**Research Article**

**Decreased ALKBH5, FTO, and YTHDF2 in Peripheral Blood Are as Risk Factors for Rheumatoid Arthritis**

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ALKBH5 (alkylation repair homolog protein 5), FTO (fat mass and obesity-associated protein), and RNA N6-methyladenosine (m6A) demethylase, are essential for the m6A mRNA modification. YTHDF2 (YT521-B homology domains 2) called m6A "readers" can recognize m6A modification. As the key enzymes of m6A methylation modification, ALKBH5, FTO, and YTHDF2 have been implicated in many diseases. However, little is known about the role of ALKBH5, FTO, and YTHDF2 in rheumatoid arthritis (RA). We measured the mRNA expression of ALKBH5, FTO, and YTHDF2 in RA patients and controls by quantitative real-time polymerase chain reaction, and the global m6A content was detected by an ELISA-like format. The mRNA expression of ALKBH5, FTO, and YTHDF2 in RA patients was further analyzed to investigate its correlations with disease activity. And, multivariate analysis (logistic regression) was used to analyze the risk factors. The mRNA expression of ALKBH5, FTO, and YTHDF2 in RA patients was significantly decreased compared to controls. The mRNA expression of ALKBH5 was significantly increased in RA patients that received regular treatment. The mRNA expression of FTO was associated with disease activity score 28 (DAS28), complement 3 (C3), immunoglobulin G (IgG), and lymphocyte-to-monocyte ratio (LMR), some common markers for RA disease activity. The mRNA expression of YTHDF2 was associated with RBC, L%, N%, NLR, and LMR. Furthermore, logistic regression analysis revealed that decreased expression of ALKBH5, FTO, and YTHDF2 in peripheral blood was a risk factor for RA. Moreover, the peripheral blood global m6A content was significantly increased in patients with RA compared to CON, and increased m6A contents negatively correlated with decreased mRNA expression of FTO. In conclusion, this study firstly demonstrates the critical role of ALKBH5, FTO, and YTHDF2 in RA, which provides novel insights into recognizing the pathogenesis of RA and a promising biomarker for RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic debilitating systemic autoimmune disease with permanent joint destruction, which is a highly disabling disease because of joint deformity and loss of function [1]. Due to its heterogeneity and multiplicity, the etiology of RA is still largely unknown [2]. Many studies have probed that the development of RA is attributed to genetic, infectious, environmental, and hormonal factors [3]. Accumulating studies have shown that dysregulation of the immune system, including abnormal activation T and B lymphocytes, neutrophils, mast cells, and macrophages, is involved in the mechanisms that drive the onset of RA [4, 5]. High levels of autoantibodies such as anticitrullinated protein antibodies (ACPA) generated by dysregulated B cells can cause lung destructions [6, 7]. Neutrophils and other inflammatory cells can arrive at sites of inflammation under stimulants from macrophages and mast cells, leading to joint injuries and deformity. Many current studies have probed the pathogenesis of RA, but the interference of different epigenetic alterations in RA is not fully understood.

In addition to DNA and histone modifications, epigenetic modifications of RNA have been proposed to be another layer of epigenetic regulation. Among RNA modifications, N6-methyladenosine (m6A) modification is the most prevalent in mammalian mRNA [8]. Despite m6A modification being
first reported in early 1970s [9], its role and significance in RA are largely unknown. The key enzymes for m6A methyl-
ation modification primarily include m6A methyltransferase (writer), m6A demethylase (eraser), and m6A RNA-binding pro-
teins (reader) [10]. Two well-known eraser enzymes, ALKBH5 (alkylation repair homolog protein 5) and FTO (fat mass and obesity-associated protein), are involved in mediating methylation reversal [11, 12]. It has been 
demonstrated that the role of ALKBH5 and FTO may alter in different 
tissues and cells. Evidences have found that ALKBH5 and 
FTO may promote cancer tumorigenesis [13, 14]. However, 
ALKBH5 and FTO have been reported as a tumor suppressor 
inhibiting cancer progression [15, 16]. More interestingly, 
Huang et al. have found that ALKBH5 and FTO are associated 
with inflammation [17], and Lu et al. have shown that the 
expression of ALKBH5 and FTO mRNA in the liver of piglets 
was decreased after injection of LPS, which could take a signif-
icant role in hepatic injury during inflammation [18]. 
YTHDF2 (YT521-B homology domains 2) called m6A "readers" can 
recognize m6A modification [19], and YTHDF2 has been reported 
to regulate LPS-induced inflammatory 
response [20]. Thus, the role of ALKBH5, FTO, and YTHDF2 in RA, an autoimmune and inflammatory disease, still needs 
to be explored. In this study, we investigate the expression of 
ALKBH5, FTO, and YTHDF2 in RA and its relationship with 
disease activity.

