1-1-2019

Epigenetic Silencing Rna Methylation Machinery Fto And Mettl3 Associate With Patient Survival In Renal Clear Cell Carcinoma

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by

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April,2019

Year Degree Awarded: 2019

Degree Awarded: Master of Public Health

Department: School of Public Health

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Abstract:

Purpose: RNA methylation eraser FTO and writer METTL3 play important roles in human diseases by regulating gene expression. However, the potential of FTO and METTL3 as markers in renal clear cell carcinoma (CCRCC) is still unknown. The purpose of this study is to investigate differential expression of FTO and METTL3 in CCRCC vs. normal kidney tissues, the association of FTO and METTL3 expression and methylation with and their interaction of FTO and METTL3 expression in patient survival in CCRCC.

Method: FTO and METTL3 expression and methylation, the clinicopathologic data were retrieved from a publicly accessed dataset of The Cancer Genome Atlas (TCGA) of 537 patients with primary CCRCC. Survival analysis was performed using Kaplan-Meier survival curve and multivariate cox regression model. Random-effects meta-analysis was applied to examine differential expression of FTO and METTL3 in CCRCC vs. normal kidney tissues.

Results: Significant upregulation of FTO and METTL3 expression with 1.64 (95% CI: 1.43-1.89) and 1.17 (95% CI: 1.02-1.35) folds, respectively, were observed in CCRCC vs. normal kidney tissues. Survival analysis showed that a superior survival was observed in both either high FTO expression or low methylation, and either low METTL3 expression or high methylation. The adjusted hazard ratios (HRs) were 0.67 (95% CI: 0.49-0.91, p=0.01) for high vs. low FTO expression, 2.17 (95% CI: 1.38-3.42, p=0.0008) for high vs. low FTO methylation, 1.97 (95% CI: 1.45-2.68, p<0.0001) for high vs. low METTL3 expression, and 0.49 (95% CI: 0.31-0.79, p=0.003) for high vs low METTL3 methylation, respectively. A significant interaction between FTO and METTL3 expression was observed in CCRCC patient survival (P=0.0328).

Conclusion: FTO and METTL3 expression and methylation are potential prognostic and diagnostic markers in CCRCC.

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Introduction

RNA modification takes place in all living organisms and during this post-transcriptional process, RNA nucleotides are modified. Up until now, over 100 different types of RNA modifications have been identified, most of them occur in structured RNA such as tRNA and rRNA, and they can also take place in mRNA, small and long non-coding RNAs.(LncRNAs) [1]These RNA modifications are critical in modulating gene expression,[2] and consequently impacting many essential biological processes.[3]Loss of regulation for RNA modifications could cause relevant diseases.[4]

N6-methyladenosine (m6A) refers to the RNA modification during which the adenosine at the nitrogen-6 position is methylated, and it is the most abundant modification in mRNAs and LncRNAs. m6A has been demonstrated to be associated with a series of fundamental cellular functions such as splicing,[5]stability,[6]translation,[7]circadian clock,[8]stem cell differentiation[9], and innate immune response.[10] The abnormal m6A level has been reported to link to diverse cancer types, such as leukemia, breast cancer, cervical cancer, glioblastoma.[11]

m6A methylation is a dynamic and reversible modification through the orchestration of a set of proteins “writers” (methyltransferase), “erasers” (demethylase) and “readers” (binding proteins). [12] METTL3 is a major member of N6-adenosine-methyltransferase, which is encoded by *METTL3* gene on chromosome 14. The depletion of this enzyme results in significant reduction in m6A level in mRNAs. [13] METTL3 has been reported to be associated with the pluripotency and differentiation of embryonic stem cells (ESCs). [14] METTL3 has also been shown to

participate in many fundamental biological processes, such as formation of the hematopoietic system, T cell homeostasis, and neural stem cells differentiation. [14] It has been shown that the METTL3 depletion in human myeloid leukemia cells is associated with increased differentiation and apoptosis, which has been found to have delayed leukemia in experimental mice in vivo. [15] Hua-Bing Li and colleagues have used METTL3 knockout mice to demonstrate that the lack of METTL3 can affect the homeostasis and differentiation of T cell, and as a result, the T cells would stay in the naïve state and lose the ability to respond to various stimuli.[10] The upregulation of METTL3 has been found in many tumors, including liver cancer, breast cancer, colorectal cancer, prostate cancer, etc.[16] It has been found that METTL3 can facilitate the translation and expression of several critical oncogenes such as EGFR and TAZ in human cancer cells, and hence can boost the growth and invasion of the cancer cells. The depletion of METTL3 was observed to be related to remarkable reduction in growth, invasion of cancer cells, and increased cell apoptosis has also been found. In contrast, the overexpression of METTL3 has totally opposite effects on cancer cells.[17]

Fat mass and obesity-associated protein FTO is the first identified demethylase of m6A, which is encoded by the FTO gene on chromosome 16. It has been demonstrated that the depletion of *FTO* can result in the elevated level of m6A in mRNA, while the overexpression of *FTO* is associated with decreased m6A level in human cells. [18] FTO has been found to be involved in many physiological processes, such as transcriptome regulation and translation. [19] It has been reported that FTO can target pre-mRNAs and act as an mediator in the process of alternative splicing and 3'end processing. [5] At present, FTO is mostly considered to be associated with obesity. It has been reported that, FTO, as an eraser of m6A, influences fat metabolism and

mitochondrial content by regulating m6A level in liver cells. Decreased levels of m6A and mitochondrial content with increased triglyceride (TG) accumulation have been observed along with the high expression of FTO.[20] Since obesity is a risk factor for many cancers, the association between FTO and cancers has attracted more and more attention recently. Accumulated evidence has shown the overexpressed FTO in several cancers, such as AML, endometrial cancer and gastric cancer. [19] It has been demonstrated to act as an oncogene in several cancers, and FTO can also promote the growth and transformation of cancer cells.[19]

Recently, it has been reported that the cross-talk among m6A writers, readers and erasers can modulate the growth and progression of cancers by controlling m6A level and gene expression in cancer cells.[21] Another critical finding about m6A modification is that it can cause a structural switch between double-stranded RNAs (dsRNA) and single-stranded RNAs (ssRNA) in the secondary structure of RNAs.[22] m6A RNA modification inhibited the innate immune response because it could cause the decrease of dsRNA, which acts as a stimulus to the innate immunity.[23]Kidney cancer has been proven to be an immunogenic tumor and almost all kidney cancers are associated with dysfunctional immunity. [24]The essential role that Innate immunity has played in the development and progression of renal cell carcinoma has also been identified.[25] Thus in this study, we aimed to investigate the association between RNA methylation machinery FTO and METTL3 expression and their interaction, as well as their promoter DNA methylation, with patient survival in kidney renal clear cell carcinoma, and to further explore the correlations between both FTO and METTL3 and dsRNA and innate immunity-related genes.

