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Remimazolam relieved the injury of hypoxia/reperfusion treated human embryo liver cell line through the targeting METTL3 mediated m6A modification of P53

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ABSTRACT

Background: This study was performed to explore the role of Re in liver IRI progression. Hypoxia and reperfusion (H/R) treated human embryo liver cell line (L-02) was used to establish a liver IRI model.

Materials and methods: Cell behaviors were detected using CCK-8, flow cytometry and TUNEL staining assays. The m6A content was detected using m6A dot blot assay. RT-qPCR and western blot assays were used to assessed the relative mRNA and protein levels. MeRIP assay was conducted to determine the m6A levels of P53. The relationship between METTL3 and P53 was demonstrated using RIP and dual-luciferase reporter assays.

Results: The results showed that Re treatment significantly decreased the cell apoptosis and promoted the cell viability in the H/R treated L-02 cells. Besides, H/R treatment increased the METTL3 and m6A levels in the L-02 cells, and Re treatment decreased them. Additionally, METTL3 overexpression reversed the role of Re in the H/R treated L-02 cells. Mechanistically, METTL3 overexpression enhanced the m6A levels and mRNA stability and expressions of P53. The combination of METTL3 and P53 was further confirmed.

Conclusion: In conclusion, this study demonstrated that Re treatment relieved the H/R induced injury in the L-02 cells through decreasing the METTL3 levels. METTL3 enhanced the mRNA stability and expressions of P53 through m6A modification. Re-METTL3-P53 axis might a new direction for the treatment of liver IRI in the future.

1. Introduction

Ischemia reperfusion injury (IRI) refers to the injury of tissue cells caused by hypoxia and the aggravation of tissue damage after the restoration of oxygen supply, involving two evolutionary processes of local ischemic injury and inflammation mediated reperfusion injury [1,2]. Liver transplantation is the only effective way to treat end-stage liver diseases. During the operation, the process of liver transplantation from donor to recipient must undergo ischemia and reperfusion [3]. In addition, many liver operations also need to block the liver blood vessels, leading to the ischemia and reperfusion of hepatic cells, resulting in liver IRI [4]. At present, liver IRI has

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become a bottleneck restricting the use of liver donors and the development of hepatectomy. The pathological factors/pathways involved in the pathogenesis of liver I/R injury include anaerobic metabolism, mitochondrial dysfunction, oxidative stress injury, intracellular calcium overload injury, and cytokine and chemokine storms [4]. However, there is still a lack of effective prevention or treatment methods for liver I/R.

Remimazolam (Re), a derivative of midazolam, is a new type of ultra short acting benzodiazepine sedative. Re is another product under the concept of "soft drug" after remifentanil (Sneyd JR et al., 2020). It has the characteristics of rapid onset, rapid recovery, less impact on functions and hemodynamics of liver and kidney [5]. Previous study showed that hepatocyte metabolism was stable within 5 days after continuous exposure to clinically relevant concentrations of Re, which indicated that Re exposure has no harmful effect on the integrity and metabolic activity of human primary hepatocytes in vitro [6]. Recently, SHI et al.(2019) found that Re can effectively improve the neurological dysfunction, reduce the volume of cerebral infarction and the damage of cortical neurons in cerebral IRI mice, which suggested that Re played a protective role in cerebral IRI. However, whether Re also plays a therapeutic role in liver IRI remains unclear.

As a reversible chemical modification on RNA, N6 methyl adenosine (m6A) is a new way of post-transcriptional regulation, which participates in many biological processes such as lipid accumulation, energy metabolism, etc [7]. Methyltransferase like 3 (METTL3), as a classical methylation enzyme, is reported to regulate gene expression by promoting the methylation level of target genes in many diseases, and ultimately participate in the occurrence and development of diseases [8–10]. METTL3-mediated m6A modification is demonstrated to participate in the progression many Liver disease, such as nonalcoholic steatohepatitis [11], nonalcoholic fatty liver disease [11], liver fibrosis [12], and liver cancer [13], by regulating mRNA stability, mRNA splicing, and translational efficiency. However, the role of METTL3 in liver IRI and the relationship between Re and METTL3 remains unclear.

Therefore, this study used the hypoxia and reperfusion (H/R) treated human embryo liver cell line (L-02) to establish a liver IRI model. We hypothesized that Re might relieved the liver IRI through targeting the METTL3 mediated m6A modification.

2. Materials and methods

2.1. Cell culture and treatment

The human embryo liver cell line (L-02) was purchased from the Procell Life Science&Technology Co.,Ltd. (Wuhan, China). The cells were cultured in the DMEM/F12 cell medium (Gibco, USA) containing 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin and 100 μ g/ml streptomycin at 37 °C and 5% CO2. After cultured for 24 h, for the establish the hypoxia and reperfusion model, the cells were transferred into DMEM/F12 medium without glucose and serum and cultured in a three-gas incubator at 37 °C (5% CO2, 94% N2, and 1% O2). 24 h later, the cells were cultured under normal conditions in DMEM/F12 medium with glucose and FBS for 18 h. One hour before H/R model establishment, the cells were treated with Re (25, 50, 100, 150 μg/ml).

