachusetts, US) overnight at 4 °C. Then, they were incubated with a secondary antibody of goat antirabbit HRP conjugate (Cell Signaling Technology, Beverly, MA, USA) for 1 h at 25°C. A DAB solution was used for brown color development.

PCa survival analysis of *METTL3*

490 PCa patients from TCGA cohort were used to evaluate the prognostic value of *METTL3*, and 515 PCa patients from the cohort integrated by TCGA cohort and ICGC cohort were used for verification. PFS analyses were performed via the Kaplan-Meier and uni-variable Cox algorithm respectively.

Analysis of the mechanism of METTL3 closely related to the prognosis of PCa

By using TCGA cohort and RNA modification data, three main steps were included in this part. Firstly, the m6A methyltransferase METTL3 target genes which were significantly related to the prognosis of PCa patients were selected. These genes should meet the following criteria: (1) the target genes should be significantly co-expressed with *METTL3* in PCa (the absolute value of the Pearson's correlation coefficient should be more than 0.3, and *P* value should be less than 0.05.); (2) the target genes should have the m6A methyltransferase METTL3 protein binding site; (3) the target genes should be significantly related to the prognosis of PCa (*P* value should be less than 0.05.). Secondly, in order to eliminating the influence of confounding factors, the multi-variable PFS analysis via Least absolute shrinkage and selector operation (LASSO) Cox regression was performed to establish METTL3-related risk model (M-RM) by using “glmnet” R package [16], which was followed by the verification by using 515 PCa patients from the cohort integrated by TCGA cohort and ICGC cohort. Thirdly, ssGSEA was performed to evaluate the activity of the KEGG pathways by calculating their estimate score (ES). Pearson's correlation test was performed to identify the KEGG pathways that were closely related to the M-RM (the absolute value of the correlation coefficient should be more than 0.3, and *P* value should be less than 0.05.).

Nomogram construction and evaluation

A nomogram that integrated the *METTL3* and other clinical risk factors was constructed with TCGA cohort using the ‘rms’ R package [17]. Calibration plot was drawn to describe the degree to which the predicted PFS were consistent with the observed PFS. A concordance index (C-index) was calculated to determine the discrimination of the nomogram via a bootstrap method with 1000 re-samples.

Statistical analysis

Statistical analyses were performed with R software (version 4.1.2). Pearson's correlation test was performed to determine the correlation between two variables. The *t*-test was used for the comparison between two groups of variables, and the variance test was used for the comparison among three groups of variables. The “survival” R package [18] was used for Kaplan-Meier curve and uni-variate Cox regression analysis. The “maxstat” R package [19] was used to determine the optimal cutoff value of *METTL3* for Kaplan-Meier curve. Log Rank (Mantel-Cox) test was used to evaluate long-term PFS, and Breslow (Generalized Wilcoxon) test was used to evaluate short-term PFS. The “pROC” R package [20] was used to draw the time-depend receiver operating characteristic (ROC) curve to evaluate the diagnostic performance for the PFS events of PCa. *P* value < 0.05 was regarded as statistical significance.

Results

Up-regulated METTL3 was significantly related to PCa as well as the high risk PCa

A total of 496 PCa tissues and 51 normal prostate tissues from TCGA dataset were used to analyze the relationship between *METTL3* and PCa. The result showed that the expression level of *METTL3* in the PCa tissues was significantly higher than that in the normal prostate tissues (Figure 2A). According to GS, the 496 PCa tissues were divided into three groups including the low-risk (GS<7) (45) group, the moderate-risk (GS=7) (246) group and the high-risk (GS>7) (205) group. We compared the expression levels of *METTL3* in these three groups and found that there was a significant difference among them ($P<0.001$). As can be seen in Figure 2B, the expression level of *METTL3* in the high-risk group was significantly higher than that in the low-risk group ($P<0.01$) as well as the moderate-risk group ($P<0.001$). Furthermore, through observing the expression level of *METTL3* protein by using the PCa TMA dataset, a similar result was got. It was not only that the expression level of *METTL3* in the PCa tissues was visibly higher than that in the normal tissues and the AD tissues, but also that the expression level of *METTL3* in the high risk PCa tissues (GS>7) was obviously higher than that in the low-moderate risk PCa tissues (GS≤7) (Figure 2C). Taken together, these results suggest that the up-regulated *METTL3* is showed close relationship to PCa as well as the high risk PCa.