2. Materials and Methods

2.1. Patient Variables. Patients (n = 79) who fulfilled the 
revised American College of Rheumatology (ACR) 2010 
criteria for RA [21] were consecutively enrolled in the First 
Affiliated Hospital of Nanchang University between October 
2018 and March 2019. Those RA patients accompanied by 
other autoimmune or inflammatory diseases, hormonal 
diseases, cancers, or mental disorders were excluded. All 
patients had new-onset RA and had not received cortico-
steroids or immunosuppressive drugs prior to recruitment. 
Then, 9 new-onset RA cases were administered therapeu-
tic regimens with corticosteroids and immunosuppressive 
medications over at least 15 days. The information on disease activity score 
28 (DAS28), swollen joint count (SJC), tender joint count (TJC), patient visual analogue scale (VAS), erythrocyte sedi-
don factor (ESR), C-reactive protein (CRP), anti-cyclic citrullinated peptide antibodies (Anti-CCP), rheumatoid 
factor (RF), white blood cell count (WBC), red blood cell count (RBC), hemoglobin, hematocrit (HCT), platelet count (PLT), lymphocyte count (L), lymphocyte percentage (L%), monocyte count (M), monocyte percentage (M%), neutrophil count (N), neutrophil percentage (N%), neutrophil 
to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and lymphocyte-to-monocyte ratio (LMR) was col-
clected. Health control (CON) (n = 61) without autoimmune 
or inflammatory diseases and who were also unrelated to 
patients with autoimmune diseases were randomly enrolled 
in the First Affiliated Hospital of Nanchang University 
between October 2018 and March 2019. All study protocols 
were approved by the Ethics Committee of the 

**Table 1: The amplification primers sequences.**

| Gene name | Sequence (5'-3') |
|-----------|-----------------|
| METTL3    | F: AAGCTGCACTTCAGACGAAT  |
|           | R: GGAAATCACCTCCGACACTC |
| METTL14   | F: AGAAACTTGGACGGCTTCTCT |
|           | R: TCTCTCTCATAATGGCAAATTTTCTT |
| WTAP      | F: GGCGGAAATGTCGAAATGCT |
|           | R: CCAACTGCTGGCGTGTCGTT |
| ALKBH5    | F: CGACACCCGAATTGGCTTGAG |
|           | R: CCTCTTCAGGGCCTTTCAC |
| FTO       | F: GGACAGCTGAAGTTGGG |
|           | R: CCTCTTCAGGGCCTTAC |
| YTHDF2    | F: CTATTGGAAGGCACGATGTGA |
|           | R: TGCCACCAACCTGCTTTCG |
| GAPDH     | F: GGATGGACGTGTGTCATGAG |

2.2. Collection of Peripheral Blood and Total RNA Extraction. 
Peripheral blood samples (2 ml) were collected into EDTA-
2K containing tubes, and total RNA was extracted as soon 
as possible by using TRIZol reagent (Invitrogen, USA) 
according to the manufacturer’s protocol. The concentration 
and integrity of the RNA was assessed by a NanoDrop 
ND-1000 spectrophotometer (Agilent Technologies, Inc.).