2.2. Cell transfection

For METTL3 overexpression. The METTL3 overexpressed vector (oeMETTL3) and empty vector (oeNC) was purchased from GenePharma Pharmaceutical Technology Co., Ltd (Shanghai, China). The vectors were transfected into the cells using lipofectamine 3000 (Invitrogen, CA, United States) according to the manufacturer's protocols.

2.3. Cell counting kit (CCK)-8 assay

The L-02 cells were seeded into 96-well plates at a density of 2×10^4 cells/well, followed by incubating for 48 h; Then, the cells were treated with 10 μL CCK-8 (KeyGEN) and cultured for 4 h. Finally, the absorbance was determinated at 450 nm using a microplate reader (BioTek).

2.4. Cell apoptosis determination

Flow cytometry and TUNEL staining were performed to detect the cell apoptosis and death. The Annexin V-FITC/PI kit (Elabscience Biotechnology Co.,Ltd, Wuhan, China) was purchased to measure the apoptotic cells, and all operations were carried out according to the manufacturer's instruction.

For the TUNEL staining, the cells were rinsed with PBS. The fixation and permeabilization were carried out with 4% paraformaldehyde and 0.1 Triton X-100, individually. Subsequently, cells were fostered with a TUNEL reaction mixture (Roche, Germany) in the dark, followed by incubation with DAPI for 10 min. Finally, the TUNEL positive cells were tracked using a fluorescence microscope (Olympus, Tokyo, Japan). The TUNEL positive cells were quantified using ImageJ Software (NIH, USA).

2.5. RT-qPCR

The cells were mixed with TRIzol® reagent (Invitrogen, USA) to extract the total RNA. Then A Reverse Transcription Kit (Takara, China) was used to reserve transcription. All primers used For PCR was provided by Genscript Biotech (Nanjing, China). Then the RT-qPCR was conducted using SYBR Green Master Mix (Takara, China) in CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The reaction system is set to 20 μL. The PCR amplification procedure was as follows: predenaturation at 95 °C for 30 s; 95 °C for 5 s, 60 °C

for 30 s (40 cycles). GAPDH was selected as an internal reference. Finally, the relative quantification method ($2^{-\Delta\Delta Ct}$ method) was used to calculate the relative mRNA expressions of related genes. For normalization, the gene expression level of the control group was set to one, and the gene expression level of the experimental group was a multiple of the control group. Addtiionally, for the determination of mRNA stability of P53, the cells were treated with 1 mg/ml of Actinomycin D for 2, 4, 8, 12 h. After that, the mRNA levels of P53 were detected with RT-qPCR respectively. The primer sequences (5' -> 3′) were listed as follows.

METTL3: Forward Primer TTGTCTCCAACCTTCCGTAGT, Reverse Primer CCAGATCAGAGAGGTGGTGTAG.
METTL14: Forward Primer GAACACAGAGCTTAAATCCCCA, Reverse Primer TGTCAGCTAAACCTACATCCCTG.
RBM15: Forward Primer ACGACCCGCAACAATGAAG, Reverse Primer GGAAGTCGAGTCCTCACCAC.
WTAP: Forward Primer CTTCCCAAGAAGGTTCGATTGA, Reverse Primer TCAGACTCTCTTAGGCCAGTTAC.
VIRMA: Forward Primer AAGTGCCCCTGTTTTCGATAG, Reverse Primer ACCAGACCATCAGTATTCACCT.
FTO: Forward Primer ACTTGGCTCCCTTATCTGACC, Reverse Primer TGTGCAGTGTGAGAAAGGCTT.
ALKBH5: Forward Primer CGGCGAAGGCTACACTTACG, Reverse Primer CCACCAGCTTTTGGATCACCA.
P53: Forward Primer GAGGTTGGCTCTGACTGTACC, Reverse Primer TCCGTCCCAGTAGATTACCAC.
GAPDH: Forward Primer GAGGTTGGCTCTGACTGTACC, Reverse Primer TCCGTCCCAGTAGATTACCAC.

2.6. Immunofluorescence staining

The L-02 cells were harvested after treatment. After washed with PBS, the L-02 cells were fixed with 4% paraformaldehyde for 20 min at 4 °C. Next, the cells were blocked with goat serum and incubated with anti-METTL3 or anti-P53 for 2 h at 37 °C. After washed for three times, the cells were incubated with secondary antibodies for 1 h at 37 °C. Finally, the stained cells were mounted and observed using fluorescence microscope (100× magnification).

2.7. m6A RNA immunoprecipitation (MeRIP) assay

The m6A levels of P53 were detected using Magna MeRIP m6A Kit (Millipore, USA). Briefly, the RNA was incubated with 3 μg of anti-m6A antibody and protein A/G magnetic beads (Thermo Scientific, USA) overnight. And then the antibody-combined methylated RNA were incubated with the antibody in immunoprecipitation buffer with RNase and protease inhibitor. Finally, the interacting RNAs were isolated and m6A enrichment of P53 was analyzed using qRT-PCR.