Up-regulated *METTL3* was closely related to the poor PFS of the PCa patients

490 PCa tissues from TCGA cohort were used to evaluate the prognostic value of *METTL3* in PCa. PFS analysis via uni-variable Cox regression algorithm showed that the up-regulated *METTL3* was significantly related to the poor PFS of the PCa patients (Hazard ratio (HR): 1.58; 95% confidence interval (CI): 1.022-2.449; $P<0.05$). According to the optimal cutoff value (4.98) of *METTL3*, PCa patients were divided into two groups, the group of the patients with high expression level of *METTL3* and the group of the patients with low expression level of *METTL3*. Kaplan-Meier curve showed that the patients with high expression level of *METTL3* had poorer both short-term and long-term PFS than those with low expression level of *METTL3* (Log Rank (Mantel-Cox): $X^2=6.957$, $P<0.01$; Breslow (Generalized Wilcoxon): $X^2=5.562$, $P<0.05$; Figure 2D). 515 PCa patients from the cohort integrated by TCGA cohort and ICGC cohort were used for verification. Consistently, the similar results were got (Figure 2E). Above all, we suggest that *METTL3* have the potential to be the prognostic predictor for PCa.

Mechanism of *METTL3* that was closely related to the prognosis of PCa

In order to uncover the mechanism of *METTL3* involved in the prognosis of PCa, firstly, twenty-five m6A methyltransferase *METTL3* target genes were identified (S-Table 1) [see Additional file 1], which met the criteria mentioned in “Materials and Methods”. Then, these target genes were used to perform the PFS analysis via LASSO Cox regression with 490 PCa tissues from TCGA cohort to establish the M-RM, which was consist of seven m6A methyltransferase *METTL3* target genes including small nuclear ribo-

nucleoprotein U1 sub-unit 70 coding gene (*SNRNP70*), claudin 15 coding gene (*CLDN15*), non-SMC condensin I complex sub-unit H coding gene (*NCAPH*), DExD-box helicase 39A coding gene (*DDX39A*), RAD54 like coding gene (*RAD54L*), essential meiotic structure-specific endonuclease subunit 2 coding gene (*EME2*), and melanocortin 1 receptor coding gene (*MC1R*) (Figure 3A). The formula of the M-RM score for each patient was: M-RM score = (0.221 * expression level of *SNRNP70*) + (0.233 * expression level of *CLDN15*) + (0.008 * expression level of *NCAPH*) + (0.001 * expression level of *DDX39A*) + (0.516 * expression level of *RAD54L*) + (0.014 * expression level of *EME2*) + (0.324 * expression level of *MC1R*). Subsequently, the preliminary evaluation of these seven m6A methyltransferase *METTL3* target genes and the M-RM were performed. These seven target genes were all moderately positively correlated to *METTL3*, and significantly related to the PFS of PCa (Figure 3B). The time-dependent ROC showed that the M-RM had a good diagnostic performance for 1-year, 3-year and 5-year PFS events with the area under the curve (AUC) of 0.78 (95% CI: 0.71-0.85), 0.78 (95% CI: 0.72-0.84), and 0.74 (95% CI: 0.65-0.82) (Figure 3C). According to the optimal cutoff value of the M-RM score, the PCa patients were divided into the high M-RM score group and the low M-RM score group. As shown in Figure 3D, the gene expression levels of variables in the M-RM and the number of the PFS events in the same period of time in the high M-RM score group were both obviously higher than those in the low M-RM score group. In addition, Kaplan-Meier curve showed that the patients with high RM score had poorer both short-term and long-term PFS than those with low PFS score (Log Rank (Mantel-Cox): $X^2=46.131$, $P<0.0001$; Breslow (Generalized Wilcoxon): $X^2=40.082$, $P<0.0001$; Figure 3E). This result was confirmed by using the cohort integrated by TCGA cohort and ICGC cohort subsequently (Log Rank (Mantel-Cox): $X^2=22.123$, $P<0.0001$; Breslow (Generalized Wilcoxon): $X^2=18.510$, $P<0.0001$; Figure 3F). Thus, we believed that *METTL3* might affect the prognosis of PCa mainly through regulating the expression of these seven m6A methyltransferase *METTL3* target genes in the M-RM. Finally, in order to further understand how the M-RM affected the prognosis of PCa, ssGSEA was performed. And based on it, we found that the M-RM score was significantly positively correlated to the activity of the cell cycle related KEGG pathways, such as cell cycle, DNA replication, base excision repair, etc. (Figure 4). Taken together, we suggest that *METTL3* may affect the prognosis of PCa mainly through regulating the expression levels of their target genes which were closely related to the process of cell cycle.