Materials and Methods

Gene expression, methylation and clinicopathologic data

A CCRCC dataset from The Cancer Genome Atlas (TCGA), which is available at TCGA provisional (www.cbioportal.org) was used. The upper quartile normalized RNA-Seq by Expectation Maximization (RNA Seq V2 RSEM) data for FTO expression, the data for FTO methylation (HM450), the data for dsRNA related factors expression (TLR3, TLR7, TLR8, DDX58, IFIH1, NLRP3), the data for innate immunity related factors expression (CD274, CD80, CD86, FCGR3A) of 537 patients were extracted. The clinicopathologic data was also retrieved and combined with the gene expression and methylation data.

Differential expression of FTO and METTL3 in renal clear cell carcinoma vs. normal kidney tissues

Gene expression dataset OncomineTM (www.oncomine.org) (ThermoFisher Scientific, MA, USA) was used to compare the differential expression of FTO/METTL3 in CCRCC vs. normal kidney tissues. The filters used for searching were Gene: FTO/METTL3, Analysis Type: Cancer vs. Normal Analysis, Cancer Type: Kidney Cancer, Data Type: mRNA and Sample Type: Clinical Specimen. Only renal clear cell carcinoma studies were included for the meta-analysis, other types of kidney cancer, for example, renal papillary cell carcinoma were all excluded from the analysis. Eight studies for FTO and seven studies for METTL3 were included in the final analysis. Random-effects Meta-analysis was performed to investigate the differences in FTO/METTL3 expression between CCRCC vs. normal kidney tissue. The fold-change values from raw data were first transformed into Log2 fold-change to perform the Meta-analysis. The

final Fold-change value was calculated by transforming the summarized Log2 fold change back using formula: summarized Fold-change = $2^{\text{summarized log2 Fold-change.}}$

Statistical Analysis

SAS version 9.4 (SAS Institute, Inc., NC, USA) and R version 3.5.1 were used to perform the statistical analysis. The overall survival time in months was calculated as the time from the first diagnosis of CCRCC to the occurrence of death or the last follow-up. Kaplan-Meier survival curve analysis (log-rank test) was first performed to test the association between the overall survival and either FTO or METTL3 expression and methylation, respectively. All the 537 patients in the study were first divided into three groups based on the tertile distribution of the gene expression and methylation, respectively. Then the pairwise tests were used to determine which group is significantly different from one another for each variable. For the comparison that didn't have a significant result, these two groups would be combined as one group, then comparing to the other group. At last, the patients have been categorized as High FTO group and Low FTO group based on the expression of FTO, High FTO Methylation group and Low FTO Methylation group based on the FTO methylation level, High METTL3 group and Low METTL3 group based on the expression of METTL3, and High METTL3 Methylation group and Low METTL3 Methylation group based on the METTL3 methylation level. The Kaplan-Meier survival curves have been constructed based on the final grouping described above. Then the Multivariate Cox regression model was used to adjust for the potential confounders, backward elimination strategy was used to obtain the final model. Wald test was performed to test the interaction effect between gene FTO and gene METTL3. The HRs and 95% CI were estimated after adjusting for age and tumor stage. The Spearman correlation analysis was

performed to evaluate the correlation between the FTO/METTL3 expression and dsRNA related genes, and also for the correlation between FTO/METTL3 expression and innate immunity related genes. A p-value less than 0.05 was considered as significant.

Results

1. Clinicopathologic characteristics of CCRCC patients

The characteristics of the 537 patients with primary Renal Clear Cell Carcinoma (CCRCC) are shown in table 1. The average age for the patients in this study was 61-years old with a range from 25 to 90-years old. There were 64.4% male and 35.6% female in this study. Among the 530 patients whose race information was available, the majority of them were Caucasian (87.9%), followed by African American (10.6%), and Asian (1.5%). The information for tumor stage was known for 534 patients and 50.4% of them had stage I tumor, followed by stage III (23.4%), stage IV (15.5%), and stage II (10.7%). During the follow-up, 33.0% of the CCRCC patients deceased, with an average overall survival time of 44.3 months, ranging from 0 to 149.1 months.

Table 1. Clinicopathologic Characteristics of CCRCC Patients

Characteristic	N	%
Race	530	
Asian	8	1.5
Black or African American	56	10.6
White	466	88.0
Sex	537	
Male	346	64.4
Female	191	35.6

Tumor Stage	534		
Stage I	269	50.4	
Stage II	57	10.7	
Stage III	125	23.4	
Stage IV	83	15.5	
Death	537		
Yes	177	33.0	
No	360	67.0	
		mean ± SD	Range
Age (years)	537	60.6±12.2	26-90

2. Correlations for FTO expression vs. METTL3 expression, FTO expression vs. FTO methylation, METTL3 expression vs. METTL3 methylation

The spearman correlation test was performed to test the correlations. The results showed that there was a significant negative correlation between the expression of FTO and METTL3 ($p < 0.0001$), the correlation coefficient was -0.32, with a 95% CI of (-0.39, -0.24); There was a significant negative correlation between FTO expression and FTO methylation level ($p < 0.0001$), the correlation coefficient was -0.42, with a 95% CI of (-0.51, -0.33); There was also a significant negative correlation between METTL3 expression and METTL3 methylation level ($p < 0.0001$), the correlation coefficient was -0.31, with a 95% CI of (-0.40, -0.20). The scatterplots with regression line and 95% boundary have been shown in figure 1.2.3.