2.8. Dual-luciferase reporter assay

In order to explore the binding specificity between METTL3 and P53, cDNAs containing partial CDS sequence near stop codon and full-length 3′UTR of P53 were cloned into pGL3-control vectors (Promega) which was comprised of firefly luciferase(F-luc). For mutant reporter plasmids, the 4 adenosine (A) in m6A motif were replaced by cytosine (C). Then the wild and mutant type of P53 3′-UTR were inserted into the reporter plasmids. The L-02 cells were seeded in 24-well plate and co-transfected with plasmids and oeMETTL3 using lipofectamine 3000. After 48 h, the luciferase activities were detected using the Dual Luciferase Reporter Assay System (Promega, USA).

2.9. RNA immunoprecipitation (RIP) assay

The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) was utilized to confirmed the relationship between METTL3 and P53. In brief, after transfected with oeMETTL3, L-02 cells were lysed using the RIP lysis buffer. Then the obtained cell extract was incubated with IgG antibody and RIP buffer solution for 1 h. Then the cells were treated with magnetic beads. Next, the protein was digested with proteinase K and the immunoprecipitated RNA was extracted. Finally, the RT-qPCR was performed to detect the P53 expressions.

2.10. Western blot assay

Total proteins were collected from L-02 cells using RIPA lysis buffer (Thermo, USA), and the protein concentration was detected by a BCA Protein Assay Kit (Beyotime, Shanghai, China). Then the obtained protein was separated using 10% SDS-PAGE for 1.5 h at 120 V and transferred onto PVDF membranes (Millipore, USA) for 2 h at 200 mA. Aftet that, the membranes were incubated with primary antibodies (METTL3, 1:1500, Abcam; P53, l: 800, Abcam; GAPDH, 1:2500, Abcam) at 4 °C overnight. Next, the membranes were washed and incubated with a secondary antibody for 90 min. Finally, the bands were observed using a BeyoECL Plus ECL kit (Beyotime) and the gray value was analyzed by Image Lab software (Bio-Rad, Hercules, USA). GAPDH was used as internal reference.

2.11. M6A dot blot assay

After total RNA was extracted by Trizol method, mRNA was separated with mRNA purification kit (YEASEN Biology, China). The mRNA quality was tested by Nano Drop (Thermo, USA). Then the mRNA was denatured at 95 °C for 5 min and immediately placed on ice. 200 ng mRNA was added on nylon membrane (GE, USA) fixed on Biodot microporous filter (Bio-Rad, USA). After natural air drying, the nylon membrane was removed and placed on a clean plate. Then the plate was placed in the ultraviolet crosslinking

instrument (Thermo, USA) to promote the combination of mRNA and nylon membrane. Next, the membrane was sealed in 5% skimmed milk for 2 h, and then incubated with m6A antibody (1: 1000) at 4 °C overnight. After washing with PBST for three times, the membrane was incubated with goat anti rabbit IgG secondary antibody at room temperature for 1 h. The chemiluminescence imaging system was used to luminescence the nylon film, and the m6A dot imprint image was obtained.

2.12. Statistical analysis

SPSS v.20.0 (IBM, SPSS, USA) was used for statistical analysis. Data were presented as mean ± SD. Each experiment was independently repeated three times. All data conformed to the Normal distribution test. Then the difference between two group were evaluated using Student's t-test and one-way ANOVA followed by Tukey's multiple comparison test for multiple group. In addition, for the analysis of the relationship between METTL3 and P53, the GSE24430 data set was downloaded from GEO database (https://www.ncbi.nlm.nih.gov/geo/info/overview.html) and analyzed by R software. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Re treatment enhanced the cell viability of the H/R treated L-02 cells

The chemical structural formula of Re was showed in Fig. 1A. Firstly, we choose the appropriate concentration of Re through CCK-8 assay. As shown in Fig. 1B, we found that different concentration of Re treatment showed no effect on the cell viability of normal L-02 cell, but significantly increased the cell viability of H/R stimulated L-02 cell. In addition, we found that there was no significant difference between 150 μg/ml Re and 100 μg/ml Re in the promotion of cell viability in H/R stimulated L-02 cell. Therefore, 100 μg/ml Re was selected for the next experiments.

3.2. Re treatment inhibited the cell apoptosis rate on the H/R treated L-02 cells

Then, we found that after H/R treatment, the cell apoptosis rate (Fig. 2A) and TUNEL positive cells (Fig. 2B) of the L-02 cells were significantly increased. Additionally, Re treatment significantly decreased the cell apoptosis rate (Fig. 2A) and TUNEL positive cells (Fig. 2B) of the H/R treated L-02 cells, and showed no effect on the cell apoptosis of normal L-02 cells.