Nomogram construction and evaluation

METTL3 and the clinical risk factors including age, GS, pathological T (pT) staging and pathological N (pN) staging were used for the PFS analysis via multi-variable Cox regression algorithm. The risk model (RM) ($X^2=33.03$, $P<0.0001$) integrated the *METTL3* and pT staging for was established (S-Table 2) [see Additional file 1]. PFS analyses via Kaplan-Meier and uni-variate Cox regression algorithm showed that the RM was significantly related to the prognosis of PCa patients. The patients with high RM score had poorer both short-term and long-term PFS than those with low RM score (Log Rank (Mantel-Cox): $X^2=22.951$, $P<0.0001$; Breslow (Generalized Wilcoxon): $X^2=15.925$, $P<0.0001$; Figure 5A). The nomogram based on the RM was drawn to provide the quantitative approach to predict the probability of 1-year, 3-

year and 5-year PFS for the PCa patients (Figure 5B). The C-index of the RM was 0.729 (95% CI: 0.651-0.808; $P < 0.0001$). And the calibration curve showed that the RM-based nomogram-predicted 3-year and 5-year PFS of the PCa patients was highly fit with the observed ones (Figure 5C).

Discussion

METTL3 is a critical m6A methyltransferase, which can participate in diverse pathological processes through maintaining the homeostasis of m6A methylation by methylating its target mRNAs [5–7]. Recently, a few studies revealed that the up-regulation of METTL3 was significantly related to the poor OS of the PCa patients by using TCGA cohort [12–13]. However, there were only ten OS events out five hundred cases in TCGA cohort, so that, according to the recommendations of TCGA-CDR, PFS was identified to be more suitable than OS for PCa survival studies [14]. Thus, in this study, by using TCGA database, PFS event was used as the substitute for OS to further evaluate the prognostic value of METTL3 for the PCa patients, and the results showed that the patients with high expression level of *METTL3* had poorer both short-term and long-term PFS than those with low expression level of *METTL3*. Furthermore, the result of the PFS analysis by using 515 PCa patients from the cohort integrated by TCGA cohort and ICGC cohort confirmed it. Taken together, these results suggest that *METTL3* had a good predictive ability for the prognosis of PCa, which is consistent with previous studies [12–13]. In addition, in order to provide a quantitative approach to predict the probability of 1-year, 3-year and 5-year PFS for the PCa patients, the nomogram based on the RM which integrated by the *METTL3* and pT was constructed with TCGA cohort. The RM had a high ability to distinguish the occurrence of the PFS events, and the RM-based nomogram-predicted 3-year and 5-year PFS of PCa patients was also highly fit with the observed ones, indicating the good predictive ability of it for the prognosis of PCa.