2.3. Reverse Transcription-Quantitative PCR (RT-qPCR) Analysis. 
Reverse transcription (RT) and quantitative PCR 
(qPCR) were carried out with the PrimeScript™ RT kit 
(Takara Bio Inc.) and SYBR Premix Ex Taq™ II (Takara 
Bio Inc.), respectively. RT-qPCR was performed on an ABI 
7500 Real-Time PCR System (Applied Biosystems; Thermo 
Fisher Scientific, Inc.) with the following PCR thermocycler 
protocol: initial denaturation step at 95°C for 5 min, followed 
by 40 cycles of 95°C for 15 sec (denaturation), 60°C for 1 min 
(annealing and elongation), and 72°C for 2 min (final exten-
sion). GAPDH was used as an internal control. The primers 
used in RT-qPCR are listed in Table 1. The data were analyzed using the 2^ΔΔCt method [22].

2.4. m6A RNA Methylation Analysis. Total RNA that was iso-
lated from the peripheral blood of RA patients and CON was 
used to detect m6A RNA methylation by The EpiQuik™ 
m6A RNA Methylation Quantification Kit (Colorimetric) 
according to the manufacturer’s protocol.

2.5. Statistical Analysis. Statistical analysis and graphic 
presentation were carried out with GraphPad Prism 5.0 (Graph-
Pad Software, Inc.) and SPSS version 17.0 (SPSS Inc.). A 
Student’s t-test was used between two groups where the sam-
ples passed the normality test; otherwise, the nonparametric
Mann–Whitney test was used to analyze the data. The paired t-test was performed for the evaluation of changes in treatment. Spearman’s method was used for correlation analysis. Multivariate regression analysis (logistic regression) was used to analyze the risk factors. P < 0.05 was considered to indicate statistically significant differences.

3. Results

3.1. Characteristics of Study Subjects. A total of 140 subjects were enrolled in the present study, including 79 patients with RA and 61 CON. RA patients were classified into screening and validation cohorts. The screening cohort included 20 RA patients and 20 CON. An independent cohort consisting of 59 RA patients and 41 CON was enrolled in the validation set for evaluation of abnormal genes. The characteristics of the study subjects are summarized in Table 2. There were no significant differences between RA patients and CON regarding age or sex. No correlation between the mRNA expression of ALKBH5, FTO, and YTHDF2 in the peripheral blood, and age or sex was observed in RA or CON (data not shown).

3.2. Decreased mRNA Expression of ALKBH5, FTO, and YTHDF2 in the Peripheral Blood from RA Patients. To identify the mRNA expression of METTL3, METTL14, WTAP, ALKBH5, FTO, and YTHDF2 in the peripheral blood from RA patients and CON, we used qRT-PCR to assess these gene expressions in screening testing set consisting of 20 RA patients and 20 CON. Data showed that although these gene expressions in screening testing set consisting of 59 RA patients and 41 CON was enrolled and determined for their ALKBH5, FTO, and YTHDF2 levels. From the data of all the RA patients and CON, the mRNA expression of ALKBH5, FTO, and YTHDF2 in the peripheral blood from 79 RA patients was significantly lower compared to 61 CON (all P < 0.050) (Figures 2(a)–2(c)).

3.3. Correlation of ALKBH5, FTO, and YTHDF2 Expression in the Peripheral Blood with Clinical Features of RA. To determine whether the mRNA expression of peripheral blood ALKBH5, FTO, and YTHDF2 from RA patients could reflect the activity of the disease, clinical features including DAS28-ESR, DAS28-CRP, RF, ESR, CRP, C3, C4, IgG, WBC, RBC, HGB, HCT, PLT, L, M, M%, N, N%, PLR, NLR, LMR, and duration were collected, and analysis was performed to assess the correlation between the clinical features of RA and the mRNA expression of the peripheral blood ALKBH5, FTO, and YTHDF2. As shown in Figure 3, the mRNA expression of peripheral blood FTO correlated with DAS28-ESR, DAS28-CRP, C3, IgG, LMR, and the mRNA expression of the peripheral blood YTHDF2 correlated with RBC, L%, N%, NLR, and LMR. However, no correlation was found between these clinical features of RA and the mRNA expression of peripheral blood ALKBH5 (data no shown).