3.3. Re treatment decreased the METTL3 mediated m6A levels in the H/R treated L-02 cells

Next, through the m6A dot blot analysis, we found the total m6A levels in the L-02 cells were significantly increased after H/R treatment, while Re treatment significantly decreased it (Fig. 3A). Then we analyzed the m6A related genes levels in the L-02 cells. After H/R treatment, the METTL3, METTL14 and WYTAP levels were significantly increased, while FTO and ALKBH5 levels were significantly decreased; in addition, Re treatment just significantly decreased the METTL3 levels and increased ALKBH5 levels in the H/R treated L-02 cells. Moreover, the inhibition role of Re in METTL3 expression is better than the promotion role in ALKBH5 expression (Fig. 3B). Therefore, METTL3 was considered to be the key gene of RE regulating m6A level in H/R treated L-02 cells. The immunofluorescence results also showed that H/R treatment increased the METTL3 levels in the L-02 cells and RE treatment significantly decreased it (Fig. 3C).

3.4. METTL3 overexpression reversed the role of Re in the H/R treated L-02 cells

The transfection efficiency of oeMETTL3 was tested by RT-qPCR and western blot. The results showed that oeMETTL3 significantly increased the METTL3 levels in the L-02 cells at both mRNA (Figrue 4A) and protein levels (Fig. 4B). Then, we found that after oeMETTL3 transfection, the increase of cell viability induced by Re treatment in the H/R treated L-02 cells were significantly decreased (Fig. 4C). Besides, after oeMETTL3 transfection, the decrease of cell apoptosis (Fig. 4D) and TUNEL positive cells (Fig. 4E) induced by

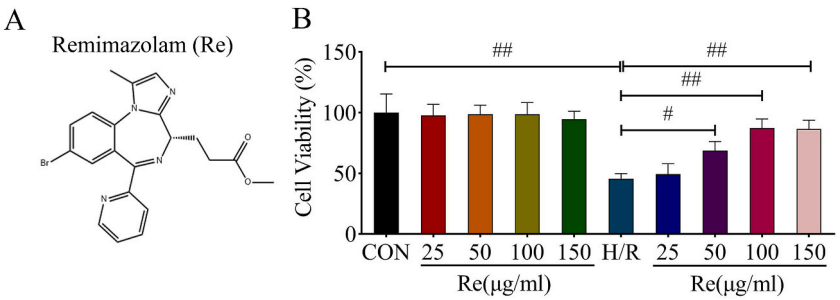

Fig. 1. Re treatment enhanced the cell viability of the H/R treated L-02 cells (A) The chemical structural formula of Re. (B) The cell viability of the normal and H/R treated L-02 cells were tested using CCK-8 assay. #P < 0.05, ##P < 0.01.

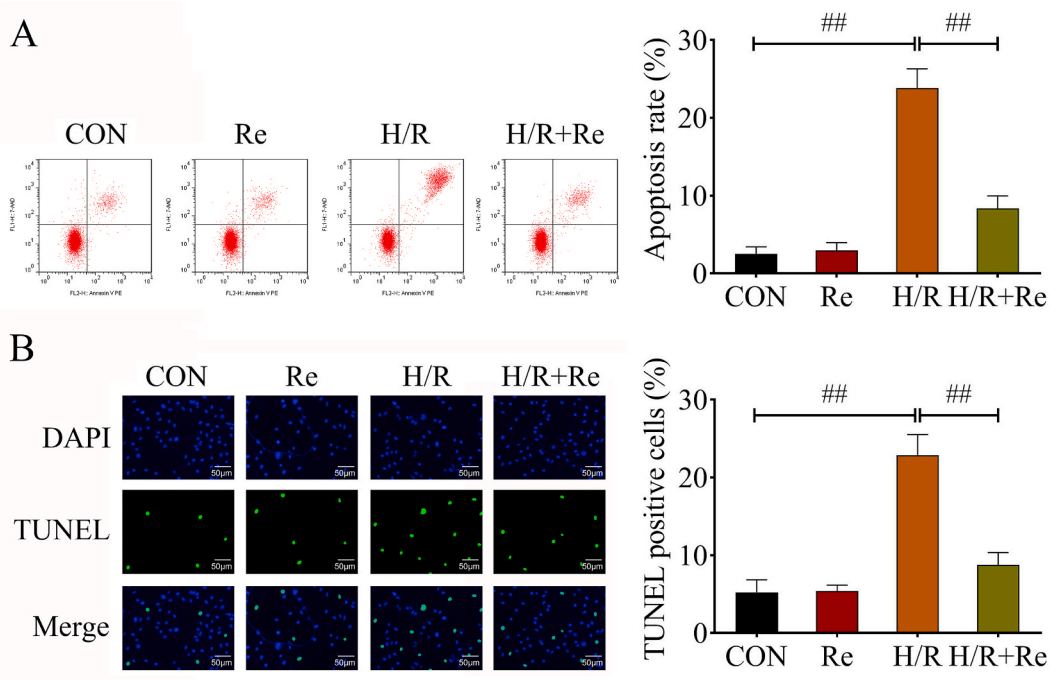

Fig. 2. Re treatment inhibited the cell apoptosis rate on the H/R treated L-02 cells The H/R treated L-02 cells were treated by Re. (A) Flow cytometry and (B) TUNEL staining were performed to detect the cell apoptosis and death. ##P < 0.01.

