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Decreased ALKBH5 in neutrophil correlates with disease activity in rheumatoid arthritis and ALKBH5 modulates neutrophil autophagy

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Neutrophils are key immune components in rheumatoid arthritis (RA), but the role of ALKBH5 in neutrophil function and RA progression remains unclear. METTL3, METTL14, WTAP, ALKBH5, FTO, YTHDF2, autophagy related gene were investigated by RT-qPCR. Autophagy, LC3B were respectively detected by flow cytometry analysis, Western blotting. Correlation analyses between ALKBH5 levels and RA disease activity, autophagy were performed, and receiver operating characteristic (ROC) curves were constructed to evaluate its predictive value. Univariate analysis and multivariate regression analysis were used to analyze the risk factors and construct predictive model. Sh-ALKBH5 lentiviral vectors was constructed and used to infect HL-60 cell line. The role of ALKBH5 in autophagy regulation was explored using RNA immunoprecipitation with next generation sequencing (RIP-Seq), m^{6}A RNA Methylation Analysis, m^{6}A immunocoprecipitation-quantitative polymerase chain reaction (MeRIP-qPCR) and Actinomycin D treatment. Results showed significantly reduced *ALKBH5* levels in RA neutrophils compared to healthy controls (HC) and ankylosing spondylitis (AS) patients, correlating with RA disease activity. A novel predictive model incorporating *ALKBH5*, hemoglobin (HGB), and lymphocyte percentage (L%) exhibited enhanced efficacy in distinguishing RA patients from HC (AUC = 0.937) and AS patients (AUC = 0.943), and could reflect RA severity and activity. Additionally, autophagy levels in neutrophils were highest in RA synovial fluid, followed by RA peripheral blood, and lowest in HC peripheral blood, with *ALKBH5* expression showing the opposite trend. Moreover, *ALKBH5* negatively correlated with neutrophil autophagy and the silencing of ALKBH5 in HL-60 enhanced the autophagy. Silencing ALKBH5 increased m^{6}A level of *ATG7* mRNA and *ATG7* mRNA stability. Mechanistically, ALKBH5 inhibited neutrophil autophagy through mediating m^{6}A modification of the *ATG7* mRNA. In conclusion, these findings demonstrate that ALKBH5 regulates neutrophil autophagy in RA, and the *ALKBH5*-HGB-L% model holds potential as a diagnostic and disease activity indicator for RA.

Keywords ALKBH5, Autophagy, Rheumatoid arthritis, Neutrophil, Diagnosis

Abbreviations

ACR	American College of Rheumatology
ALKBH5	Alkylation repair homolog protein 5
Anti-CCP	Anti-cyclic citrullinated peptide antibodies
AS	Ankylosing spondylitis
ATG7	Autophagy related 7

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AUC	Area under the curve
DAS28-CRP	Disease activity score 28-C reactive protein
DAS28-ESR	DAS28-erythrocyte sedimentation rate
dNLR	Derived neutrophil-lymphocyte ratio
FTO	Fat mass and obesity-associated protein
HC	Health control
HCT	Hematocrit
HGB	Hemoglobin
L	Lymphocyte counts
L%	Lymphocyte percentages
LMR	Lymphocyte-to-monocyte ratio
M	Monocyte counts
M%	Monocyte percentages
m^6A	N6-methyladenosine
MeRIP-qPCR	m6A immunocoprecipitation-quantitative polymerase chain reaction
METTL3	Methyltransferase-like 3
MNR	Monocyte-to-neutrophil ratio
MPV	Mean platelet volume
N	Neutrophil counts
N%	Neutrophil percentages
NLR	Neutrophil-to-lymphocyte ratio
PCT	Plateletcrit
PDW	Platelet distribution width
PLR	Platelet-to-lymphocyte ratio
PLT	Platelet count
PMR	Platelet-to-monocyte ratio
PNR	Platelet-to-neutrophil ratio
RA	Rheumatoid arthritis
RBC	Red blood cell counts
RF	Rheumatoid factor
RIP-Seq	RNA immunoprecipitation with next generation sequencing
ROC	Receiver operating characteristic
RT-qPCR	Real-time quantitative polymerase chain reaction
SII	Systemic immune-inflammation index
SJC	Swollen joint count
TJC	Tender joint count
VAS	Patient visual analogue scale
WBC	White blood cell counts
WTAP	Wilms tumor 1 associated protein
YTHDF2	YT521-B homology domains 2

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disorder characterized by persistent synovitis and joint destruction, leading to significant morbidity[1]. While the precise etiology remains unclear, dysregulated immune responses in RA primarily involve aberrant neutrophil activation alongside other immune cells (e.g., T cells, B cells, synovial fibroblasts and macrophages)[1,2]. Neutrophils, the most abundant immune cells in the synovial fluid of RA patients, contribute to RA pathology by releasing inflammatory mediators, reactive oxygen species, and granule proteases, promoting autoantibody production, and modulating the functions of other immune cells (macrophages, NK cells, dendritic cell, T cells and B cells)[2,3]. However, the specific roles of neutrophils in RA pathogenesis, particularly the mechanisms regulating their function, are incompletely understood. Due to this limited understanding of RA pathogenesis, specific diagnostic and prognostic markers for early RA are still lacking.

RNA N6-methyladenosine (m^6A) is considered as the most common modification in eukaryotic organism[4]. m^6A modification plays a critical role in regulating RNA processing, splicing, nucleation, translation, and stability, which is closely associated with numerous physiological and pathological processes[5]. M6A modification is dynamically regulated by methyltransferases ("writers"), demethylases ("erasers"), and binding proteins ("readers"). Methyltransferase-like 3 (METTL3), METTL14, and Wilms tumor 1 associated protein (WTAP) are the core members of writers involving transferring methyl groups[6,7]. Then, m^6A is recognized by readers, such as YT521-B homology domains 2 (YTHDF2)[8]. Alkylation repair homolog protein 5 (ALKBH5) and fat mass and obesity-associated protein (FTO) function as erasers, catalyzing m6A demethylation[9,10]. Growing evidence indicates that m^6A modification and dysregulated m^6A regulators hold potential as novel diagnostic, therapeutic, and prognostic targets for human diseases[11–13]. And, m^6A methylation-related regulators play crucial roles in peripheral blood mononuclear cell, macrophage, and FLSs in RA[14]. Recently, evidence from Ou et al. showed that neutrophil promote breast cancer glycolysis through WTAP-dependent m^6A methylation of ENO1[15]. However, the feature of m^6A modification and the role in neutrophil in RA remain unclear.

In this study, we characterized m6A modification in neutrophil from patients with new onset RA and evaluated the clinical significance of the decreased expression of ALKBH5 in neutrophil. Then, we explored the relationship between ALKBH5 and neutrophil autophagy by clinical samples and cell lines. Our findings demonstrate that *ALKBH5* and the *ALKBH5*-HGB-L% predictive model hold promise as diagnostic and prognostic indicators for RA, and that ALKBH5 modulates neutrophil autophagy by regulating m^6A modification of ATG7 mRNA.

Materials and methods

Subjects and samples

Potential subject patients from the First Affiliated Hospital of Nanchang University were consecutively enrolled between November 2019 and September 2020. RA and ankylosing spondylitis (AS) patients met the 2010 revised American College of Rheumatology (ACR) criteria[16] and modified 1984 New York criteria[17], respectively, and were excluded if they had other chronic disorders (e.g., cancer, iron deficiency, vitamin B12 deficiency). At research start, a total of 81 RA and 20 AS subjects were evaluated. All RA patients were new-onset that is first time diagnosis of RA and no history of use corticosteroids or/and immunosuppressive drugs before blood sample collection. 20 AS patients including 9 new-onset, 8 individuals with suboptimal treatment outcomes and 3 relapse patient after drug discontinuance. Concurrently, 66 age-matched healthy controls (HCs) without chronic disorders were randomly recruited from the same hospital. All subjects sampling procedures complied with the principles outlined in the Declaration of Helsinki and were approved by the Institutional Ethics Committee of our hospital. The need for informed consent was exempted by "the Ethics Committee of The First Affiliated Hospital of Nanchang University" as discarded specimens from patients was used for the study.