Figure 1. Correlation between FTO Expression vs. METTL3 Expression

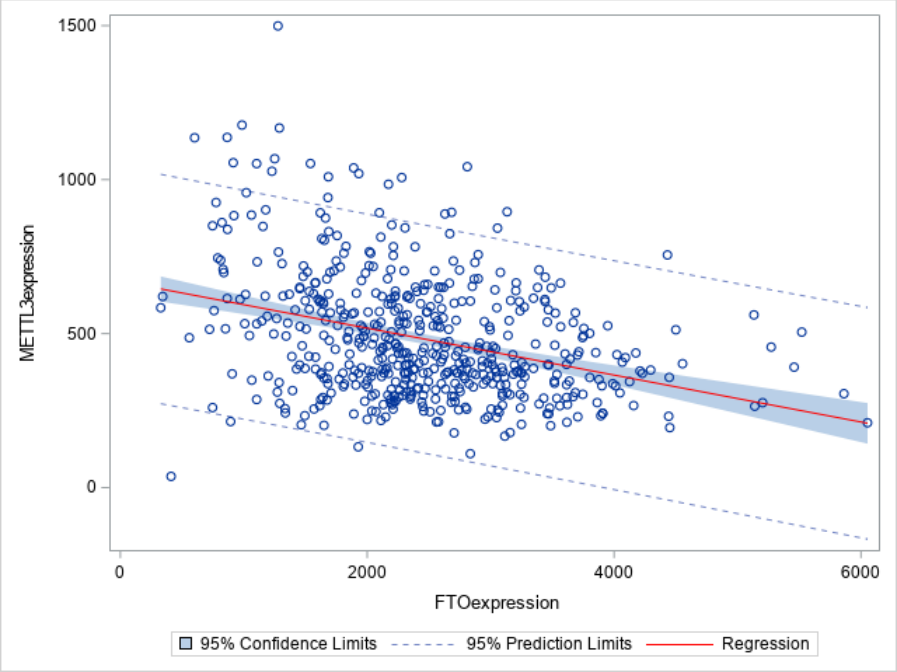


Figure 2. Correlation between FTO Expression vs. FTO Methylation

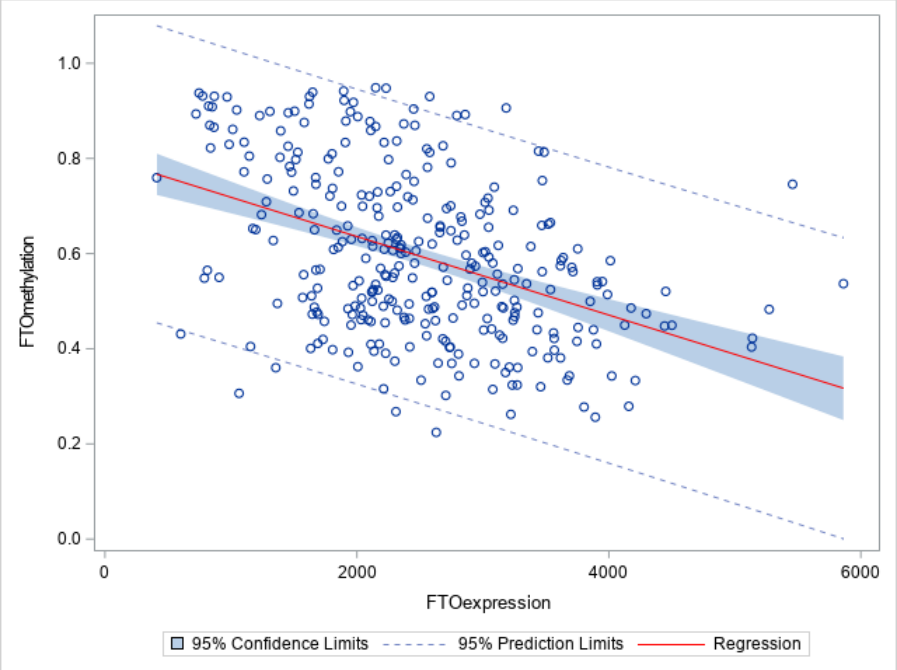
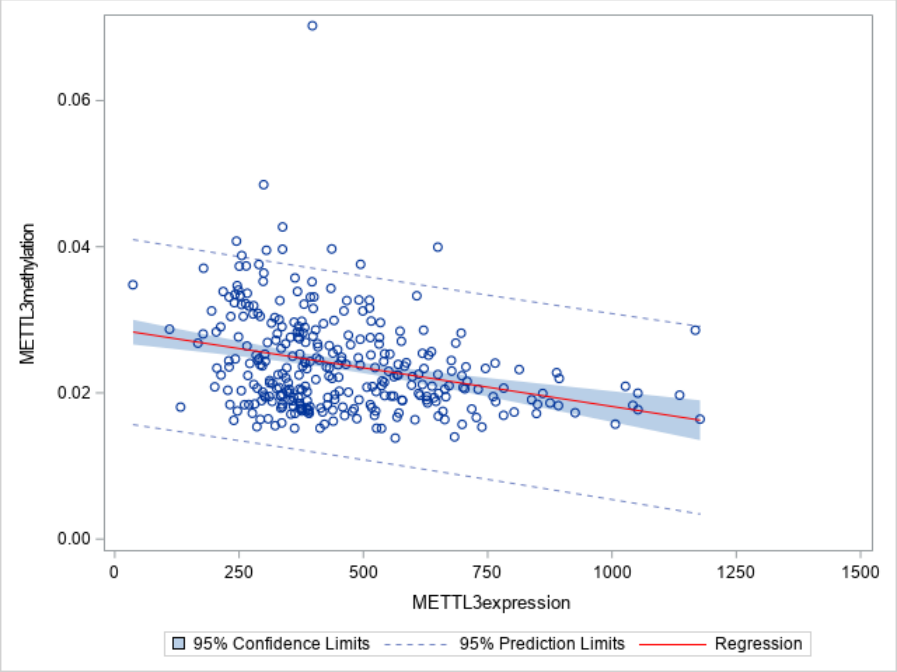


Figure 3. Correlation between METTL3 Expression vs. METTL3 Methylation



3. Association between FTO expression and overall patient survival in CCRCC

The log-rank test showed a significant difference in overall survival time between patients with high or low FTO expression (log-rank p=0.003). The patients in high-level FTO group had a superior overall survival comparing to the patients in FTO low-level group. The Kaplan-Meier survival curves are shown in figure 4.