It has been reported that the role of METTL3 in the process of tumorigenesis and tumor development is tumor-specific [22–27]. Some studies showed that METTL3 played an oncogenic role in myeloid leukaemia [22], breast cancer [23], bladder cancer [24], colorectal carcinoma [25], etc. Other studies indicated that METTL3 played a tumor suppressor in renal cell carcinoma [26] and glioblastoma [27]. In PCa, a recent study suggested that METTL3 was an oncogenic factor [28]. However, the mechanism of METTL3 that affected the prognosis of PCa were still unclear. Our results revealed that after eliminating the interference of confounding factors, m6A methyltransferase METTL3 target genes *SNRNP70*, *CLDN15*, *NCAPH*, *DDX39A*, *RAD54L*, *EME2*, and *MC1R* were identified to significantly affect the prognosis of PCa patients. So far there was no related studies to illuminate the mechanism of these seven genes in PCa. However, we noticed that except *CLDN15*, the rest m6A methyltransferase METTL3 target genes might be closely related to the process of cell cycle. *SNRNP70* and *DDX39A* participated in transcription, *NCAPH* participated in DNA replication, *RAD54L* and *EME2* participated in DNA repair, as well as *MC1R* promoted the process of DNA repair in skin [29]. Furthermore, by using ssGSEA and Pearson's correlation test, we noticed that the activity of some cell cycle related KEGG pathways such as cell cycle, DNA replication, and basic resection repair was significantly enhanced with the increasing of the M-RM scores calculated from the expression levels of these seven m6A methyltransferase METTL3 target genes.

These results suggest that METTL3 might participate in PCa progression mainly through regulating the expression of their target genes which were closely related to cell cycle.

In conclusion, *METTL3* had the potential to be developed as a PCa prognostic predictor. The process of cell cycle may be the possible mechanism of it in the prognosis of PCa. Our outcomes will be helpful to improve the prognosis of patients, as well as reduce the mortality of PCa.

Declarations

Ethics approval and consent to participate

This study was performed in accordance with the Declaration of Helsinki and approved by Ethics Committee of Qingpu Branch of Zhongshan Hospital Affiliated to Fudan University(2020-19).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

The authors declare that they have no competing interests.

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Authors' contributions

SYT and FXW conceived and designed the study and were responsible for the writing and critical reading of the manuscript. SYT contributed to bioinformatical analysis. FXW contributed to sample collection and experiment implementation. WZQ contributed to theoretical and experimental guidance.

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Figures

Figure 1

Detail analysis strategy

Figure 2

Prognostic value of the m6A methyltransferase *METTL3*

(A) Comparison between the expression level of *METTL3* in the PCa tissues and that in the normal prostate tissues by using TCGA dataset; (B) Comparison among the expression level of *METTL3* in the low-risk PCa tissues (GS<7), the moderate-risk PCa tissues (GS=7) and the high-risk PCa tissues (GS>7) by using TCGA dataset; (C) *METTL3* protein expression level in the normal prostate tissue, the AD prostate tissues, the low-moderate risk PCa tissues (GS≤7) and the high risk PCa tissues (GS>7) by using TMA dataset; (D) Kaplan-Meier curve of *METTL3* for the PFS of the PCa patients by using TCGA cohort; (E) Kaplan-Meier curve of *METTL3* for the PFS of the PCa patients by using the cohort integrated TCGA cohort and ICGC cohort.

Figure 3

Prognostic power of the m6A methyltransferase METTL3 targeted genes

(A) LASSO Cox regression to establish the M-RM; (B) Forest plot of the seven m6A methyltransferase METTL3 target genes in the M-RM: "Cor. (P value)" was the correlation coefficient and P value between *METTL3* and each of the variables in the M-RM; (C) Time-dependent ROC of the M-RM score; (D) Preliminary evaluating the prognostic values of the M-RM and the m6A methyltransferase METTL3 target genes in its formula; (E) Kaplan-Meier curve of the M-RM score for the PFS of the PCa patients by using TCGA cohort; (F) Kaplan-Meier curve of the M-RM score for the PFS of the PCa patients by using the cohort integrated TCGA cohort and ICGC cohort.

Figure 4

Correlations between the activities of KEGG pathways and the M-RM score

(Top) Heatmap of the top ten KEGG pathways that were significantly correlated to the M-RM score;
 (Bottom) Scatter diagrams of the top ten KEGG pathways that were significantly correlated to the M-RM score.

Figure 5

Correlations between the activities of KEGG pathways and the M-RM score

(Top) Heatmap of the top ten KEGG pathways that were significantly correlated to the M-RM score;
(Bottom) Scatter diagrams of the top ten KEGG pathways that were significantly correlated to the M-RM score.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [STable1.xlsx](#)
- [STable2.docx](#)