Subsequently, the mRNA expression of peripheral blood ALKBH5, FTO, and YTHDF2 was detected in 9 new-onset RA cases pre- and posttreatment. Notably, the mRNA expression of peripheral blood ALKBH5 in 7 of the RA patients increased following the treatment when compared

### Table 2: Clinical details of the patients with RA and HC.

| Clinical characteristic | RA   | CON  |
|-------------------------|------|------|
| Number of subjects      | 79   | 61   |
| Sex, male/female        | 15/64| 12/49|
| Age, years              | 51.35 ± 12.26 | 48.66 ± 14.09 |
| Duration, day           | 1439.82 ± 2246.33 |  |
| DAS28-ESR               | 6.12 ± 1.38 |      |
| DAS28-CRP               | 5.43 ± 1.31 |      |
| SJC                     | 11.26 ± 7.10 |      |
| TJC                     | 13.64 ± 6.93 |      |
| VAS                     | 54.35 ± 28.52 |      |
| RF, IU/ml               | 507.39 ± 613.34 |      |
| Anti-CCP, RU/ml         | 614.87 ± 772.44 |      |
| ESR, mm/h               | 57.34 ± 33.78 |      |
| CRP, mg/l               | 35.03 ± 37.63 |      |
| IgG, g/l                | 16.54 ± 4.12  |      |
| C3, g/l                 | 0.98 ± 0.21   |      |
| C4, g/l                 | 0.23 ± 0.09   |      |
| WBC, 10⁹/l              | 7.90 ± 2.40 * | 5.69 ± 0.95 |
| RBC, 10¹²/l             | 4.37 ± 0.51 * | 4.55 ± 0.36 |
| HGB, g/l                | 122.68 ± 18.37 * | 136.98 ± 11.08 |
| HCT, l/l                | 0.38 ± 0.05 * | 0.41 ± 0.03 |
| PLT, 10⁹/l              | 334.92 ± 135.74 * | 236.51 ± 41.34 |
| L, 10⁹/l                | 1.61 ± 0.56 * | 1.97 ± 0.38 |
| L, %                    | 21.42 ± 8.26 * | 35.13 ± 6.94 |
| M, 10⁹/l                | 0.43 ± 0.18 * | 0.35 ± 0.08 |
| M, %                    | 5.71 ± 2.07   | 6.22 ± 1.41 |
| N, 10⁹/l                | 5.70 ± 2.26 * | 3.24 ± 0.80 |
| N, %                    | 70.67 ± 9.77 * | 56.10 ± 7.01 |
| PLR                     | 242.55 ± 178.36 * | 123.65 ± 28.00 |
| NLR                     | 4.23 ± 3.12 * | 1.72 ± 0.58 |
| LMR                     | 4.56 ± 4.97 * | 5.97 ± 1.63 |

*P < 0.05 RA compared to CON. Anti-cyclic citrullinated peptide antibodies (Anti-CCP), health control (CON), C-reactive protein (CRP), disease activity score (DAS28), erythrocyte sedimentation rate (ESR), hematocrit (HCT), hemoglobin (HGB), lymphocyte count (L), lymphocyte percentage (L%), lymphocyte-to-monocyte ratio (LMR), monocyte count (M), monocyte percentage (M%), neutrophils count (N), neutrophils percentage (N%), neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), platelet count (PLT), tender joint count (TJC), rheumatoid arthritis (RA), red blood cell count (RBC), rheumatoid factors (RF), swollen joint count (SJC), Visual Analogue Scale (VAS), and white blood cell count (WBC).
Figure 1: Continued.
with those prior to treatment, and 2 RA patients had decreased mRNA expression of peripheral blood ALKBH5. As shown in Figure 4, after treatment, there was a significant difference; however, there was no difference between pre- and posttreatment levels in the mRNA expression of the peripheral blood FTO and YTHDF2.