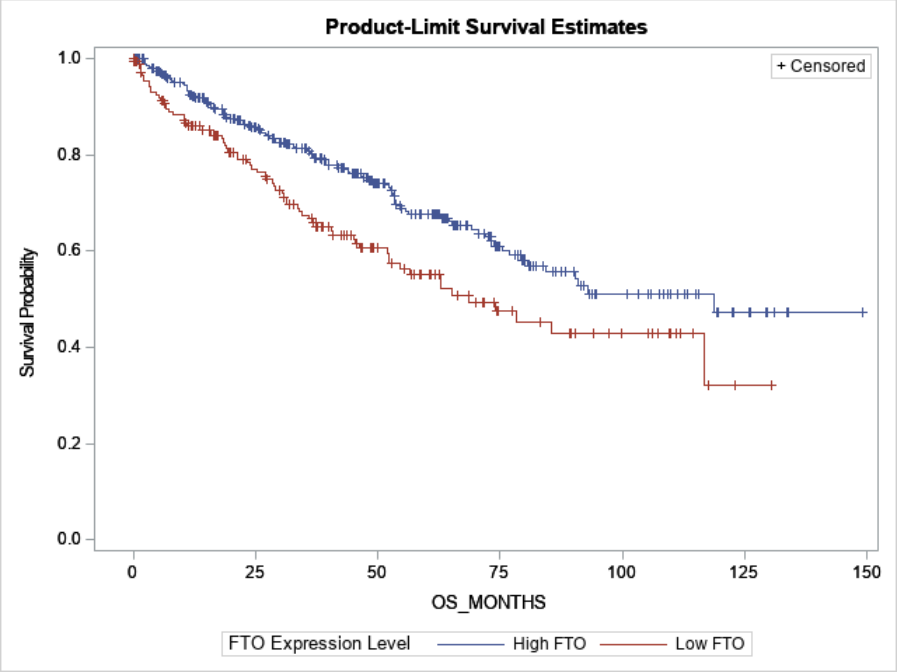
To adjust for the potential confounders, the Multivariate Cox regression model was constructed, starting with the full model with all potential confounders (race, sex, tumor stage, age) available in the dataset, then used the backward elimination strategy to arrive at a parsimonious model. Three variables were retained in the final model (at p-value < 0.1), variable race and sex were finally dropped. The result is shown in table 2. After adjusting for tumor stage and age, the significant association between the FTO expression and death risk still remained. The high-level

FTO expression group decreased the risk of death comparing to the low-level group. (HR:0.67, 95% CI: 0.49-0.91, p=0.01)

Table 2. Association between FTO Expression and CCRCC Patient Survival

Variable	Death		
FTO	HR	95% CI	P
Low	Reference		
High	0.67	0.49-0.91	0.01
Tumor Stage			
Stage I	Reference		
Stage II	1.21	0.65-2.24	0.553
Stage III	2.38	1.59-3.58	<0.0001
Stage IV	6.75	4.60-9.89	<0.0001
Age	1.04	1.02-1.05	<0.0001

Figure 4. Kaplan-Meier Survival Curves by FTO Expression



4. Association between FTO Methylation level and overall patient survival in CCRCC

The log-rank test was performed to test if there was any difference in patient survival between the two groups of patients with different levels of FTO methylation. It turned out that there was a borderline significant difference between the two groups (log rank p= 0.0682). Patients with Lower FTO methylation level seemed to have better overall survival comparing to those with higher FTO methylation level. The Kaplan-Meier survival curves constructed for these two groups have been shown in figure 5.

In order to adjust for the potential confounders, the Multivariate Cox regression model was used, similar to the model for FTO expression, the construction of the model also started with all potential confounders inside (race, sex, tumor stage, age), and then backward elimination strategy was applied to arrive at the final parsimonious model. Two variables (race, sex) were

dropped during this process, and three variables were retained in the final model (at p-value <0.1). The results have been shown in table 3. After adjusting for tumor stage and age, there was a significant association between the FTO methylation level and the patient survival in CCRCC. The high-level FTO methylation group has relative elevated risk of death comparing to the low-level group. (HR: 2.17, 95% CI: 1.38-3.42, p= 0.0008)

Figure 5. Kaplan-Meier Survival Curves by FTO Methylation Level

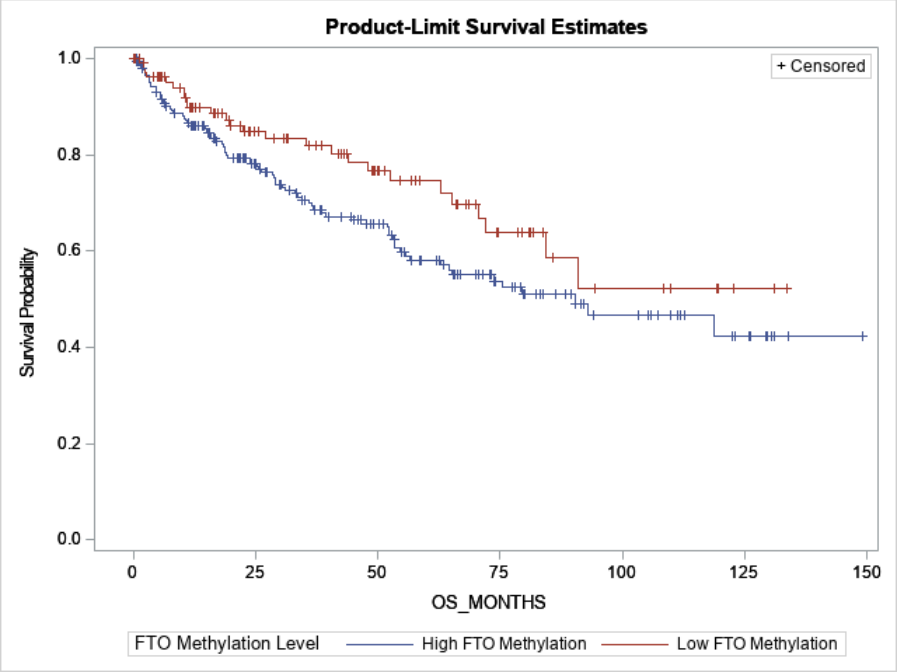


Table 3. Association between FTO Methylation and CCRCC Patient Survival

Variable	Death		
FTO Methylation	HR	95% CI	P
Low	Reference		
High	2.17	1.38-3.42	0.0008
Tumor Stage			
Stage I	Reference		
Stage II	1.46	0.65-3.31	0.3607
Stage III	3.21	1.84-5.62	<0.0001
Stage IV	10.34	6.08-17.60	<0.0001
Age	1.04	1.02-1.06	<0.0001

5. Association between METTL3 expression and overall patient survival in CCRCC

The log-rank test was performed for the two groups of patients based on the differentiated METTL3 expression level. The significant difference in patient survival has been observed between the two groups ($p < 0.0001$), patients with higher METTL3 expression have inferior overall survival compared to those with lower METTL3 expression, which is opposite to the association between FTO expression and the overall survival. The Kaplan-Meier survival curves constructed for these two groups have been shown in figure 6.