Isolation of neutrophil and RNA extraction

Peripheral blood (81 RA, 20 AS and 66 HC) or synovial fluid (24 RA) neutrophil, from the elbow vein into EDTA-coated of all subjects were isolated from the sediment with the erythrocytes fraction by a Ficoll-density gradient[18]. Briefly, anticoagulated peripheral blood or synovial fluid was mixed with sterile PBS at a 1:1 ratio, then layered slowly over FicollPaque gradient (volume ratio 1:1:1 for blood/PBS/Ficoll) along the tube wall. Horizontal centrifugation was performed at 400×g for 30 min at 20℃, resulting in three layers: upper (plasma/PBS), middle (FicollPaque/PBMC), and lower (erythrocytes/granulocytes). After removing the supernatants, the neutrophil and red blood cells were treated with lysing buffer twice to eliminate erythrocytes, and neutrophil were collected[19]. Then, the neutrophil purity was analysed by flow cytometry based on CD45 (Beckman Coulter, Brea, CA, USA) and the neutrophil purity could reach 96%. After detection of neutrophil level in each isolated sample, RNA was extracted from 3×10^6 neutrophil/patient using 0.75 mL TRIzol reagent (Invitrogen Bio, Waltham, USA) according to the manufacturers' instructions. A260/A230, A260/A280 ratios in a NanoDrop ND-1000 spectrophotometer (Invitrogen Bio, Waltham, USA) was used to determine the purity and concentration of each isolated total RNA.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Prior to PCR, 1 μg total RNA was treated to remove the DNA of the gene by gDNA Eraser reagent (Takara Bio, Tokyo, Japan) following the manufacturers' instructions. And then, cDNA was synthesized from 1 μg total RNA/isolate with a PrimeScript™ RT reagent kit (Takara Bio, Tokyo, Japan) following the manufacturers' instructions. In turn, the product from reverse transcription was used as a template for PCR and PCR was performed using the SYBR Green detection method (Takara Bio, Tokyo, Japan) to quantify the mRNA in an ABI 7500 Real-time PCR System (Invitrogen Bio, Waltham, USA). The PCR primers sequences for *METTL3*, *METTL14*, *WTAP*, *ALKBH5*, *FTO*, *YTHDF2*, autophagy related 7 (*ATG7*) and *GAPDH* (reference gene) are presented in Supplementary Table 1. The results of above genes were counted using the $2^{-\Delta\Delta Ct}$ method[20].

Clinical assessments and laboratory measurements

In the process of the research, trained staff collected clinical and laboratorial information of all subjects. RA disease activity was assessed using DAS28-CRP/DAS28-ESR scores, incorporating tender joint count (TJC), swollen joint count (SJC), patient visual analogue scale (VAS), and CRP or ESR levels[21,22]. Serum levels of CRP and rheumatoid factor (RF) were evaluated by scatter turbidimetry using an IMMAGE800 system (Beckman Coulter, San Jose, CA). The ESR was measured using an Automatic esR analyzer TEST1 system (ALIFAX Bio, Udine, Italy). Anti-cyclic citrullinated peptide antibodies (Anti-CCP) was determined by iFlash-3000-C (YHLO, Shenzhen, China). Blood routine parameters, including the platelet count (PLT), mean platelet volume (MPV), plateletcrit (PCT), platelet distribution width (PDW), white and red blood cell counts (WBC, RBC), hemoglobin (HGB), hematocrit (HCT), lymphocyte counts and percentages (L, L%), monocyte counts and percentages (M, M%), neutrophil counts and percentages (N, N%) were examined by a Sysmex Xe-2100 analyzer (Sysmex Bio, Kobe, Japan). And lymphocyte-to-monocyte, neutrophil-to-lymphocyte, platelet-to-lymphocyte, derived neutrophil-lymphocyte, platelet-to-monocyte, platelet-to-neutrophil, monocyte-to-neutrophil ratio (LMR, NLR, PLR, dNLR, PMR, PNR, MNR, respectively) and systemic immune-inflammation index (SII), were calculated based on some blood routine parameters.

Flow cytometry analysis

Isolation of peripheral blood or synovial fluid neutrophil was drawn and analyzed immediately for the autophagy level of neutrophil, using flow cytometry. Briefly, 50 μL of peripheral blood neutrophil (10^6/ml) were incubated with 100 μL Autophagy Green™ working solution (AAT Bioquest bio. California, USA) in 37 °C, 5% CO_2 incubator for 30 min. Cells were washed three times with Wash Buffer, then resuspended in 100 μL Wash Buffer. All flow samples were analyzed with a CYTOMICS FC 500 flow cytometer (Beckman Coulter Inc., Brea, CA, USA) and associated software programs (CXP).

Cell culture

HL-60 cell line was cultured in RPMI 1640 medium with alone with 10% FBS (Sigma-Aldrich, UAS), 100 U/mL penicillin and 100 μg/mL streptomycin and incubated at 37℃ in a humidified atmosphere with 5% CO_2. Experiments were performed using third-passage HL-60 cells.

Cell transfection

The double-strand oligonucleotides corresponding to the target sequences were synthesized by Jikai Gene (Shanghai, China). The following sequences were targeted to human ALKBH5 small interfering RNA (siRNA): ALKBH5-1: 5′-CATCGTGTCCGTGTCCTTCTT-3′; ALKBH5-2: 5′-TAGCTTCAGCTCTGAGAACTA-3′; ALKBH5-3: 5′-ACGGATCCTGGAGATGGACAA-3′ and NC (negative control) siRNA: 5′-TTCTCCGAAC GTGTCACGT-3′. The lentiviral vectors expressing shRNA targeting ALKBH5 and NC (named sh-ALKBH5 and sh-GFP), and the ALKBH5-lentiviral expression vector (named oe-ALKBH5) were provided by Jikai Gene (Shanghai, China).

Detection of protein expression by Western blotting

The expressions of ALKBH5, LC3B and Tubulin proteins were analyzed by Western blotting. HL−60 were lysed in RIPA lysis buffer (Solarbio, Beijing, China). Protein concentrations were measured using a BCA protein assay kit (Solarbio, Beijing, China). Equal amounts of protein (30 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, USA); subsequently, the membranes were blocked with 5% non-fat milk for 2 h and probed with antibodies against ALKBH5 (Abcam, Cambridge, USA), LC3B (MeRck, Darmstadt, Germany), or Tubulin (Abcam, Cambridge, USA) overnight at 4℃. Thereafter, the membranes were washed with TBS-Tween buffer and further incubated with an HRP-conjugated goat anti-rabbit IgG Ab (absin, Shanghai, China) at room temperature for 2 h. Protein bands were visualized by an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, USA) and evaluated the band density on a gel imaging system.

RNA Immunoprecipitation with next generation sequencing (RIP-Seq)

RIP-Seq was executed by Seqhealth Technology Co., LTD (Wuhan, China). Briefly, The HL-60 was treated with cell lysis buffer. 10% of the lysate was stored as "input," 80% was used for immunoprecipitation with anti-ALKBH5 antibody (Proteintech, Wuhan, China) ("IP"), and 10% was incubated with rabbit IgG (Cell Signaling Technology) as a negative control ("IgG"). The RNA of input and IP was extracted using TRIzol reagent (Invitrogen Bio, Waltham, USA). The stranded RNA sequencing library was constructed by using KC-DigitalTM Stranded mRNA Library Prep Kit for Illumina® (Wuhan Seqhealth Co., Ltd. China) following the manufacturer's instruction. Library fragments (200–500 bp) were enriched, quantified, and sequenced on a DNBSEQ-T7 sequencer (MGI Tech Co., Ltd.) using the PE150 mode. Gene ontology (GO) analysis and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis for annotated genes were both implemented by KOBAS software (version: 2.1.1) with a corrected P-value cutoff of 0.05 to judge statistically significant enrichment.

m^{6}A RNA methylation analysis

Total RNA that was isolated from sh-ALKBH5 and sh-GFP was used to detect m^{6}A RNA methylation by The EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Colorimetric) according to the manufacturer's protocol.

m^{6}A immunocoprecipitation-quantitative polymerase chain reaction (MeRIP-qPCR)

The purified mRNA was incubated with anti-m^{6}A antibody (Bersinbio, Guangzhou, China) or rabbit IgG in immunoprecipitation (IP) wash buffer for 2 h at 4℃. Protein A beads (Bersinbio, Guangzhou, China) were then added, and the mixture was incubated at 4 °C for another 2 h. After incubation, the immunoprecipitated beads-m 6A antibody-mRNA complex was extensively washed with IP wash buffer. TRIzol reagent (Invitrogen Bio, Waltham, USA) was added to elute m^{6}A nucleotide. The PrimeScript™ RT reagent kit (Takara Bio, Tokyo, Japan) was used for cDNA synthesis. All qPCRs were run on ABI 7500 Real-time PCR System (Invitrogen Bio, Waltham, USA) using SYBR Green detection method (Takara Bio, Tokyo, Japan).

Actinomycin D treatment

Sh-ALKBH5 and sh-GFP were treated with 100nmol/L Actinomycin D (Cell signalling technology, Danvers, MA, USA) to block the RNA transcription. After treatment with Actinomycin D, qRT-PCR was performed to determine the expression level of ATG7 at indicated time points.

Statistical analysis

All data are presented in forms of means ± standard error of the mean (SEM). For the analyses, Statistical comparisons were performed using unpaired *t* test or Mann-Whitney U-test among two groups according to the normality, and One-way analysis of variance or Kruskal-Wallis test among three groups according to the normality. For evaluation of changes between the peripheral blood and synovial fluid in the same patients, paired *t* tests were performed. The Spearman method was used for correlation analysis. Risk factors were identified via univariate and multivariate logistic regression analyses. And ROC was used to evaluate the predictive value for RA. All data were analyzed using SPSS v.16.0 (SPSS Bio, Chicago, USA) and Prism v.5.0 (GraphPad Bio, San Diego, USA) software. Prism v.5.0 (GraphPad Bio, San Diego, USA) software was also used to plot figures. *P*-value ≤ 0.05 was considered to be a statistically significant difference.