Re treatment in the H/R treated L-02 cells were significantly increased.

3.5. METTL3 participated in the injure of H/R treated L-02 cells through targeting P53

Finally, through analyzing the GSE24430 data set, we found that the expression of METTL3 and P53 was positively correlated in liver IRI (Figrue 5A). Besides, the relative m6A (Figrue 5B), mRNA (Figrue 5C) and protein (Figrue 5D) expression levels of P53 was significantly increased in the H/R treated L-02 cells. RIP assays demonstrated that METTL3 could be combined with P53, and METTL3 overexpression promoted the combination of METTL3 and P53 (Fig. 5E). Additionally, dual-luciferase reporter assay showed that METTL3 overexpression significantly increased the luciferase activity of WT-P53, which further confirmed that combination of METTL3 and P53 (Fig. 5F). Besides, the RT-qPCR (Fig. 5G) and Immunofluorescence (Fig. 5H) results showed that METTL3 overexpression significantly increased the P53 levels. What's more, METTL3 overexpression significantly increased mRNA stability of P53 (Fig. 6A). The mechanism diagram of Re in liver IRI was shown in Fig. 6B.

4. Discussion

Here, we demonstrated that Re treatment significantly prevented the cell apoptosis and promoted the cell viability of the H/R treated L-02 cells through decreasing the METTL3 levels. Furthermore, METTL3 participated in the injure of H/R treated L-02 cells through regulating the m6A levels of P53, which further promoted the mRNA degradation and decreased the P53 expressions levels. Re-METTL3-P53 axis might be a key target for liver IRI treatment.

The process of IRI is accompanied by the death of a large number of liver cells, which leads to a series of pathological changes in the physiological structure of the liver(20). For the treatment of various liver diseases, prevention and treatment of the injury caused by IRI is the top priority [14]. Re, a new water-soluble ultra short acting intravenous sedative, has been proved to have a certain repair effect on cell damage [15]. demonstrated that Re exhibited a better outcome in the LPS treated BV2 microglia cells via regulating the autophagy and increasing the cell viability through the BDKRB1/RAS/MEK signalling pathway [16]. suggested that Re inhibited the inflammatory responses and promoted the cell growth in the LPS treated macrophage, which indicated that Re was beneficial to septic patients. All these evidence showed Re played a promoting role in the cell growth in many diseases. In this study, we first confirmed that Re has no effect on cell growth of normal L-02 cells. Additionally, Re treatment significantly promoted the cell viability and inhibited the cell apoptosis of the H/R treated L-02 cells. Similarly, Shi et al.(2022) found that Re was an ideal anesthetic drugs for cerebral IRI through suppressing pyroptosis and promoting the cell growth of the rat cardiomyocytes.

Subsequently, we found that Re treatment significantly decreased the total m6A content in the H/R treated L-02 cells. With further analysis, we found that METTL3 is the key methylation enzyme for Re to regulate the m6A modification of HR treated cells. Despite the increasing cognition of the biological significance of METTL3-mediated m6A modification, the mechanisms on how m6A regulates