To adjust for the potential confounders, the Multivariate Cox regression model was applied, similar to the model for FTO expression, the construction of the model also started with all potential confounders inside (race, sex, tumor stage, age), and then the final parsimonious model

was obtained using the backward elimination strategy. Two variables (race, sex) were dropped during this process, and three variables were retained in the final model (at p-value <0.1). The result has been shown in table 4. After adjusting for tumor stage and age, there was a significant association between the METTL3 expression level and the overall patient survival in CCRCC. The elevated METTL3 expression has raised the risk of death for the CCRCC patients. (HR: 1.97, 95% CI: 1.45-2.68, $p < 0.0001$)

Figure 6. Kaplan-Meier Survival Curves by METTL3 Level

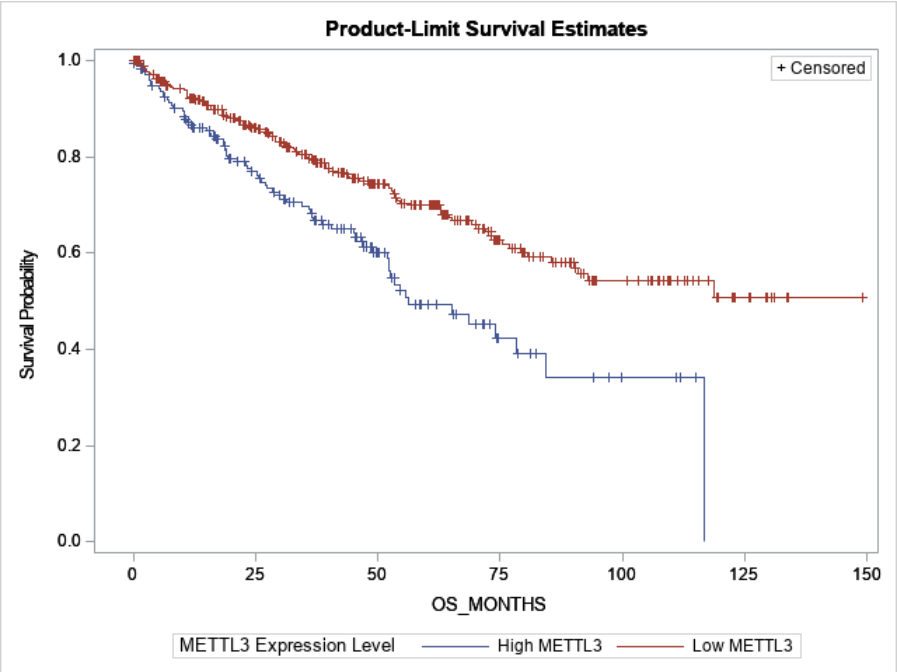


Table 4. Association between METTL3 Expression and CCRCC patient survival

Variable	Death		
METTL3	HR	95% CI	P
Low	Reference		
High	1.97	1.45-2.68	< 0.0001
Tumor Stage			
Stage I	Reference		
Stage II	1.20	0.65-2.22	0.5682
Stage III	2.52	1.68-3.79	<0.0001
Stage IV	7.05	4.81-10.32	<0.0001
Age	1.03	1.02-1.05	<0.0001

6. Association between METTL3 methylation level and overall patient survival in CCRCC

The log-rank test was performed to test if there was any difference in patient survival between the two groups of patients with different levels of METTL3 methylation. Kaplan-Meier showed a significant difference between the two groups (log rank p=0.0004). Patients with high METTL3 methylation level have superior overall survival comparing to those with low METTL3 methylation level. The Kaplan-Meier survival curves constructed for these two groups have been shown in figure 7.

In order to adjust for the potential confounders, the Multivariate Cox regression model was used, similar to the model for METTL3 expression, the construction of the model also started with all potential confounders inside (race, sex, tumor stage, age), and then backward elimination

strategy was applied to arrive at the final parsimonious model. Two variables (race, sex) were dropped during this process, and three variables were retained in the final model (at p-value <0.1). The result has been shown in table 5. After adjusting for tumor stage and age, there was a significant association between the METTL3 methylation level and patient survival in CCRCC. The high-level METTL3 methylation group has relative reduced risk of death comparing to the low-level group. (HR: 0.49, 95% CI: 0.31-0.79, p= 0.0008)

Figure 7. Kaplan-Meier Survival Curves by METTL3 Methylation Level

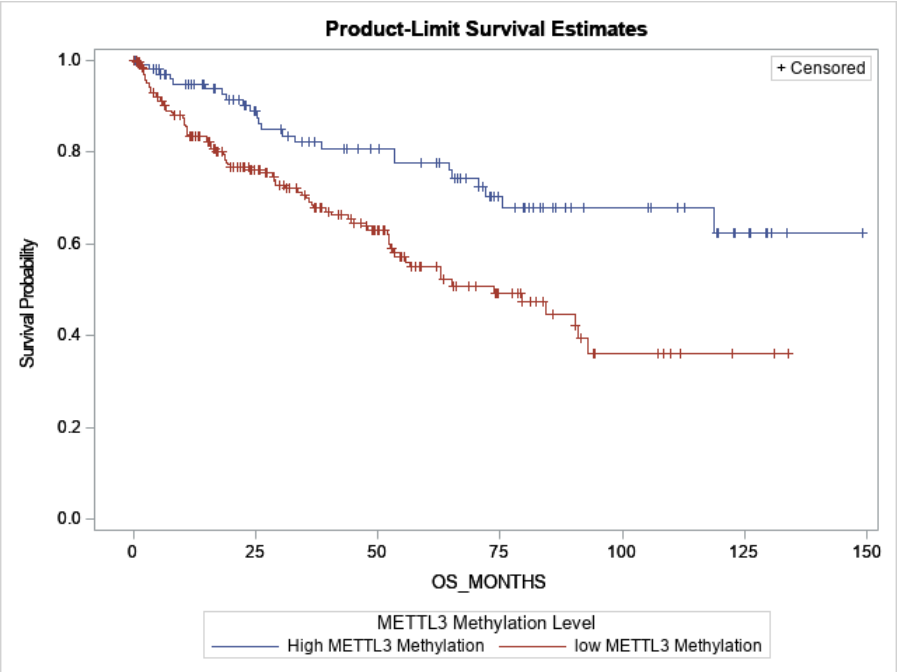


Table 5. Association between METTL3 Methylation and CCRCC patient survival

Variable	Death		
Mettl3 Methylation	HR	95% CI	P
Low	Reference		
High	0.49	0.31-0.79	0.0031
Tumor Stage			
Stage I	Reference		
Stage II	1.44	0.64-3.26	0.379
Stage III	2.59	1.47-4.55	0.001
Stage IV	8.67	5.14-14.63	<0.0001
Age	1.04	1.02-1.06	0.0003