Results

Characteristics of the participants

81 new-onset RA patients, 20 AS patients and 66 HC were recruited in the present study. RA patients and HC were classified into screening (34 RA and 24 HC) and validation cohorts (47 RA and 42 HC). The participants characteristics are detailed in Table 1. The new-onset RA group and HC group was matched regarding age, while no matched regarding sex. The new-onset RA group and AS group was no matched regarding sex and age. Moreover, no association between the mRNA expression level of ALKBH5 in neutrophil and age or sex was found in subjects (data not shown). Patients with RA were classified into non-severe active group (DAS28-

Clinical characteristic	RA (81)	HC (66)	AS (20)
Sex, female/male	63/18*	62/4	4/16#
Age, years	54.27 ± 12.98	51.09 ± 11.49	33.35 ± 15.23#
Duration, days	365.00 (6.00-9125)	-	-
DAS28-CRP	4.80 ± 0.98	-	-
DAS28-ESR	5.11 ± 1.04	-	-
SJC	7.00 (1.00–18.00)	-	-
TJC	10.56 ± 5.63	-	-
VAS	6.00 (3.00–9.00)	-	-
RF, IU/ml	105.00 (< 20-4350)	-	-
Anti-CCP, U/ml	136.00 (0.50–800.00)	-	-
ESR, mm/h	44.00 (4.00-138.00)	-	20.00 (2.00–74.00)#
CRP, mg/l	20.60 (1.08–180.00)	-	4.84 (0.2-38.58)#
WBC, 10^9/l	6.70 (3.58–14.59)*	6.03 ± 1.32	7.44 ± 2.22
RBC, 10^{12}/l	4.18 ± 0.50*	4.50 ± 0.30	4.97 ± 0.63#
HGB, g/l	118.05 ± 17.15*	135.00 ± 8.91	141.21 ± 15.68#
HCT, l/l	0.37 ± 0.05*	0.41 ± 0.02	0.42 ± 0.05#
PLT, 10^9/l	296.00 (96.00-691.00)*	235.90 ± 47.92	264.00 (171.00-460.00)#
MPV, fl.	10.10 (1.08–14.90)*	10.95 ± 1.12	9.91 ± 0.90
PCT, %	0.30 (0.12–0.74)*	0.26 ± 0.05	0.26 ± 0.05#
PDW, fl.	11.70 (8.40–25.20)*	13.54 ± 2.29	16.10 (10.70–16.60)#
L, 10^9/l	1.70 (0.49–3.44)*	2.18 ± 0.63	2.07 ± 0.58#
L%	24.00 (7.80–54.30)*	36.39 ± 6.83	28.73 ± 6.97#
M, 10^9/l	0.40 (0.12–1.19)	0.39 ± 0.13	0.44 ± 0.19
M%	5.80 (2.10–11.50)*	6.54 ± 1.64	5.94 ± 1.44
N, 10^9/l	4.61 (1.45–11.91)*	3.30 ± 0.90	4.74 ± 1.64
N%	66.90 (37.40–81.60)*	54.76 ± 7.50	62.99 ± 7.78
LMR	4.33 (1.20-15.62)*	5.91 ± 1.74	4.60 (2.78–11.81)
NLR	2.76 (0.69–10.51)*	1.44 (0.87–3.58)	2.20 (1.03–4.89)#
PLR	164.10 (52.17–769.40)*	109.80 (56.79–328.30)	138.24 ± 46.57#
SII	777.20 (223.00-4065.00)*	350.80 (124.60–1067.00)	657.44 ± 318.30#
dNLR	2.03 (0.60–4.44)*	1.19 (0.62–2.67)	1.58 (0.83–3.78)
PMR	712.10 (150.00-4238.00)*	661.59 ± 242.45	680.67 ± 207.92
PNR	63.99 (14.98–222.80)*	75.67 ± 22.21	58.52 (35.43–132.60)
MNR	0.09 (0.03–0.22)*	0.12 ± 0.04	0.09 (0.05–0.19)

Table 1. Clinical details of the patients with new-onset RA, AS and HC. *$P < 0.0500$, RA compared to HC; #$P < 0.0500$, AS compared to RA. Anti-CCP: anti-cyclic citrullinated peptide antibodies, AS: Ankylosing spondylitis, C-reactive protein (CRP), DAS28: disease activity score, dNLR: derived neutrophil-lymphocyte ratio, ESR: erythrocyte sedimentation rate, HC: health control, HCT: hematocrit, HGB: hemoglobin, L: lymphocyte count, L%: lymphocyte percentage, LMR: lymphocyte-to-moncyte ratio, M: monocyte count, M%: monocyte percentage, MLR: monocyte-to-lymphocyte ratio, MNR: monocyte-to-neutrophil ratio, MPV: mean platelet volume, N: neutrophil count, N%: neutrophil percentage, NLR: neutrophil-to-lymphocyte ratio, PCT: plateletcrit, PDW: platelet distribution width, PLR: platelet-to-lymphocyte ratio, PLT: platelet count, PMR: platelet-to-monocyte, PNR: platelet-to-neutrophil ratio, TJC: tender joint count, RA: rheumatoid arthritis, RBC: red blood cell, RF: rheumatoid factors, SJC: swollen joint count, SII: systemic immune-inflammation index, VAS: visual analogue scale, WBC: white blood cell.

CRP < 5.1) and severe active group (DAS28-CRP ≥ 5.1) according to DAS28. The characteristics of non-severe active group and severe active group are detailed in Table 2. In addition, the mRNA expression level of ALKBH5 in new-onset AS and non-new-onset AS was not statistically significant (data not shown).

Expression of ALKBH5 in neutrophil from RA patients and the correlation with disease activity

We used RT-qPCR to evaluate the mRNA expression levels of m^{6}A regulators in neutrophil from screening cohorts including 34 RA patients and 24 HC. *METTL3*, *METTL14*, *WTAP*, and *YTHDF2* expression did not differ between RA and HC (all $P > 0.05$; Fig. 1A), but *ALKBH5* and *FTO* showed significant differences: *ALKBH5* was downregulated in RA ($P < 0.0001$; Fig. 1B), and *FTO* was upregulated ($P = 0.0199$; Fig. 1D). In the validation cohort (47 RA patients, 42 HC), *ALKBH5* remained significantly lower in RA ($P = 0.0010$; Fig. 1B), while *FTO* expression was comparable between groups ($P = 0.1450$; Fig. 1D). Overall, neutrophil *ALKBH5* was significantly

Clinical characteristic	DAS28-CRP ≥ 5.1 RA [32]	DAS28-CRP < 5.1 RA [47]
Sex, female/male	26/7	37/11
Age, years	54.14 ± 20.51	55.00 ± 13.52
Duration, days	730.00 (6.00-7300.00)	180.00 (15.00-9125.00)
DAS28-CRP	5.73 ± 0.44#	4.30 (2.80-5.0)
DAS28-ESR	5.88 ± 0.76#	4.57 ± 0.87
SJC	12.33 ± 4.11#	5.34 ± 2.68
TJC	13.76 ± 5.78#	8.00 (1.00–16.00)
VAS	7.00 (4.00–9.00)#	5.00 (3.00–8.00)
RF, IU/ml	190.0 (1.90–4350.00)	84.00 (20-1090.00)
Anti-CCP, U/ml	149.00 (0.50–800.00)	193.27 ± 180.05
ESR, mm/h	68.34 ± 33.64#	30.00 (4.00-121.00)
CRP, mg/l	61.21 ± 42.14#	7.75 (1.08–106.00)
WBC, 10^9/l	7.75 ± 2.95	6.61 (3.58–13.17)
RBC, 10^{12}/l	3.93 (3.15–5.40)#	4.32 ± 0.46
HGB, g/l	108.42 ± 16.87#	126.5 (76.00-155.00)
HCT, l/l	0.34 ± 0.04#	0.40 (0.27–0.46)
PLT, 10^9/l	333.39 ± 107.93	286.50 (96.00-691.00)
MPV, fl.	9.81 ± 0.90#	10.40 (1.08–14.90)
PCT, %	0.33 ± 0.09	0.30 (0.12–0.74)
PDW, fl.	11.75 ± 2.50	11.80 (8.40–25.20)
L, 10^9/l	1.61 ± 0.59	1.76 (0.77–3.44)
L%	22.13 ± 7.77#	25.80 (15.00-54.30)
M, 10^9/l	0.41 (0.12–1.19)	0.40 (0.13–1.07)
M%	6.37 ± 2.23	5.70 (2.10–11.50)
N, 10^9/l	4.65 (2.28–11.91)	4.15 (1.45–9.35)
N%	69.33 ± 8.02#	65.40 (37.40–81.50)
LMR	3.40 (1.20-11.83)	4.59 (1.71–15.62)
NLR	3.13 (1.22–10.51)#	2.63 (0.69–6.97)
PLR	209.50 (85.00-769.40)	157.60 (52.17–751.10)
SII	994.10 (268.60–4065.00)#	675.10 (223.00-3553.00)
dNLR	2.46 ± 0.95#	1.89 (0.60–4.39)
PMR	804.29 ± 339.62	699.50 (150.00-4238.00)
PNR	69.94 (24.07–163.20)	63.65 (14.98–222.80)
MNR	0.08 (0.04–0.22)	0.09 (0.03–0.21)