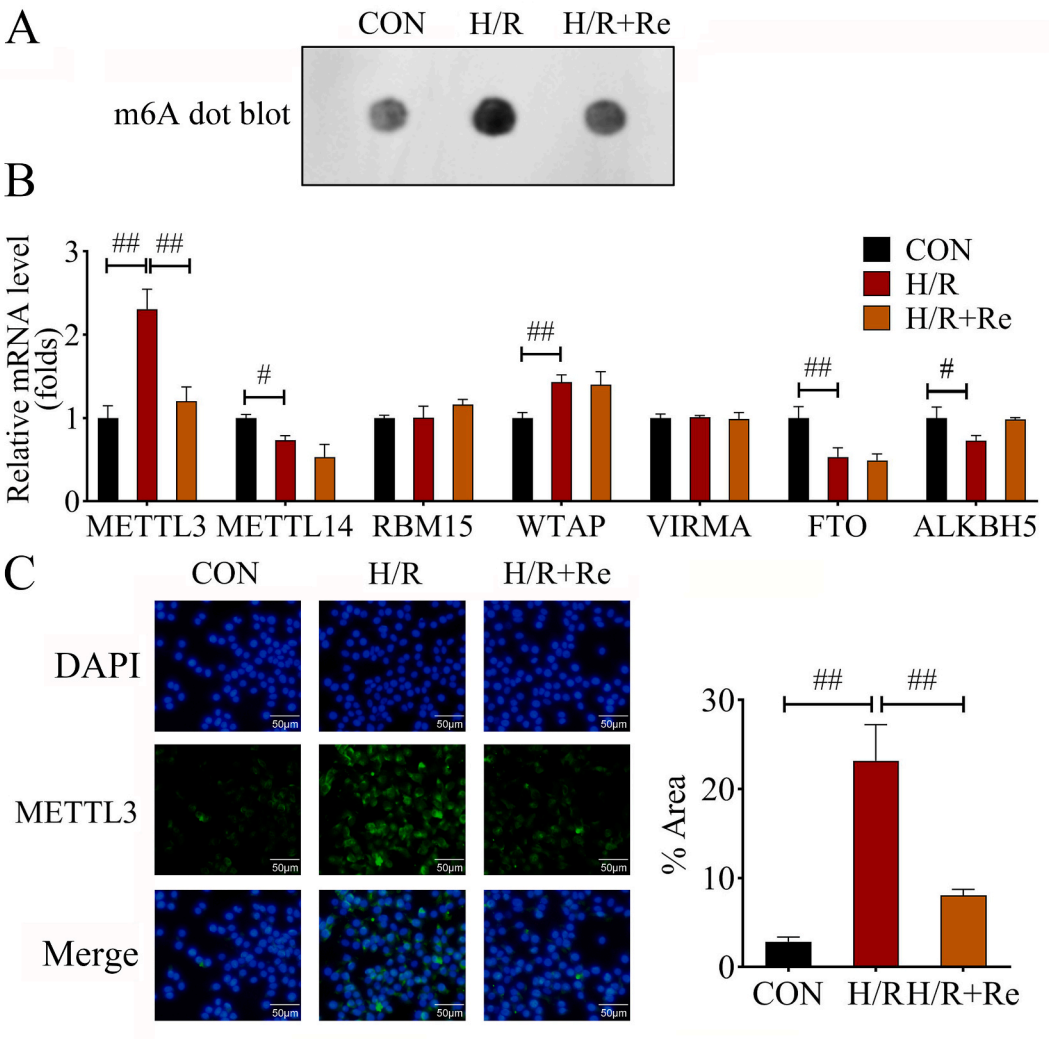

Fig. 3. Re treatment decreased the METTL3 mediated m6A levels in the H/R treated L-02 cells The H/R treated L-02 cells were treated by Re. (A) The m6A dot blot analysis was conducted to detect the total m6A levels. (B) The m6A related genes levels were assessed by RT-qPCR. (C) Immunofluorescence staining was performed to analyze the METTL3 levels. #P < 0.05, ##P < 0.01.

gene expression in liver IRI remain poorly understood [17]. found that when METTL3 expression was suppressed, m6A levels was decreased in the H/R treated NRK-52E cells, which further regulated the forkhead box D1 levels and suppressed cell apoptosis. Song et al.(2019) found that METTL3 is the primary factor involved in abnormal m6A levels in myocardial IRI, and METTL3 knockdown promoted the autophagic flux and inhibitd apoptosis in H/R treated cardiomyocytes. Here, we found that Re treatment significantly decreased METTL3 levels, and METTL3 overexpression reversed the effects of Re on the cell viability and apoptosis in the H/R treated L-02 cells. These results indicated that Re treatment might alleviate the injury of H/R treated L-02 cellsthrough regulating METTL3 mediated m6A modification.

P53, as a very important tumor suppressor, is the junction of many signal transduction pathways in cells [18]. At the same time, P53 is also the central regulating switch to control cell proliferation and apoptosis. When organs or cells are damaged, P53 expression will increase, and then transcriptionally activated the expressions of downstream mRNA, which further resulted in the cell cycle arrest and cell apoptosis [19,20]. Previous studies indicated that downregulation of P53 effectively relieved the liver injury in many diseases [21]; Song L et al., 2019; [22]. In this study, we demonstrated that P53 was significantly increased in the H/R treated L-02 cells. Besides, the RIP and dual-luciferase reporter assays demonstrated the combination of METTL3 and P53 in the L-02 cells. METTL3 overexpression increased the m6A levels, and promoted the mRNA stability and expression levels of P53. All these results implied that METTL3 mediated m6A modification of P53 was a key target for Re to participate in the injury of H/R treated L-02 cells.

However, there were still some limitations in this study. This study only involved in vitro experiments and lacks validation from in vivo experiments. Due to limitations in hospital conditions, we did not conduct clinical outcome analysis. These will serve as the focus of our future research to comprehensively refine our conclusions.