7. Effects of FTO-METTL3 Interaction on Patient Survival in CCRCC

Wald test was performed to explore the interaction effect of FTO-METTL3 on patient survival in CCRCC. Patients have been divided into two groups based on the median distribution of FTO expression, and then also have been categorized into two groups based on the median distribution of the METTL3 expression. There were 266 patients in the Low FTO group, with the FTO expression ranging from 327.91 Reads Per Kilobase of transcript per Million mapped reads (RPKM) to 2349.60 RPKM, comparing to the 267 patients in the High FTO group, with the FTO expression ranging from 2350.79 RPKM to 6053.67 RPKM. For the two METTL3 groups, there were 266 patients in the Low METTL3 group, with the METTL3 expression ranging from 36.73 RPKM to 437.52 RPKM, compared to the 267 patients in the High METTL3 group, with the METTL3 expression ranging from 438.51 RPKM to 1499.30 RPKM. The Wald test result has

shown a significant interaction between the FTO expression and METTL3 expression with the adjustment of age and tumor stage. (W^2=4.5559, P=0.0328) The stratified result for FTO and METTL3 has been shown in table 6.

Table 6. Stratified Hazard Ratios for FTO and METTL3

Strata	Death	
	HR	95% CI
FTO High vs. Low at Low METTL3	1.15	0.71-1.88
FTO High vs. Low at High METTL3	1.97	1.45-2.68
	HR	95% CI
METTL3 High vs. Low at Low FTO	2.19	1.38-3.50
METTL3 High vs. Low at High FTO	1.10	0.71-1.70

8. Correlation between FTO expression and dsRNA related genes

Spearman Correlation results are shown in table 7. Significantly positive correlations were observed between FTO and TLR3, TLR7, TLR8, DDX58, IFIH1, NLRP3. (all $p<0.0001$) Correlation coefficients were 0.60 (95% CI: 0.54-0.65) for TLR3, 0.35 (95% CI: 0.27-0.42) for TLR7, 0.31 (95% CI: 0.23-0.38) for TLR8, 0.35 (95% CI:0.28-0.43) for DDX58, 0.40 (95% CI: 0.32-0.47) for IFIH1, and 0.24 (95% CI: 0.16-0.32) for NLRP3.

Table 7. Spearman Correlation between FTO Expression and dsRNA Related Genes

Gene	N	Correlation Coefficient	95% CI	P
TLR3	533	0.60	0.54-0.65	<0.0001
TLR7	533	0.35	0.27-0.42	<0.0001
TLR8	533	0.31	0.23-0.38	<0.0001
DDX58	533	0.35	0.28-0.43	<0.0001
IFIH1	533	0.40	0.32-0.47	<0.0001
NLRP3	533	0.24	0.16-0.32	<0.0001

9. Correlation between FTO expression and innate immunity related genes

Spearman Correlation results are shown in table 8. Significantly positive correlations were observed between FTO and CD80, CD86, FCGR3A. Correlation coefficients were 0.10 (95% CI: 0.02-0.19) for CD80 ($p=0.0162$), 0.17 (95% CI: 0.09-0.25) for CD86 ($p<0.0001$), 0.23 (95% CI: 0.15-0.31) for FCGR3A ($p<0.0001$). While significantly negative correlations were observed between FTO and CD274. Correlation coefficients was -0.09 (95% CI: -0.17, 0.00) ($p=0.0498$).

Table 8. Spearman Correlation between FTO Expression and Innate Immunity Related Genes

Gene	N	Correlation Coefficient	95% CI	P
CD274	533	-0.09	-0.17, 0.00	0.0498
CD80	533	0.10	0.02, 0.19	0.0162
CD86	533	0.17	0.09, 0.25	<0.0001
FCGR3A	533	0.23	0.15, 0.31	<0.0001

10. Correlation between METTL3 expression and dsRNA related genes

Spearman Correlation results are shown in table 9. Significantly negative correlations were observed between METTL3 and TLR3, TLR7, TLR8, DDX58, IFIH1, NLRP3. (all $p<0.0001$ except for NLRP3 $p=0.02$) Correlation coefficients were -0.40 (95% CI: -0.46, -0.32) for TLR3, -0.28 (95% CI: -0.36, -0.20) for TLR7, -0.23 (95% CI: -0.31, -0.15) for TLR8, -0.25 (95% CI:-0.33, -0.17) for DDX58, -0.18 (95% CI: -0.26, -0.10) for IFIH1, and -0.10 (95% CI: -0.18, -0.02) for NLRP3.

Table 9. Spearman Correlation between METTL3 Expression and dsRNA Related Genes

Gene	N	Correlation Coefficient	95% CI	P
TLR3	533	-0.40	-0.46, -0.32	<0.0001
TLR7	533	-0.28	-0.36, -0.20	<0.0001
TLR8	533	-0.23	-0.31, -0.15	<0.0001
DDX58	533	-0.25	-0.33, -0.17	<0.0001
IFIH1	533	-0.18	-0.26, -0.10	<0.0001
NLRP3	533	-0.10	-0.18, -0.02	0.02

11. Correlation between METTL3 expression and innate immunity related genes

Spearman Correlation results are shown in table 10. Significantly negative correlations were only observed between METTL3 and CD86, FCGR3A. Correlation coefficients were -0.19 (95% CI: -0.27, -0.11) for CD86 ($p<0.0001$), -0.20 (95% CI: -0.28, -0.12) for FCGR3A ($p<0.0001$).