Table 2. Clinical details of the patients with non-severe active and severe active. #$P < 0.0500$, DAS28-CRP ≥ 5.1 RA compared to DAS28-CRP < 5.1 RA. Anti-CCP: anti-cyclic citrullinated peptide antibodies, C-reactive protein (CRP), DAS28: disease activity score, dNLR: derived neutrophil-lymphocyte ratio, ESR: erythrocyte sedimentation rate, HCT: hematocrit, HGB: hemoglobin, L: lymphocyte count, L%: lymphocyte percentage, LMR: lymphocyte-to-moncyte ratio, M: monocyte count, M%: monocyte percentage, MLR: monocyte-to-lymphocyte ratio, MNR: monocyte-to-neutrophil ratio, MPV: mean platelet volume, N: neutrophil count, N%: neutrophil percentage, NLR: neutrophil-to-lymphocyte ratio, PCT: plateletcrit, PDW: platelet distribution width, PLR: platelet-to-lymphocyte ratio, PLT: platelet count, PMR: platelet-to-monocyte, PNR: platelet-to-neutrophil ratio, TJC: tender joint count, RA: rheumatoid arthritis, RBC: red blood cell, RF: rheumatoid factors, SJC: swollen joint count, SII: systemic immune-inflammation index, VAS: visual analogue scale, WBC: white blood cell.

reduced in 81 RA patients compared to HC and AS patients ($P < 0.0001$; Fig. 1C), whereas *FTO* levels did not differ between RA ($n = 81$) and HC ($n = 66$; $P = 0.9101$; Fig. 1D). In addition, Western blotting confirmed that ALKBH5 protein expression was also significantly lower in RA neutrophils than in HC (Fig. 1E).

To determine if neutrophil *ALKBH5* mRNA expression correlates with RA disease activity or severity, we analyzed the association between the expression of mRNA *ALKBH5* in neutrophil and DAS28-CRP, DAS28-ESR, VAS, TJC, SJC, CRP, ESR, RF, anti-CCP, PLT, MPV, PCT, PDW, RBC, HGB, HCT, WBC, L, L%, M, M%, N, N%, LMR, NLR, PLR, dNLR, SII, MNR, PMR, PNR. The expression of mRNA *ALKBH5* in neutrophil from RA patients with DAS28-CRP ≥ 5.1 was significantly lower than that in RA patients with DAS28-CRP < 5.1 ($P = 0.0081$, Fig. 1F), and the expression of mRNA *ALKBH5* in neutrophil displayed a negative correlation with DAS28-CRP (r_s = -0.2599, $P = 0.0191$, Fig. 1G), ESR (r_s = -0.2252, $P = 0.0460$, Fig. 1H) and N% (r_s = -0.2206, $P = 0.0478$, Fig. 1I), whereas the expression of mRNA *ALKBH5* in neutrophil displayed a positive correlation

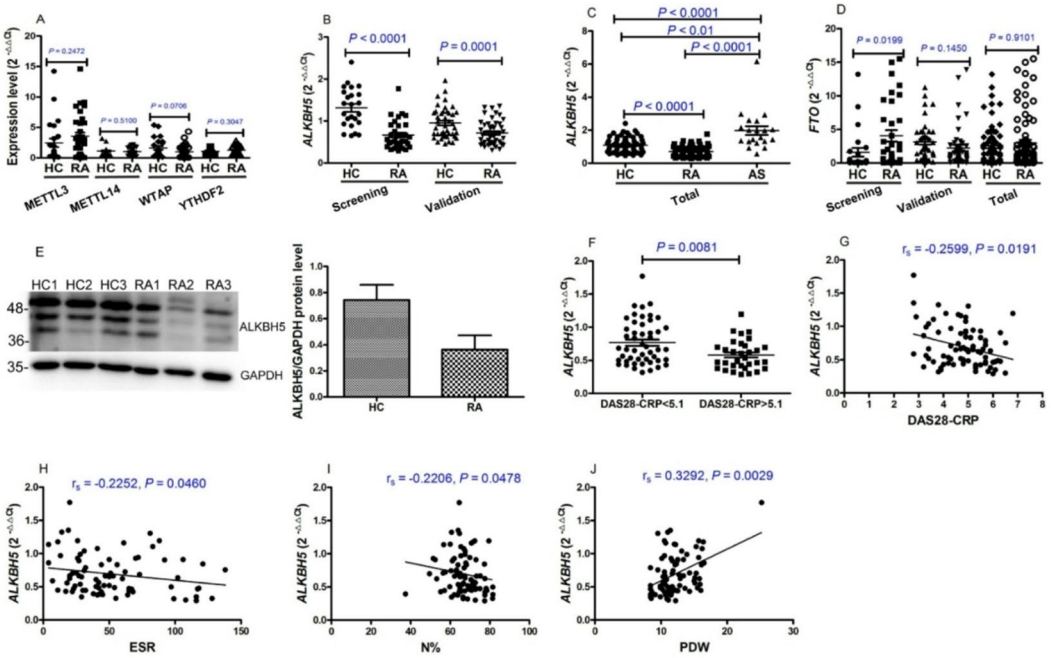

Fig. 1. Deceased expression of ALKBH5 in neutrophil from RA correlated with disease activity. **(A)** The expression levels of *METTL3*, *METTL14*, *WTAP* and *YTHDF2* were the same in neutrophil from screening cohorts including 34 RA patients and 24 HC. (**B**) The expression levels of *ALKBH5* were significantly differential expression in neutrophil from RA patients when compared to HC from screening cohorts and validation cohorts. (**C**) The expression level of *ALKBH5* was significantly low in neutrophil from 81 RA patients than that in HC and AS patients. (**D**) The expression level of *FTO* in neutrophil was significantly increased in 34 RA patients than that in 24 HC from screening cohorts, while it was the same in neutrophils from validation cohorts and total cohorts. (**E**) The expression level of ALKBH5 was low in neutrophil from RA patients than that in HC (original blots/gels are presented in Supplementary Fig. 2). (**F**) The expression of mRNA *ALKBH5* in neutrophil from RA patients with DAS28-CRP ≥ 5.1 was significantly low than that in RA patients with DAS28-CRP < 5.1. (**G**) The expression of mRNA *ALKBH5* in neutrophils displayed a negative correlation with DAS28-CRP. (**H**) The expression of mRNA *ALKBH5* in neutrophils displayed a negative correlation with ESR. (**I**) The expression of mRNA *ALKBH5* in neutrophils displayed a negative correlation with N%. (**J**) The expression of mRNA *ALKBH5* in neutrophils displayed a positive correlation with PDW. ALKBH5: alkylation repair homolog protein 5, AS: Ankylosing spondylitis, CRP: C-reactive protein, DAS28: disease activity score, ESR: erythrocyte sedimentation rate, FTO: fat mass and obesity-associated protein, HC: health control, METTL3: methyltransferase-like 3, METTL14: methyltransferaselike 14, N%: neutrophil percentage, PDW: platelet distribution width, RA: rheumatoid arthritis, WTAP: Wilms tumor 1-associating protein, YTHDF2: YT521-B homology domains 2.

with PDW (r_s = 0.3292, P = 0.0029, Fig. 1J). However, no correlation was found between the expression of mRNA *ALKBH5* in neutrophil and other indicators for the activity and severity of RA (data no shown).

Construction of predictive model based on ALKBH5-HGB-L% and its clinical relevance

Given that the expression of *ALKBH5* in neutrophil was associated with disease activity, we selected this molecule to evaluate its predictive value in RA by ROC analysis. As shown in Supplementary Fig. 1, the AUC of mRNA *ALKBH5* in neutrophil for distinguishing RA from HC was 0.775, with a cut-off of < 0.6643, a sensitivity of 55.56% and a specificity of 83.33%, the AUC of mRNA *ALKBH5* in neutrophil for distinguishing RA from AS was 0.941, with a cut-off of < 1.203, a sensitivity of 95.06% and a specificity of 85.00%, and AUC of mRNA *ALKBH5* in neutrophil for distinguishing RA with DAS28-CRP ≥ 5.1 from RA with DAS28-CRP < 5.1 was 0.675, with a cut-off of < 0.838, a sensitivity of 87.50% and a specificity of 40.82%.

Blood routine parameters and derived ratios differed significantly between RA patients and HC (Table 1). To confirm the predictive model based on the combination of mRNA *ALKBH5* and routine laboratory measurements for distinguishing RA patients from HC, univariable and multivariable analyses were used to screen potential indicators. *ALKBH5*, hemoglobin (HGB), and lymphocyte percentage (L%) were selected as variables for the model based on regression analyses (Table 3). A model for predict RA patients from HC were built based on regression coefficients as follow: P = 21.953–1.949**ALKBH5*-0.106*HGB-0.207*L%. P, predictive value. The predictive value of all samples were reckoned, and a higher predictive value would indicate greater probability for RA (P < 0.0001, Fig. 2A).