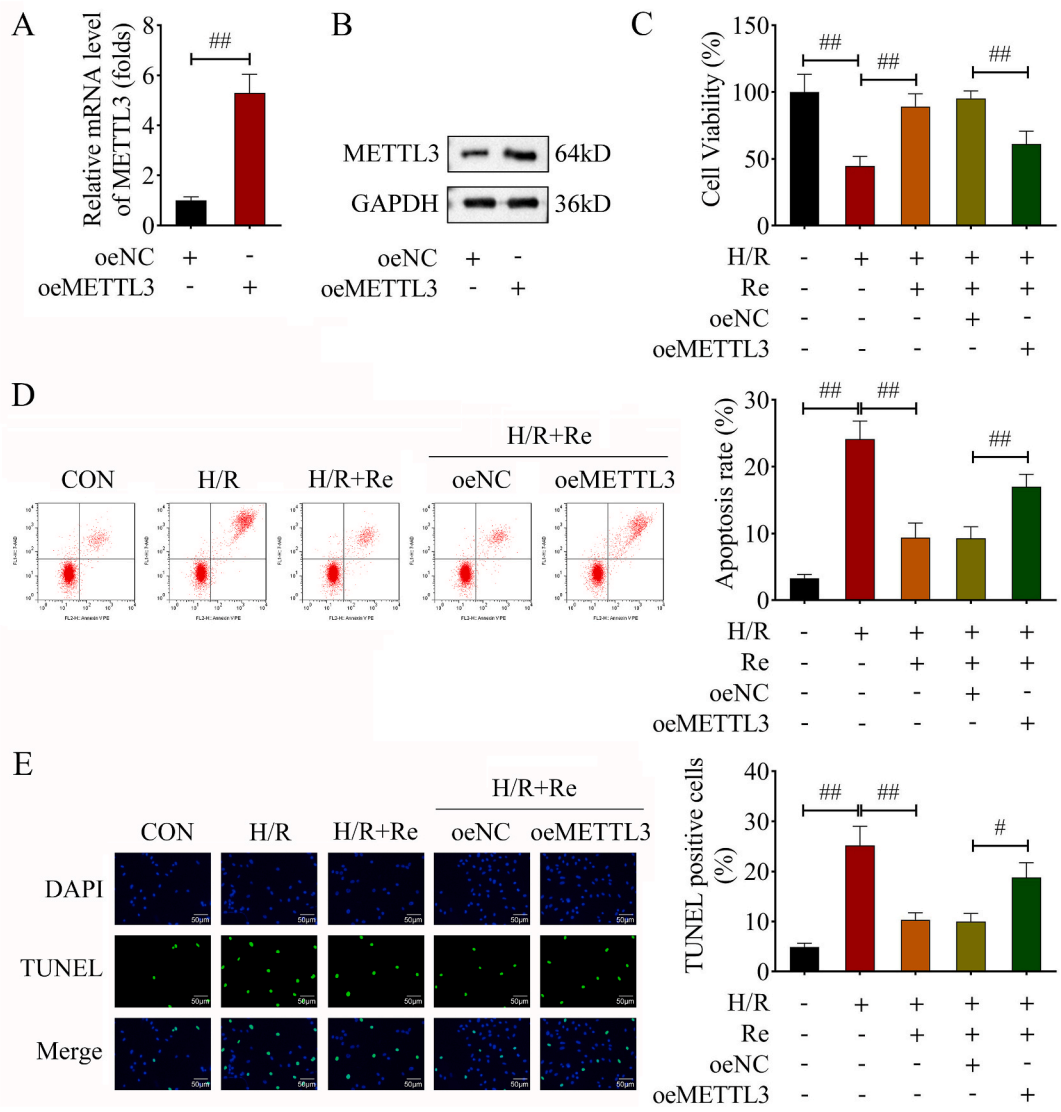

Fig. 4. METTL3 overexpression reversed the role of Re in the L-02 cells The transfection efficiency of oeMETTL3 was tested by (A) RT-qPCR and (B) western blot. The H/R treated L-02 cells were treated with Re and transfected with oeMETTL3. (C) The cell viability was tested using CCK-8 assay. (D) Flow cytometry and (E) TUNEL staining were performed to detect the cell apoptosis and death. #P < 0.05, ##P < 0.01.

In conclusion, this study demonstrated that Re, a new water-soluble ultra short acting intravenous sedative, effectively relieved the injury of H/R treated L-02 cells through targeting the METTL3 mediated m6A modification of P53. Our research provided a new perspective for the promotion and application of Re and the treatment of IRI in future.

Author contribution statement

Weixing Ding: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper. Huijuan Peng: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Jianyou Tian; Siyan Wang: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