Table 10. Spearman Correlation between METTL3 Expression and Innate Immunity Related Genes

Gene	N	Correlation Coefficient	95% CI	P
CD274	533	0.04	-0.05,0.12	0.3749
CD80	533	0.02	-0.06,0.11	0.6094
CD86	533	-0.19	-0.27, -0.11	<0.0001
FCGR3A	533	-0.20	-0.28, -0.12	<0.0001

12. Differential expression of FTO in CCRCC vs. normal kidney tissues

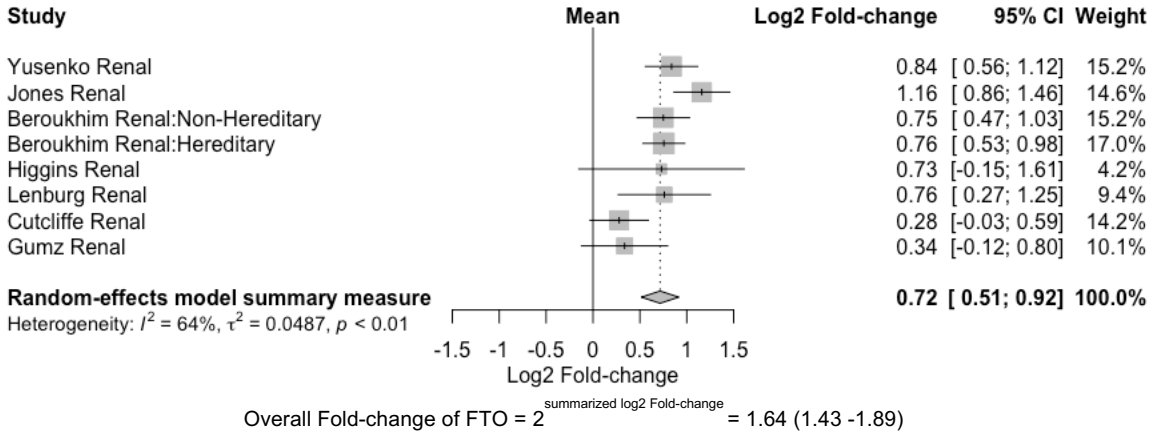
The summary result of fold change in FTO expression in CCRCC comparing to normal kidney tissues from random effect Meta-analysis has been shown in table 11. Eight studies have met the including criteria described above in the method section, and fold-change, p-value, t-statistics, number of carcinoma samples, number of normal samples were extracted from these studies to perform the random-effects Meta-analysis. All of these eight studies have an upregulation of FTO expression in CCRCC comparing to that in normal kidney tissues, with fold-change greater than 1. the summary FTO expression from the random-effects Meta-analysis across these eight studies showed 1.64 folds upregulation in CCRCC compared to that in normal kidney tissues, with 95% CI ranging from 1.43 to 1.89. The forest plot has been shown in Figure 8.

Table 11. Random Effects Meta-analysis of Differential FTO Expression in CCRCC vs. Normal Kidney Tissues

Study	Fold-change	P-value	t-statistics	N_{case}	N_{normal}
Yusenko Renal					
Clear Cell Renal Cell Carcinoma vs. Normal	1.787	1.30E-06	5.884	26	5
Jones Renal					
Clear Cell Renal Cell Carcinoma vs. Normal	2.235	9.06E-10	7.635	23	23
Beroukhim Renal					
Non-Hereditary Clear Cell Renal Cell Carcinoma vs. Normal	1.682	4.22E-06	5.267	27	11

Hereditary Clear Cell Renal Cell Carcinoma vs. Normal	1.688	6.08E-07	6.635	32	11
Higgins Renal					
Clear Cell Renal Cell Carcinoma vs. Normal	1.659	0.116	1.623	24	3
Lenburg Renal					
Clear Cell Renal Cell Carcinoma vs. Normal	1.694	0.005	3.031	9	9
Cutcliffe Renal					
Clear Cell Sarcoma of the kidney vs. Normal	1.213	0.07	1.754	14	3
Gumz Renal					
Clear Cell Renal Cell Carcinoma vs. Normal	1.263	0.089	1.436	10	10
Summary Fold-change (95% CI)	1.64 (1.43, 1.89)				

Figure 8. Forest Plot for Random Effects Meta-analysis of Differential FTO Expression in CCRCC vs. Normal Kidney Tissues



13. Differential expression of METTL3 in CCRCC vs. normal kidney tissues

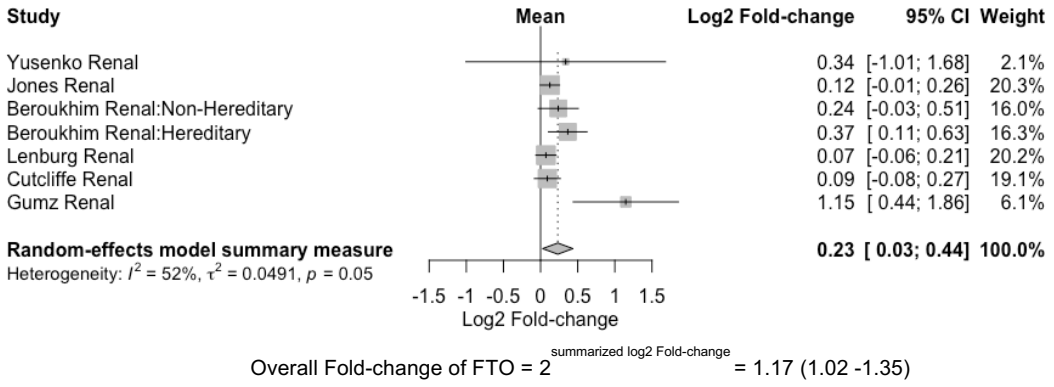
The summary result of fold change in METTL3 expression in CCRCC comparing to normal kidney tissues from random effect Meta-analysis has been shown in table 12. Seven studies were included, and fold-change, p-value, t-statistics, number of carcinoma samples, number of normal samples were extracted from these studies to perform the random-effects Meta-analysis. All of these seven studies have an upregulation of METTL3 expression in CCRCC, comparing to that in normal kidney tissues, with fold-change greater than 1. The summary METTL3 expression from the random-effects meta-analysis across these seven studies showed 1.17 folds upregulation in CCRCC compared to that in normal kidney tissue, with 95% CI ranging from 1.02 to 1.35. The forest plot has been shown in Figure 9.

Table 12. Random Effects Meta-analysis of Differential METTL3 Expression in CCRCC vs. Normal Kidney Tissues

Study	Fold-change	P-value	t-statistics	N_{case}	N_{normal}
Yusenko Renal					
Clear Cell Renal Cell Carcinoma vs. Normal	1.26	0.322	0.49	26	5
Jones Renal					
Clear Cell Renal Cell Carcinoma vs. Normal	1.09	0.036	1.86	23	23
Beroukhim Renal					
Non-Hereditary Clear Cell Renal Cell Carcinoma vs. Normal	1.18	0.051	1.73	27	11
Hereditary Clear Cell Renal Cell Carcinoma vs. Normal	1.29	0.007	2.77	32	11

Lenburg Renal					
Clear Cell Renal Cell Carcinoma vs. Normal	1.05	0.159	1.04	9	9
Cutcliffe Renal					
Clear Cell Sarcoma of the kidney vs. Normal	1.07	0.162	1.02	14	3
Gumz Renal					
Clear Cell Renal Cell Carcinoma vs. Normal	2.22	0.003	3.18	10	10
Summary Fold-change (95% CI)	1.17 (1.02, 1.35)				