The AUC of predictive model based on *ALKBH5*-HGB-L% was 0.937 (95% CI, 0.900-0.975) (Fig. 2B), which was superior to mRNA *ALKBH5* (Supplementary Fig. 1A), HGB, L% and other routine laboratory measurements

	Univariate analysis			Multivariate analysis		
	OR	95% CI	P-value	OR	95% CI	P-value
ALKBH5	−2.848	0.019–0.176	< 0.0001	−1.949	0.031–0.665	0.0130
WBC	0.348	1.150–1.744	0.0010			
RBC	−1.842	0.065–0.388	< 0.0001			
HGB	−0.111	0.859–0.933	< 0.0001	−0.106	0.850–0.952	< 0.0001
HCT	−30.366	0.000–0.000	< 0.0001			
PLT	0.011	1.006–1.017	< 0.0001			
MPV	−0.671	0.360–0.725	< 0.0001			
PCT	10.647	170.542–10000000.038	< 0.0001			
PDW	−0.218	0.696–0.928	0.0040			
L	−1.481	0.114–0.453	< 0.0001			
L%	−0.223	0.745–0.860	< 0.0001	−0.207	0.750–0.880	< 0.0001
M%	−0.113	0.750–1.065	0.208			
N	0.902	1.714–3.545	< 0.0001			
N%	0.197	1.143–1.298	< 0.0001			
LMR	−0.366	0.574–0.838	< 0.0001			
NLR	2.208	4.255–19.453	< 0.0001			
PLR	0.024	1.014–1.035	< 0.0001			
SII	0.006	1.004–1.009	< 0.0001			
dNLR	2.889	6.720–48.091	< 0.0001			
PMR	0.001	1.000–1.002	0.0320			
PNR	−0.006	0.983–1.005	0.299			
MNR	−17.323	0.000–0.000	< 0.0001			

Table 3. Univariable and multivariable analysis of risk factors correalted with RA. ALKBH5: alkylation repair homolog protein 5, dNLR: derived neutrophil-lymphocyte, platelet-to-monocyte, HCT: hematocrit, HGB: hemoglobin, L: lymphocyte count, L%: lymphocyte percentage, LMR: lymphocyte-to-moncyte ratio, M: monocyte count, M%: monocyte percentage, MLR: monocyte-to-lymphocyte ratio, MNR: monocyte-to-neutrophil ratio, MPV: mean platelet volume, N: neutrophil count, N%: neutrophil percentage, NLR: neutrophil-to-lymphocyte ratio, PCT: plateletcrit, PDW: platelet distribution width, PLR: platelet-to-lymphocyte ratio, PLT: platelet count, PMR: platelet-to-monocyte ratio, PNR: platelet-to-neutrophil ratio, RA: rheumatoid arthritis, RBC: red blood cell, SII: systemic immune-inflammation index.

(Supplementary Table 2), showing predictive model performed best value in distinguishing RA patients from HC. When the cut-off of predictive model as 0.4609, the sensitivity and specificity were 81.48% and 92.42%, respectively.

The *ALKBH5*-HGB-L% model score was significantly higher in RA versus AS patients ($P < 0.0001$; Fig. 2C). ROC analysis further evaluated the model's ability to distinguish RA from AS, showing an AUC of 0.943 (95% CI, 0.888–0.998) with a cutoff > 0.2232, yielding 85.19% sensitivity and 94.74% specificity (Fig. 2D). In addition, the predictive model based on *ALKBH5*-HGB-L% in RA patients with DAS28 ≥ 5.1 was obviously increased compared with that in RA patients with DAS28 < 5.1 ($P < 0.0001$, Fig. 2E), and the AUC of predictive model based on *ALKBH5*-HGB-L% was 0.818 (95% CI, 0.716–0.920), with a cut-off > 3.059, a sensitivity of 81.82% and a specificity of 77.08% (Fig. 2F).

In addition, predictive model based on *ALKBH5*-HGB-L% correlated with clinical activity and severity indicators of RA (Fig. 2G and R), including DAS28-ESR (r_s = 0.3741, $P = 0.0006$), DAS28-CRP (r_s = 0.5135, $P < 0.0001$), SJC (r_s = 0.3786, $P = 0.0005$), VAS (r_s = 0.4148, $P = 0.0001$), ESR (r_s = 0.4769, $P < 0.0001$), CRP (r_s = 0.4970, $P < 0.0001$), N (r_s=0.3064, $P = 0.0054$), N% (r_s=0.6228, $P < 0.0001$), RBC (r_s=-0.5264, $P < 0.0001$), HCT (r_s=-0.7046, $P < 0.0001$), PLT (r_s=0.3224, $P = 0.0033$), PCT (r_s=0.3086, $P = 0.0053$).

Complications of RA include interstitial lung disease, vasculitis, coronary heart disease, osteoporosis and anemia. The main complications of RA patients collected in this study were anemia, osteoporosis and interstitial lung disease (ILD). In 81 RA patients, 25 RA patients were complicated with anemia, 3 RA patients were complicated with ILD, 6 RA patients were complicated with osteoporosis, 7 RA patients were complicated with ILD or/and osteoporosis (ILD/osteoporosis). Then, we investigated the association between predictive model of RA and those complications. As shown in Fig. 2S V, the predictive model based on *ALKBH5*-HGB-L% was significantly increased in the RA patients with anemia ($P < 0.0001$), ILD/osteoporosis ($P = 0.0459$). In addition, the predictive model based on *ALKBH5*-HGB-L% tended to be increased in RA patients with osteoporosis ($P = 0.0863$), although this difference was not significant. However, the predictive model based on *ALKBH5*-HGB-L% between RA patients with ILD and without ILD was no difference ($P = 0.3499$).