3.4. The Expressions of ALKBH5, FTO, and YTHDF2 in the Peripheral Blood Were Risk Factors for RA. The aforementioned results (Figures 3 and 4) demonstrate that the decreased mRNA expression of ALKBH5 in the peripheral blood correlated with treatment, and the decreased mRNA expression of FTO and YTHDF2 in the peripheral blood correlated with disease activity. Thus, we investigated whether ALKBH5, FTO, and YTHDF2 were risk factors for RA using the “enter method” of multivariate logistic regression. As shown in Table 3, the equations about the levels of peripheral blood ALKBH5, FTO, and YTHDF2 were obtained, $Y = -87.526 \times 1 (\text{ALKBH5}) - 54.550 \times 2 (\text{FTO}) - 23.192 \times 3 (\text{YTHDF2}) + 5.929$. Importantly, multivariate regression analysis revealed that decreased mRNA expressions of ALKBH5, FTO, and YTHDF2 in peripheral blood were risk factors for RA ($P = 0.019; P = 0.029; \text{and } P < 0.001$), suggesting ALKBH5, FTO, and YTHDF2 may play prominent pathogenic roles in the development and progression of RA.

3.5. The Increased Global m6A Contents Negatively Correlated with Decreased mRNA Expression of FTO. Multivariate regression analysis showed that the decreased mRNA expressions of m6A demethylase (ALKBH5, FTO) and m6A RNA-binding proteins (YTHDF2) were all risk factors for RA. Thus, we detected the global m6A content in peripheral blood and investigated the correlations between the global m6A content and the mRNA expression of ALKBH5, FTO, and YTHDF2 in the peripheral blood. As shown in Figure 5, the peripheral blood global m6A content was significantly increased in patients with RA compared to CON ($P < 0.001$), and the increased m6A contents negatively correlated with decreased mRNA expression of FTO ($r_s = -0.5141, P = 0.014$).

4. Discussion

m6A is a methylation at the N6 position of adenosine, which is regarded as the most abundant epitranscriptomic modification of mRNA in eukaryotic cells [23]. Abnormal m6A modification may lead to dysfunction of RNA, which can
further trigger some diseases in both animals and humans [14, 24]. There are several key genes involved in m6A methylation modification, primarily including METTL3, METTL14, WTAP, FTO, ALKBH5, and YTHDF2 [25, 26]. Given the effects of these key genes involving in m6A methylation modification on the pathogenesis of many disease [14, 24], we firstly detected the expression of m6A methylation-associated genes (METTL3, METTL14, WTAP,
FTO, ALKBH5, and YTHDF2) in the peripheral blood from RA patients and showed that the expression of ALKBH5, FTO, and YTHDF2 in the peripheral blood from RA patients was significantly lower than CON, while the expression of METTL3, METTL14, and WTAP was unchanged. The reason of downregulated m6A regulators in RA compared with CON may be the environmental, infectious, and genetic factor, as well as some RA risk factors (unbalance of adaptive and innate immune). Recently, Wang and colleagues have investigated the expression of METTL3, FTO, ALKBH5, METTL14, and YTHDF2 in peripheral blood mononuclear cell from RA patients and reported conflicting results in which only METTL3 was obviously upregulated in RA [27]. The reasons for these outcomes are probably due to differences in cell type and the disease duration.

Thus, we investigated whether the expression of peripheral blood ALKBH5, FTO, and YTHDF2 from RA patients could reflect the activity of the disease and inflammatory response. We found that the expression of the peripheral blood ALKBH5 increased following the treatment when compared with those prior to treatment. We showed that the expression of peripheral blood FTO correlated with DAS28-ESR, DAS28-CRP, TJC, C3, IgG, L, PLR, and LMR, which indicated the activity of the disease. In addition, the expression of peripheral blood YTHDF2 correlated with RBC, L%, N%, NLR, and LMR. These results indicated that m6A demethylase ALKBH5, FTO, and YTHDF2 were associated with disease activity and inflammatory response. Evidences from other disease indicated that ALKBH5 and FTO might be used as prognostic markers. Yang and colleagues showed that ALKBH5 was an independent prognostic indicator of overall survival and disease-free survival in colon cancer patients [28]. Xu and colleagues have found that the expression of FTO was positively correlated with TNM stage, and the Kaplan-Meier analysis showed that high FTO expression was significantly associated with poor prognosis in GC patients [29]. Moreover, evidences have indicated that FTO and YTHDF2 are associated with inflammation [17, 20]. Our results also showed the expression of peripheral blood ALKBH5, FTO, and YTHDF2 may use as indicator of activity and inflammatory response.