Figure 9. Forest Plot for Random Effects Meta-analysis of Differential METTL3 Expression in CCRCC vs. Normal Kidney Tissues



Discussion

In this study, we demonstrated that patient survival in renal clear cell carcinoma (CCRCC) is associated with the FTO and METTL3 machinery. We found that patients with high FTO level have superior overall survival compared to those with low FTO level. The association remained

significant after adjusting for age and tumor stage. This result suggests high FTO level has played a protective role during the prognosis of CCRCC patients. This effect has also been proven by the association between FTO methylation level and the patient survival in CCRCC. It has been shown that higher level of FTO methylation was associated with borderline significantly worse survival in CCRCC. After adjusting for the age and tumor stage, a significant difference was observed. A negative correlation has also been found between FTO level and FTO methylation level. All of the evidence above points to the fact that FTO is acting as an anti-tumor factor in the CCRCC progression.

Previous studies showed that FTO might play totally different roles in the progression and prognosis of different types of cancers. Xu D and colleagues found that among gastric cancer patients, highly expressed FTO is associated with poor survival and cancer occurrence.[26]While another study has found that FTO can act as a tumor inhibitor in CCRCC through a novel FTO-PGC-1α signaling axis.[27]The reason for the distinct roles FTO has played in different cancers is still unclear, and the complicated mechanism under this dynamic process still needs further exploration.

Since almost all kidney cancers are documented to be associated with immune dysfunction, [28]and recently, m6A modification has been demonstrated to be associated with the regulation of innate immune systems,[29] we tried to investigate the association between FTO and CCRCC patient survival from the immune level.

It has been found that m6A modification can cause a structural switch from double-stranded RNA (dsRNA) to single-stranded RNA in the secondary structure of RNA, [22]since dsRNA can trigger the activation of innate immunity, the decreased amount of dsRNA resulting from this switch would result in downregulated response by innate immunity.[23] It has been proven by Katalin and colleagues that RNA with m6A modified will not activate toll-like receptors TLR3, TLR7, and TLR8, which are important TLR family members used to recognize pathogens by innate immune system. The m6A modification has been demonstrated to inhabit the activation of DCs. The secretion of cytokine and activation marker such as CD80, CD86 has been observed to be suppressed by m6A.[29]In this study, we found there was significantly positive correlation between FTO expression and TLR3, TLR7, TLR8, which is consistent with the findings from previous studies, since FTO acts as a demethylase in m6A, higher FTO expression is associated with lower m6A level, and as a result, more TLR receptors will be activated. Significantly positive correlation was also observed between FTO expression and CD80, CD86, which suggested that FTO can improve the activation and maturity of DCs. Instead, significantly negative correlation was observed between FTO expression and CD274(PD-L1), which suggested that higher FTO expression is associated with lower expression of checkpoint inhibitor. PD-L1 has been considered to act as suppressors for anti-tumor immune response and are critical in tumor progression, and it has been demonstrated to have reliable effects on treating many advanced cancers.[30] The significantly positive correlation was also observed between FTO and DDX58, IFIH1. These two genes encoded two essential innate immune receptors, RIG-1 and MDA5, which also have been demonstrated as dsRNA detectors.[31] The result is consistent with the findings that m6A can switch the structure of secondary RNA from double-stranded to single-stranded.

Based on all the evidence above, it is reasonable that high FTO level is associated with superior patient survival in CCRCC. Acting as eraser in m6A modification, FTO can remove the m6A from RNA, which will cause the increase in the amount of dsRNA, and as a stimulus for innate immunity, the immune response will be improved for CCRCC patients with high FTO level.

On the opposite, since METTL3 acts as a methylase in m6A modification, it is expected that METTL3 will have an opposite effect on patient survival in CCRCC. The results from this study is exactly the same as expected. Patients with higher METTL3 expression are associated with inferior overall survival, the association remained significant after adjusting for age and tumor stage. The association between METTL3 methylation level and patient survival also have demonstrated that METTL3 acts as an unfavorable marker in the progression of CCRCC. The association remained significant after adjusting for age and tumor stage. A significantly negative correlation was also found between the METTL3 expression level and METTL3 methylation level. Based on all the evidence above, METTL3, on the opposite of FTO, is playing an offensive role in the progression of CCRCC.

As opposite to FTO, significantly negative correlation was observed between METTL3 expression and TLR3, TLR7, TLR8, DDX58, and IFIH1, which is consistent with the theory of RNA structure switch by m6A, and the activation of innate immunity as described above. Acting as the writer in m6A, high METTL3 level will produce more m6A modified RNA, coupled with diminished dsRNA, and result in less activation of dsRNA sensors and TLR receptors. There were also significantly negative correlations between METTL3 and CD80, FCGR3A, which

suggested inhibited innate immune response by METTL3. The result is supported by another study which has found the depletion of METTL3 can result in upregulated interferon related response and as a result, inhibit viruses infection.[32] However, no significant correlation was found between METTL3 level and anti-tumor immune inhibitor PD-L1. Since m6A is a dynamic process, and it has been demonstrated to be able to regulate gene expressions through various pathways, which are still not totally understood at present, further investigations of the complicated mechanisms are needed.

Based on the opposite roles FTO and METTL3 played in the m6A modification process and also in CCRCC patient survival, at last, we also want to know whether there was an interaction between these two genes when they take effects on the prognosis of CCRCC. A study published last year has reported that the cross-talk among m6A writers, readers and erasers can regulate cancer growth and progression. They found that m6A methylase METTL14 and m6A demethylase ALKBH5 could regulate each other's level and also suppress m6A reader YTHDF3 to determine the m6A level in target genes. [33] Similarly, our study also found a significant interaction between FTO expression and METTL3 expression in CCRCC patient survival after adjusting for age and tumor stage. From the meta-analysis, both FTO and METTL3 have been shown upregulated in tumor in comparison with normal tissues, which suggested both of these two genes have oncogenic functions.

Conclusion

In summary, this study has demonstrated that epigenetic silencing RNA methylation machinery FTO and METTL3 are associated with patient survival in CCRCC, they are also associated with innate immunity response level. This finding suggests that machinery FTO and METTL3 are potential prognostic and diagnostic markers for CCRCC, and further studies in how exactly this machinery regulates the immune gene expression are still needed in order to design target immunotherapy for CCRCC patients in the future.

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