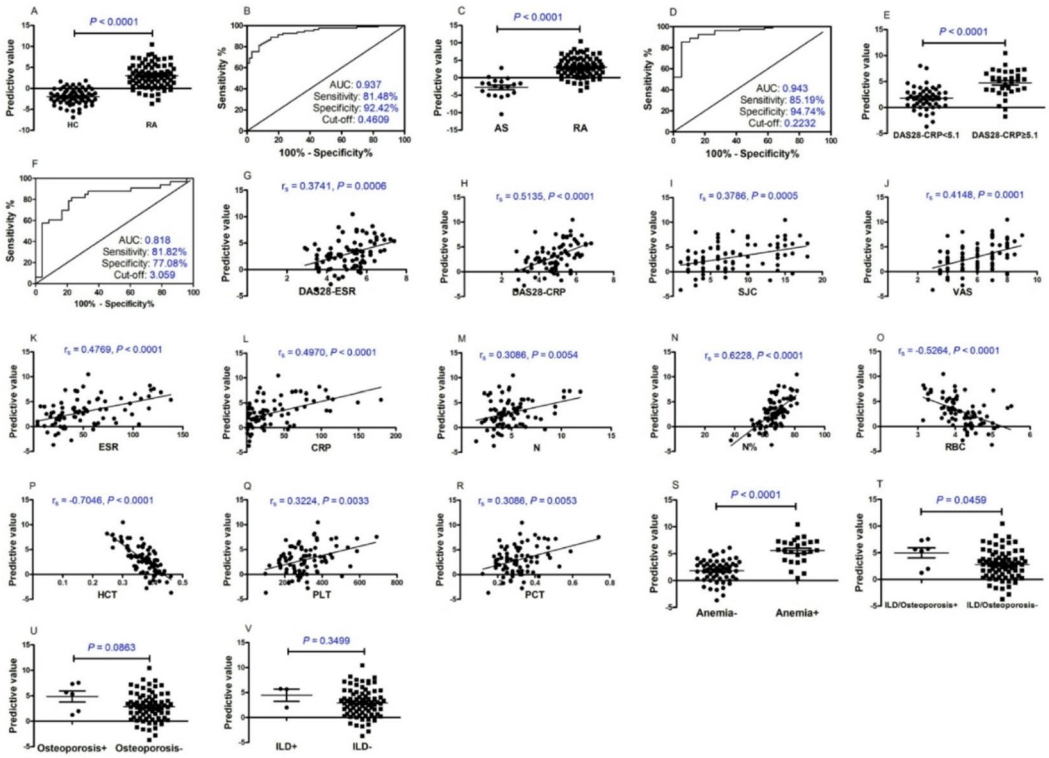

Fig. 2. Construction of predictive model based on *ALKBH5*-HGB-L% and its clinical relevance. (**A**) The value of predictive model based on *ALKBH5*-HGB-L% in RA was obviously higher than that in HC. (**B**) ROC analysis of predictive model based on *ALKBH5*-HGB-L% from RA patients and HC. (**C**) The value of predictive model based on *ALKBH5*-HGB-L% in RA was obviously lower than that in AS. (**D**) ROC analysis of predictive model based on *ALKBH5*-HGB-L% from RA patients and AS patients. (**E**) The value of predictive model based on *ALKBH5*-HGB-L% in RA patients with DAS28 ≥ 5.1 was obviously increased compared with that in RA patients with DAS28 < 5.1. (**F**) ROC analysis of predictive model based on *ALKBH5*-HGB-L% from RA patients with DAS28 ≥ 5.1 and RA patients with DAS28 < 5.1. (**G**) The predictive model based on *ALKBH5*-HGB-L% positively correlated with DAS28-ESR. (**H**) The predictive model based on *ALKBH5*-HGB-L% positively correlated with DAS28-CRP. (**I**) The predictive model based on *ALKBH5*-HGB-L% positively correlated with SJC. (**J**) The predictive model based on *ALKBH5*-HGB-L% positively correlated with VAS. (**K**) The predictive model based on *ALKBH5*-HGB-L% positively correlated with ESR. (**L**) The predictive model based on *ALKBH5*-HGB-L% positively correlated with CRP. (**M**) The predictive model based on *ALKBH5*-HGB-L% positively correlated with N. (**N**) The predictive model based on *ALKBH5*-HGB-L% positively correlated with N%. (**O**) The predictive model based on *ALKBH5*-HGB-L% negatively correlated with RBC. (**P**) The predictive model based on *ALKBH5*-HGB-L% negatively correlated with HCT. (**Q**) The predictive model based on *ALKBH5*-HGB-L% positively correlated with PLT. (**R**) The predictive model based on *ALKBH5*-HGB-L% correlated with PCT. (**S**) The predictive model based on *ALKBH5*-HGB-L% was significantly increased in the RA patients with anemia. (**T**) The predictive model based on *ALKBH5*-HGB-L% was significantly increased in the RA patients with ILD/osteoporosis ($P = 0.0459$). (**U**) The predictive model based on *ALKBH5*-HGB-L% tended to be increased in RA patients with osteoporosis, although this difference was not significant. (**V**) The predictive model based on *ALKBH5*-HGB-L% between RA patients with ILD and without ILD was no difference. ALKBH5: alkylation repair homolog protein 5, AS: Ankylosing spondylitis, AUC: area under the curve, CRP: C-reactive protein, DAS28: disease activity score, ESR: erythrocyte sedimentation rate, HC: health control, HCT: hematocrit, HGB: hemoglobin, ILD: interstitial lung disease, L%: lymphocyte percentage, N: neutrophil count, N%: neutrophil percentage, PCT: plateletcrit, PLT: platelet count, RA: rheumatoid arthritis, RBC: red blood cell, ROC: receiver operating characteristic, SJC: swollen joint count, VAS: Visual Analogue Scale.

ALKBH5 inhibited the autophagy of neutrophil

To better understand the effect of ALKBH5 in neutrophil, we detected the autophagy level of neutrophil in RA patients and HC by flow cytometry analysis (Fig. 3A). As shown in Fig. 3B, the level of autophagy in peripheral blood neutrophil was significant increased in RA patients than that in HC ($P = 0.0220$). And the decreased mRNA *ALKBH5* in peripheral blood neutrophil negatively correlated with increased autophagy in RA (r_s = -0.4142, $P = 0.0165$, Fig. 33). No associations existed between *ALKBH5* mRNA expression and ROS or degranulation markers (CD11b/CD66b) in RA peripheral blood neutrophils (Figs. 3D-F). Synovial fluid neutrophils exhibited

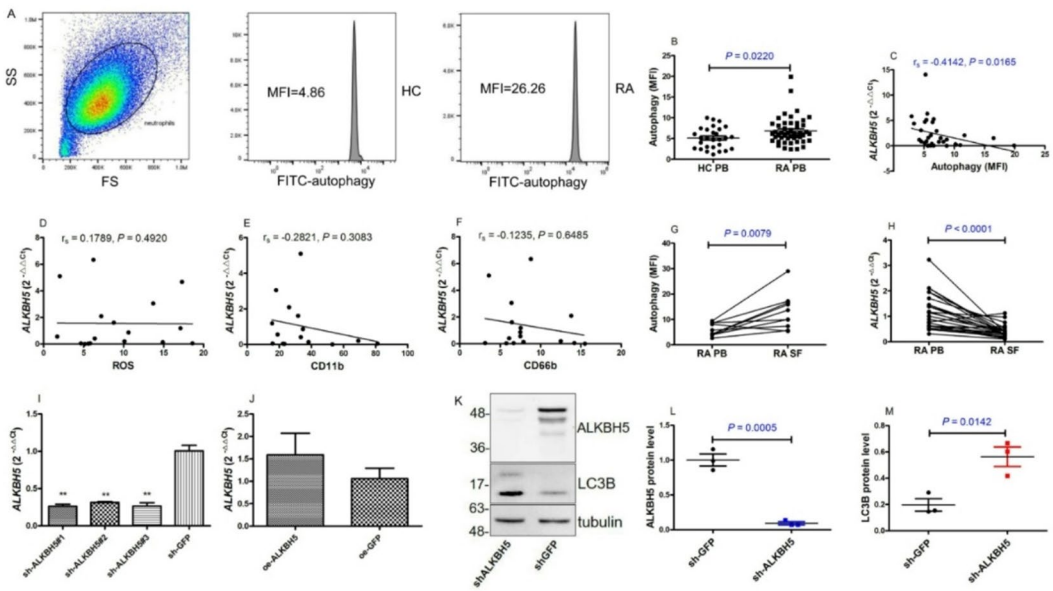

Fig. 3. ALKBH5 inhibited the autophagy of neutrophil. (**A**) Representative dot plots of population gating and autophagy level of neuttophil in RA patients and HC by flow cytometry analysis. (**B**) The level of autophagy in peripheral blood neutrophil was significant increased in RA patients than that in HC. (**C**) The decreased mRNA *ALKBH5* in peripheral blood neutrophil negatively correlated with increased autophagy in RA patients. (**D**) No association between the expression of mRNA *ALKBH5* in peripheral blood neutrophil and ROS in RA patients was found. (**E**) No association between the expression of mRNA *ALKBH5* in peripheral blood neutrophil and CD11b in RA patients was found. (**F**) No association between the expression of mRNA *ALKBH5* in peripheral blood neutrophil and CD66b in RA patients was found. (**G**) The level of autophagy in synovial fluid neutrophil was significant increased in RA patients than that in peripheral blood neutrophil. (**H**) The expression of mRNA *ALKBH5* in synovial fluid neutrophil was significant decreased in RA patients than that in peripheral blood neutrophil. (**I**) Sh-ALKBH5 significantly disturbed *ALKBH5* mRNA expression. (**J**) Oe-ALKBH5 did not enhance *ALKBH5* mRNA expression. (**K**) Western blot of ALKBH5 and LC3B in HL-60 (original blots/gels are presented in Supplementary Fig. 3). (**L**) Sh-ALKBH5 significantly disturbed ALKBH5 expression in HL-60. (**M**) Sh-ALKBH5 significantly promoted LC3B expression in HL-60. ALKBH5: alkylation repair homolog protein 5, HC: health control, PB: peripheral blood, RA: rheumatoid arthritis. SF: synovial fluid.

significantly higher autophagy ($P = 0.0079$) but lower ALKBH5 expression ($P < 0.0001$) than peripheral blood neutrophils in RA patients (Figs. 3G-H).

To further identify the role of ALKBH5 in regulating neutrophil autophagy, we transduced HL-60 cells with shRNA targeting ALKBH5 (sh-ALKBH5) or an ALKBH5 overexpression construct (oe-ALKBH5). RT-qPCR showed that sh-ALKBH5 significantly disturbed *ALKBH5* mRNA expression ($P < 0.0100$, Fig. 3I), while oe-ALKBH5 did not enhance *ALKBH5* mRNA expression (Fig. 3J). Thus, we used sh-ALKBH5 to explore the role of ALKBH5 in regulating neutrophil autophagy, Western blotting validated that sh-ALKBH5 knockdown reduced ALKBH5 protein ($P = 0.0005$) and increased LC3B expression ($P = 0.0142$) in HL-60 cells (Fig. 3K M).

ALKBH5 inhibited the autophagy of neutrophil through targeting ATG7 mRNA

To further determine the potential molecular mechanism by which ALKBH5 inhibited neutrophil autophagy, RIP-Seq was employed to identify the molecules that interact with ALKBH5. This analysis revealed a set of genes specifically bound by ALKBH5, and GO/KEGG enrichment analyses indicated these genes are involved in autophagy (Fig. 4A), including key autophagy-related genes such as ATG7, ATG4, and ATG2. Considering ATG7 is one of the classic autophagy related genes reported so far, ATG7 was selected as the target molecule of ALKBH5 in regulating autophagy. Then, Sh-ALKBH5 resulted in a rise of ATG7 mRNA level in HL-60 ($P = 0.0001$, $P = 0.0341$, Fig. 4B).

ALKBH5 is one of erasers involving in reversible process of m^6A demethylation, To determine the effect of ALKBH5 on m^6A level in neutrophil, we used m^6A RNA Methylation Analysis to detect the total m^6A level of HL-60, and data showed Sh-ALKBH5 resulted in a rise of total m^6A level ($P < 0.0001$, Fig. 44). MeRIP-qPCR was then applied to confirm the sh-ALKBH5-mediated m^6A methylation of *ATG7* mRNA. ATG7 mRNA was enriched in the anti-m^6A antibody group compared to the IgG pull-down control, and as expected, sh-ALKBH5 markedly elevated m^6A levels on *ATG7* mRNA ($P = 0.0020$; Fig. 4D).