The expression of peripheral blood ALKBH5, FTO, and YTHDF2 in peripheral blood was significantly decreased in RA patients, and the expressions of peripheral blood ALKBH5, FTO, and YTHDF2 were associated with disease activity and inflammation. Thus, we explored whether the expressions of the peripheral blood ALKBH5, FTO, and YTHDF2 were risk factors for RA. In agreement with previous results, a logistic regression analysis revealed that decreased expressions of ALKBH5, FTO, and YTHDF2 in peripheral blood were risk factors for RA. Our results suggested that ALKBH5, FTO, and YTHDF2 may play prominent pathogenic roles in the development and progression of RA.

As we have known, FTO and ALKBH5 are involved in mediating methylation reversal. Thus, we detected global m6A content in the peripheral blood from RA patients and CON. And, the results showed that the peripheral blood global m6A content was significantly increased in patients with RA compared to CON. Moreover, we investigated the correlations between the global m6A content and the mRNA expression of ALKBH5, FTO, and YTHDF2 in the peripheral blood, and we found that the increased m6A contents negatively correlated with decreased mRNA expression of FTO. Although FTO was decreased and global m6A contents were increased, we did not know which mRNA that plays an important role in RA was methylated. In addition, as the most prevalent modification of RNA, m6A methylation is a double-edged sword for many diseases, over m6A modification of certain genes could lead to alterations of mRNA behavior and expression, resulting in the acceleration of disease development, whereas the lack of m6A modification on other genes may also lead to disease progression [30]. Thus, It is possible that the downregulated m6A regulators

### Table 3: Clinical details of the patients with RA and HC.

|                | B     | SE    | Wald  | df | P    | Exp (s) |
|----------------|-------|-------|-------|----|------|---------|
| ALKBH5         | -87.526 | 37.379 | 5.483 | 1  | 0.019 | 0.000   |
| FTO            | -54.550 | 24.954 | 4.779 | 1  | 0.029 | 0.000   |
| YTHDF2         | -23.192 | 8.186  | 8.026 | 1  | 0.005 | 0.000   |
| Constant       | 3.592  | 0.656  | 29.974 | 1  | 0.000 | 36.320   |

| ALKBH5         | FTO   | YTHDF2 |
|----------------|-------|--------|
| Pretreatment   | Posttreatment |
| 0.00           | 0.05   |
| 0.01           | 0.04   |
| 0.02           | 0.03   |
| 0.05           | 0.00   |

**Figure 4:** Correlation of the mRNA expression of A-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5), fat mass and obesity-associated protein (FTO), and YT521-B homology domains 2 (YTHDF2) in rheumatoid arthritis (RA) with treatment. The mRNA expression of ALKBH5 (a) was significantly increased in RA patients that received regular treatment. The mRNA expression of FTO (b) and YTHDF2 (c) did not show any remarkable differences between pretreatment and posttreatment.
in RA positively correlated with indicators of disease progression.

In conclusion, the current study firstly measured the expression of METTL3, METTL14, WTAP, ALKBH5, FTO, and YTHDF2 in the peripheral blood from RA patients and showed that dysregulated ALKBH5 and FTO were associated with RA. In addition, we found that the expression of peripheral blood ALKBH5 and FTO was associated with autoantibody production and disease activity. The findings in this study are useful for understanding RA pathogenesis and exploring novel biomarkers for RA diagnosis and treatment.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors’ Contributions

QL, YG, ZH, and JL conceived and designed the experiments. YG, LZ, JR, YG, and ZH performed the experiments. QL, YG, JR, ZH, and JL analyzed the data. QL and JL wrote the manuscript. QL, YG, LZ, JR, YG, and ZH contributed reagents, materials, and analytical tools. All authors read and approved the final manuscript. Qing Luo and Yujie Gao contributed equally to this work.

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