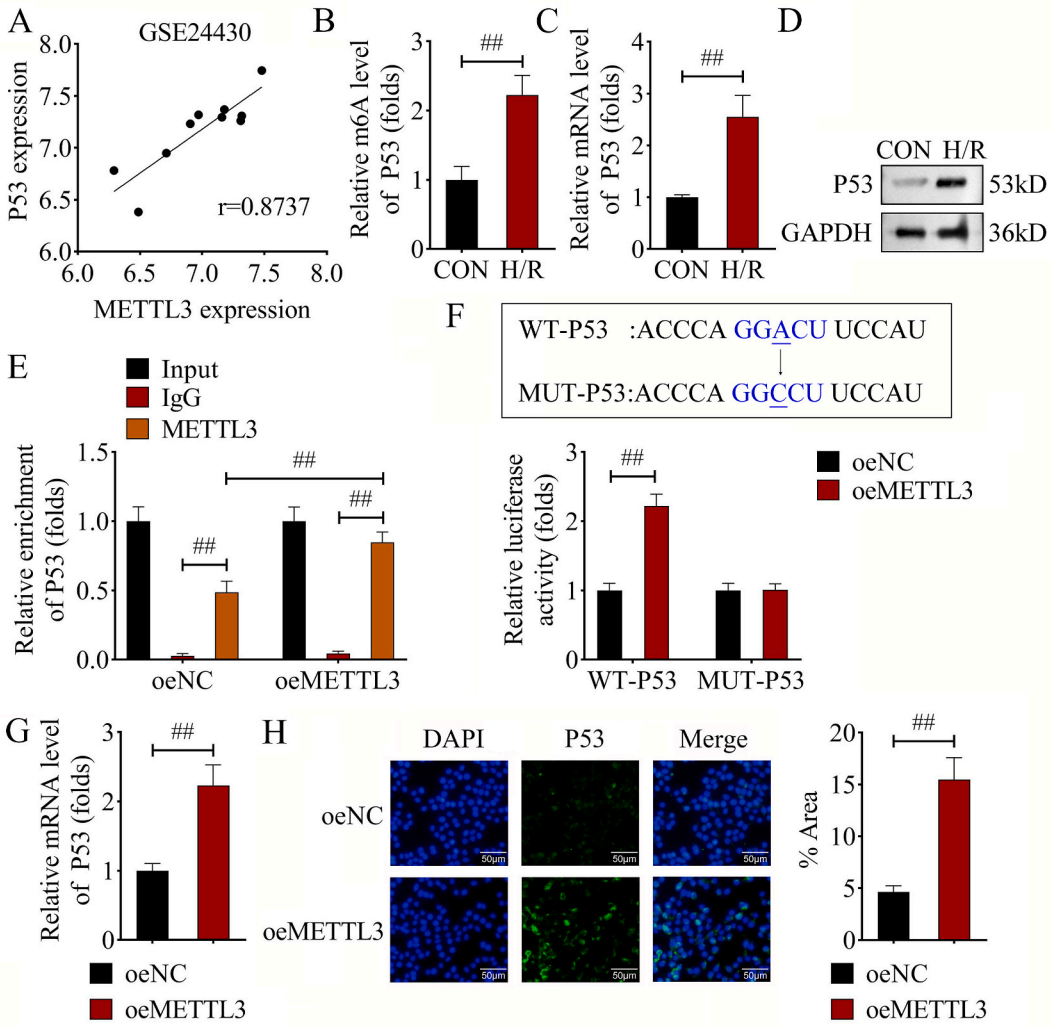

Fig. 5. METTL3 participated in the injure of H/R treated L-02 cells through targeting P53 (A) The correlation analysis between METTL3 and P53 in liver I/R. (B) The m6A levels of P53 in the H/R treated L-02 cells was detected using MeRIP assay. (C) The mRNA and (D) protein levels of P53 in the H/R treated L-02 cells was detected using RT-qPCR and western blot assay. The combination of METTL3 and P53 was demonstrated by (E) RIP and (F) dual-luciferase reporter assays. The P53 levels were determined using (G) RT-qPCR and (H) immunofluorescence assays after METTL3 over-expression. ##P < 0.01.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

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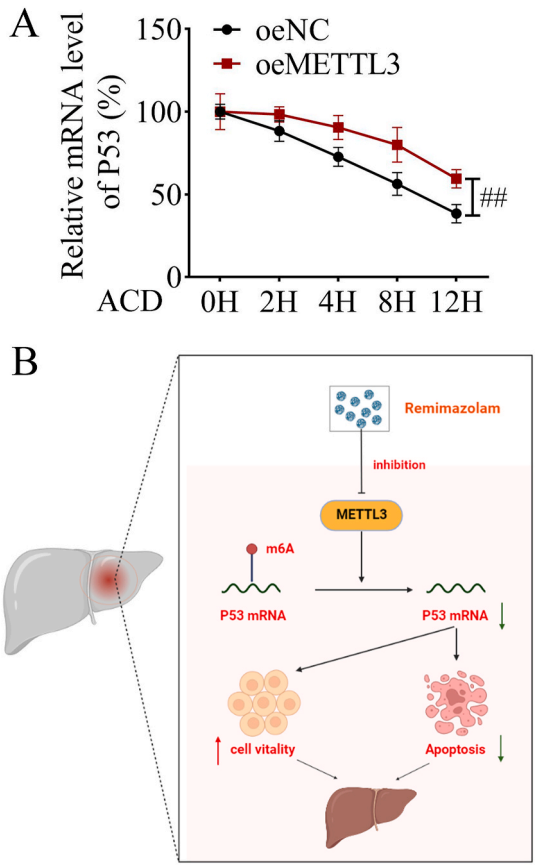

Fig. 6. METTL3 overexpression decreased the mRNA stability of P53. (A) The mRNA stability of P53 was detected using RT-qPCR assay. (B) The mechanism diagram of Re in liver IRI. ##P < 0.01.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20285.

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