To further verify the stability of *ATG7* mRNA that was methylated by m^6A, sh-ALKBH5 and sh-GFP were treated with Actinomycin D, an inhibitor of transcription. Total RNA was isolated at specified time points post-

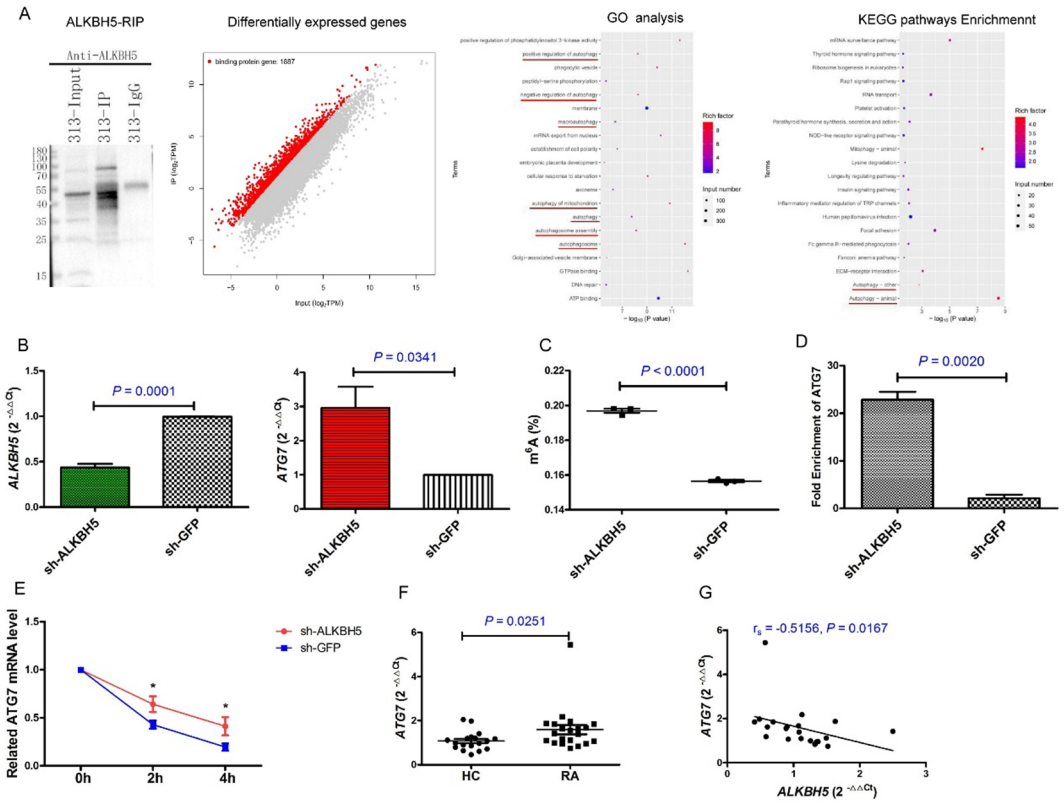

Fig. 4. (**A**) ALKBH5 inhibited the autophagy of neutrophil through targeting ATG7 mRNA. After RIP-Seq (original blots/gels are presented in Supplementary Fig. 4), GO and KEGG analysis showed the genes exhibiting specific binding to ALKBH5 involved in autophagy. (**B**) Sh-ALKBH5 resulted in a rise of ATG7 mRNA level in HL-60. (**C**) Sh-ALKBH5 resulted in a rise of total m^{6}A level in HL-60. (**D**) MeRIP-qPCR showed sh-ALKBH5 markedly induced the m^{6}A level of ATG7 mRNA. (**E**) Actinomycin D treatment indicated the *ATG7* mRNA decay rate of sh-ALKBH5 was slower than that of sh-GFP. (**F**) The mRNA expression of *ATG7* in neutrophil from RA patients was significantly increased than that in HC. (**G**) The mRNA expression of *ATG7* negatively correlated with the mRNA expression of *ALKBH5* in neutrophil from RA patients. ALKBH5: alkylation repair homolog protein 5, ATG7: Autophagy related 7, HC: health control, MeRIP-qPCR: m6A immunocoprecipitation-quantitative polymerase chain reaction, RA: rheumatoid arthritis, RIP-Seq: RNA immunoprecipitation with next generation sequencing.

treatment, and qRT-PCR was used to quantify *ATG7* mRNA levels. Results showed that the decay rate of *ATG7* mRNA was significantly slower in sh-ALKBH5 cells than in sh-GFP controls ($P < 0.0500$; Fig. 4E).

Sequentially, we explored the mRNA expression of *ATG7* in neutrophil from RA patients and HC, and analyzed its correlation with *ALKBH5* mRNA expression in RA neutrophils. Our data showed the mRNA expression of *ATG7* in neutrophil from RA patients was significantly increased than that in HC ($P = 0.0251$, Fig. 4F), and the mRNA expression of *ATG7* negatively correlated with the mRNA expression of *ALKBH5* in neutrophil from RA patients (rs = -0.5156, $P = 0.0167$, Fig. 4G).

Discussion

We showed the first line of evidence suggesting that the mRNA level of *ALKBH5* in neutrophil from new-onset RA patients was significantly lower than that in health control (HC), Ankylosing spondylitis (AS) patients and decreased *ALKBH5* correlated with disease activity of RA. Further research manifested that *ALKBH5* exhibited a preferable value and the predictive model based on *ALKBH5*-hemoglobin (HGB)-lymphocyte percentage (L%) had improvement value in distinguishing new-onset RA patients from HC, AS patients. In addition, the predictive model (*ALKBH5*-HGB-L%) serves as an indicator to reflect the severity and activity of RA. Mechanistically, the decreased mRNA *ALKBH5* negatively correlated with increased autophagy level in neutrophil and the silencing of ALKBH5 in HL-60 enhanced the autophagy. ALKBH5 inhibited the autophagy of neutrophil through targeting *ATG7* mRNA and then affecting *ATG7* mRNA stability. In summary, our findings indicate that ALKBH5 contributes to RA pathogenesis by mediating m^{6}A modification of *ATG7* mRNA to regulate neutrophil autophagy, and acts as an independent predictive marker for new-onset RA.

Growing evidence implicates RNA m^{6}A modification in regulating immune cell function through transcript stability and homeostasis[23,24]. For instance, Wang et al.[25] demonstrates that METTL3-mediated mRNA m^{6}A promotes the function of dendritic cells, facilitating the translation of target genes (CD40, CD80 and TLR4 signaling adaptor Tirap transcripts) responsible for activating T cells and strengthening TLR4/NF-κB signaling-

induced cytokine production. Zhang et al.[26] indicates that METTL3-dependent m^{6}A modification of PGC-1α mRNA regulate the monocyte inflammation response. And Zhou et al.[27] shows m^{6}A demethylase ALKBH5 decreases m^{6}A modification in CXCL2 and IFN-γ mRNA, up-regulating mRNA stability and protein expression, thus resulting in enhanced responses of CD4$^+$T cells during neuroinflammation. Moreover, m^{6}A methylation-related regulators play crucial roles in PBMCs, macrophage, and FLSs in RA[14]. Given the importance of m^{6}A modification and aberrant m^{6}A regulators in immune cells function and their role in neutrophil in RA remain unclear, it is required to establish the relationship between m^{6}A modification and neutrophil function and explore the role of m^{6}A modification in neutrophil in human RA. Thus, this study firstly outlined mRNA expression of m^{6}A regulators (METTL3, METTL14, WTAP, ALKBH5, FTO, YTHDF2) in neutrophil from RA patients and HC. The analyses indicated that the mRNA expression of ALKBH5 in neutrophil from RA patients was significantly lower than the HC in screening and validation cohorts, whereas the mRNA expression of FTO, WTAP, METTL3, METTL14 and YTHDF2 was unchanged. In addition, *ALKBH5* mRNA expression in neutrophils from RA patients was significantly lower than that in the disease control group (AS patients). These results indicate that ALKBH5 expression in RA patients differs significantly from that in HC and AS groups.

ALKBH5, the second reported demethylase, has been demonstrated to regulate immune responses in some recent studies[27,28]. And our previous study manifested that decreased peripheral blood ALKBH5 correlated with markers of autoimmune response in SLE, suggesting ALKBH5 may involve in production of autoantibodies (anti-double-stranded DNA, anti-nucleosome)[29]. To understand whether ALKBH5 in neutrophil impact the occurrence and development of RA, we investigated the association between the mRNA expression of ALKBH5 in neutrophil and clinical, laboratorial details. It was not surprising that the mRNA expression of ALKBH5 in neutrophil correlated with inflammatory state (ESR, N%), disease activity and severity (DAS28, PDW), which suggested ALKBH5 in neutrophil may involve in immune responses of RA.

Notwithstanding both ALKBH5 and FTO are key erasers, only the mRNA expression of ALKBH5 decreased in neutrophil from RA patients in this study. Inconsistent with the results that both mRNA ALKBH5 and FTO decreased in peripheral blood from RA patients in our previous study[30]. Potential reasons for this discrepancy may lie in discrepancies in cell type and/or pathology processes. It will be interesting to elucidate different functions between ALKBH5 and FTO in neutrophil or different cell type from RA.

Early recognition and diagnosis of RA are critical to selecting effective therapies, improving the survival rate and keeping the quality of life. Current diagnosis of RA is based on a combination of clinical and laboratory features according to ACR RA classification criteria. However, current diagnosis of RA have various disadvantages, especially regarding early diagnosis and overlapping symptoms with other arthritis, such as the requirement for refinement using objective and more sensitive imaging devices, subjective clinical diagnosis, and the relatively low sensitivity and specificity of auto-antibodies for RA[31,32]. In recent years, m^{6}A moderators HNRNPA2B1 and HNRNPC have used as diagnostic biomarkers for endometriosis[12]. And many studies have demonstrated that dysregulated level of key m^{6}A moderators could act as therapeutic and prognostic targets in human diseases[33]. This study investigated the potential role of decreased *ALKBH5* in neutrophil in distinguish RA patients from HC and AS patients, and data indicated that the AUC for *ALKBH5* in distinguish RA patients from HC was 0.775, the AUC for *ALKBH5* in distinguish RA patients from AS patients was 0.941, suggesting the mRNA expression of *ALKBH5* in neutrophil could be used as predictive marker for RA.

According to previous studies[31,34–36], the parameters of blood routine and their ratios differed significantly between RA patients and HC. And ROC curve analyses of these parameters found that HGB, L%, N, NLR, PLR, SII and dNLR had better predictive value for RA with AUC > 0.800. Despite extensive research on RA-associated risk factors[37], few studies have integrated these diverse factors into a combined predictive model. To our knowledge, this is the first research to build a mathematical model based on m^{6}A moderator, the blood routine parameters and the ratios for predicting RA. As wish, *ALKBH5*, HGB and L%, were brought into the mathematical model, suggesting *ALKBH5*, HGB and L% exhibit synergistic effect on predicting RA. The predictive model *ALKBH5*-HGB-L% showed better value in distinguishing RA patients from HC with AUC of 0.937, sensitivity of 81.48% and specificity of 92.42%, which was superior to *ALKBH5*, HGB, L% and other routine laboratory measures. And, the predictive model *ALKBH5*-HGB-L% could differentiate RA patients and disease control (AS patients) with AUC of 0.943. In addition, the predictive model *ALKBH5*-HGB-L% could also differentiate RA patients with DAS28 ≥ 5.1 and with DAS28 < 5.1 with AUC of 0.818. Furthermore, the predictive model *ALKBH5*-HGB-L% correlated with clinical activity and severity indicators of RA, including DAS28-ESR, DAS28-CRP, SJC, VAS, ESR, CRP, N, N%, RBC, HCT, PLT, PCT. Moreover, the predictive model *ALKBH5*-HGB-L% was associated with complications including anemia and ILD/osteoporosis. Based on these findings, we speculate the predictive model *ALKBH5*-HGB-L% not only can be used as candidate marker, but also serves as biomarker for evaluating disease activity and severity of RA.

Autophagy regulates many cells associated with RA and targeting autophagy significantly alleviates RA[38]. Among, neutrophil that is one of the most abundant immune cells, accounting for 60–70% of human peripheral leukocytes have been reported to exist autophagy[39]. Consistent with the report of An et al.[39], we also found the autophagy level of synovial fluid neutrophil in RA was significantly elevated than that in peripheral blood neutrophil, and the autophagy level of peripheral blood neutrophil in RA was significantly increased than that in HC. These results are also consistent with the RA characteristics of joint synovitis and joint destruction of the limbs, and further indicates that the stronger the neutrophil autophagy, the stronger the local inflammation. Notably, ALKBH5 can involve in autophagy of many cell, such as cancer cell[40], cardiomyocytes[41], macrophages[42]. Thus, this study explore the correlation between *ALKBH5* mRNA expression and autophagy level in neutrophil and our results showed that there was a negative correlation between them, then the ALKBH5 silencing in HL-60 enhanced the autophagy, which indicating ALKBH5 may involve in autophagy suppression of neutrophil in RA. The inhibitory effect of ALKBH5 on neutrophil autophagy is consistent with these reports in epithelial ovarian

cancer[43], hepatocellular carcinoma[44], gastric cancer[45], myocardial ischemia-reperfusion injury[46], age-related macular degeneration[47]. However, ALKBH5 has been reported to promote autophagy in many disease[40,48–51].

ALKBH5, as a well-characterized m^{6}A demethylase, has been extensively demonstrated to regulate the expression of target genes by affecting the m^{6}A level of mRNA, thus participating in diverse biological processes[52]. In our study, we observed that the differential gene binding to ALKBH5 was involved in autophagy regulation in HL-60, and these differential gene binding to ALKBH5 included ATG7, ATG4, ATG2. Notably, m^{6}A-modified mRNA transcripts are more stable due to "readers"-mediated mRNA stabilization[49,53], and we identified ALKBH5 knockdown significantly increased m^{6}A modification and promoted stabilization of *ATG7* mRNA in HL-60. Additionally, the mRNA expression of *ATG7* in neutrophil from RA patients was significantly increased than that in HC, and the mRNA expression of *ATG7* negatively correlated with the mRNA expression of *ALKBH5* in neutrophil from RA patients. These results indicated that decreased ALKBH5 in neutrophil could post-transcriptionally upregulate ATG7 expression in an m^{6}A-dependent manner to further promote stabilization of *ATG7* mRNA. ATG7 transcript has been proved to have three m^{6}A modification sites within the coding sequence region and five m^{6}A sites in the 3'-untranslated region[54]. In addition, GO and KEGG analyses indicated the molecule that binds to ALKBH5 is also involved in other pathways, suggesting that the role of ALKBH5 in neutrophils extends beyond autophagy regulation—these pathways warrant further investigation.

Recently, studies on the role of m^{6}A modification in regulating neutrophil activation and granulopoiesis, as well as neutrophil mobilization, have been reported successively. Such as, METTL3-mediated m^{6}A mRNA methylation regulates neutrophil activation through targeting TLR4 signaling[55], ALKBH5 drives emergency granulopoiesis and neutrophil mobilization by upregulating G-CSFR expression[56]. Our study complements these findings by showing that ALKBH5 may also regulate neutrophil autophagy via targeting ATG7. The studies by Luo et al.[55] and Liu[56] are in line with our findings, demonstrating that m^{6}A modification can participate in regulating neutrophil generation and function. In addition, the study by Liu[56] using murine sepsis models have reported ALKBH5-deficient septic mice exhibited lower neutrophil mobilization. The enhanced autophagy and reduced ALKBH5 expression in synovial fluid neutrophil compared to peripheral blood neutrophil from RA patients in the present study showed ALKBH5-reduced neutrophil exhibite higher mobilization. This discrepancy may stem from differences in inflammatory contexts: sepsis involves infectious inflammation, whereas RA is a non-infectious inflammatory condition. Future studies using RA animal models should explore the migratory capacity of ALKBH5-deficient neutrophils into joint cavities to clarify this relationship.

Since previous studies have reported reduced ALKBH5 expression under inflammatory conditions, such as LPS treatment[57]. Our preliminary data indicated that both RA serum and RA SF could induce low ALKBH5 expression in neutrophil, and the effect of RA SF was stronger than RA serum (data not shown), which confirmed that RA conditions might reduce ALKBH5 expression. However, the specific molecules and regulatory mechanisms of ALKBH5 downregulation exist in RA neutrophils require further investigation.

Finally, there are some limitations of this study should be acknowledged. First, limited participating institutions and the single-center study may lead to certain risk of bias. Second, the study evaluated patients at a single time point. Longitudinal analysis could assess how ALKBH5, autophagy markers and prediction model change during RA progression, treatment, or the development of complications Third, we only collected AS as disease control, which may restrict the clinical applicability in real practice. Fourth, gender distribution of the RA and HC groups were different, gender and age distribution of the RA and AS groups were different, which may lead to certain risk of bias. Fifth, further studies are needed to characterize m^{6}A reading molecules and the specific mechanism by which m^{6}A reading molecules regulates ALKBH5-ATG7-neutrophil autophagy in RA from clinical and animal model study.

In conclusion, this study reveals common m^{6}A regulators expression in neutrophil between new-onset RA and HC, revealing that decreased mRNA *ALKBH5* in neutrophil correlated with disease activity of new-onset RA. In addition, mRNA *ALKBH5* and *ALKBH5*-HGB-L% predictive model may act as novel, non-invasive predictive biomarkers and indicator of disease severity and activity for RA. Furthermore, the decreased expression of mRNA *ALKBH5* negatively correlated with autophagy in neutrophil from RA and the silencing of ALKBH5 in HL-60 enhanced the autophagy. Mechanistically, ALKBH5 inhibited neutrophil autophagy through mediating m^{6}A modification of the *ATG7* mRNA. Overall, our study identifies ALKBH5 as a candidate m^{6}A regulator, providing insights into the role of m^{6}A modification in modulating neutrophil function in RA.

Data availability

The datasets generated and/or analysed during the current study are available in the GEO repository [GSE305649].

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Author contributions

QL participated in designing the study, performed statistical analyses and drafted the manuscript. ZKH participated in the design of the study and helped to revise the manuscript. MFL carried out RT-PCR analysis, MeRIP-qPCR, Actinomycin D treatment and drafted the manuscript. ZWW carried out RT-PCR analysis, WB analysis, statistical analyses and drafted the manuscript.SQW performed m6A RNA methylation analysis, statistical analyses and drafted the manuscript. QYX performed Flow cytometry analysis, clinical data collection and drafted the manuscript. PF carried out predictive model analysis and drafted the manuscript. BQF performed data acquisition of disease activity and drafted the manuscript. YG carried out WB analysis and drafted the manuscript. CQ performed cell culture, transfection and drafted the manuscript. JML conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Declaration of ethics approval and consent to participate

The research protocol complied with the principles outlined in the Declaration of Helsinki and were approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University (approval no. (2023) CDYFYYLK(01–057)).

Additional information

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