ROUND 1

Dear Editor,

We would like to thank all the Reviewers for their critical and insightful comments on our manuscript entitled "**Genome-wide map of N6-methyladenosine circular RNAs identified in mice model of severe acute pancreatitis**" by Jun Wu and colleagues, which helped us to improve the quality of the manuscript.

We have now made major revisions to the manuscript and addressed all the concerns raised. Please find the following point-by-point responses to the Reviewers' critiques and comments below.

Thank you very much for considering our manuscript for publication in *World Journal of Gastroenterology*.

Corresponding author:
Lijun Tang, Professor.
The General Hospital of Western Theater Command (Chengdu Military General Hospital), Sichuan Province, 610083, China. Phone: 0086-028-86571858; Fax: 0086-028-86571251. E-mail: tanglj2016@163.com

(please note: Because the "Manuscript File" is automatically generated when we submit our revised manuscript, we cannot highlight our revised parts according to the Reviewer' suggestions. To make the Reviewers re-review our manuscript comfortable, we placed the revised manuscript that was highlighted the corresponding revised parts at the end of "Responses to the reviewers" in this file)

Responses to the reviewers:

Reviewer #1:

Specific Comments to Authors: Comments to the Authors In this research article, Wu et al. examined the expression of m6A circRNAs in severe acute pancreatitis (SAP), an inflammatory disease that displays a complex and unclear molecular etiology. Moreover, they conducted in silico and in vitro experiments using mice models in order to elucidate their biological roles in this disorder. The findings of the study are interesting, and shed light on the issue of circRNA epigenetics and their involvement in disease development. However, there are several issues that need to be addressed:

Response: We would like to thank you for your critical and insightful comments on our manuscript, which improve the quality of our manuscript and increase the significance of our study.

• Major issues:

1. Considering that the study was designed using animals (3 mice per group), the Authors need to specify this in the title and be more restrained in their comments regarding the Results of the study, avoiding strong arguments. In addition, generalizing of the conclusions should be avoided, since these results may not be applicable to SAP cases in humans. Authors are advised to conduct conservation analysis of the sequence of the selected circRNAs, in order to suggest that these circRNAs may have similar roles in human SAP.

Response: Thank you for your critical and insightful comments. We completely agree with your viewpoints and have revised in corresponding parts.

(1) We have specified that this study was designed using mice model in the title. The corresponding revisions are highlighted in red in the revised manuscript (Page 7, Line 2; Page 21, Line 4-5, 11-12, 25-28; Page 25, Line 1-2)

(2) To avoid strong arguments, we have adjusted the related descriptions. The corresponding revisions are highlighted in red in the revised manuscript (Page 10, Line 21-23)

(3) To explore that these circRNAs may have similar roles in human SAP, we have performed the conservation analysis of the sequence of the selected circRNAs preliminarily. Through aligning with the sequence of human circRNAs in circBase database, we found that 15/20 of the selected circRNAs that have highly similar sequences to human circRNAs (sequence identity > 80%), as shown in the following Table 1. These results suggested that these circRNAs may have similar roles in human SAP. However, there are a large number of works to explore this problem. In next study, solving this key problem is one of our main work. Thank you for your constructive comments again.

Table 1 The conservation analysis of the sequence between the selected

circRNAs and human circRNAs

	Human circRNA			
Mouse circRNA	**Human circRNA**	**Hg19 location**	**Transcript**	**Parent gene**
chr15:98656602-98658435-	**hsa_circ_0026065**	**chr12:49223538-49245957-**	**NM_004818**	**DDX23**
chr11:74928993-74990215+	**hsa_circ_0041387**	**chr17:2139785-2203958-**	**NM_001170957**	**SMG6**
chr9:108207543-108263690-	**hsa_circ_0124055**	**chr3:49514281-49548252+**	**NM_001177634**	**DAG1**
chr2:153756037-153769786+	**hsa_circ_0059811**	**chr20:31436477-31438211+**	**NM_012325**	**MAPRI**
chr19:40314443-40373578-	**hsa_circ_0094611**	**chr10:97110965-97114724-**	**ENST00000371247.2**	**SORBS**
chr16:94611419-94694141+	**hsa_circ_0115989**	**chr21:38792600-38888974+**	**ENST00000338785.3**	**DYRK1**
chr7:63891679-63938495-	**hsa_circ_0034321**	**chr15:31619082-31670102+**	**NM_015995**	**KLF13**
chr9:107847268-107860459-	**hsa_circ_0065768**	**chr3:50000008-50114685+**	**NM_005777**	**RBM6**
chr1:150413021-150442180+	**hsa_circ_0111511**	**chr1:186294895-186325581-**	**NM_003292**	**TPR**
chr1:13298706-13325802-	**hsa_circ_0113369**	**chr1:42166586-42254891-**	**ENST00000247584.5**	**HIVEP3**
chr6:119951703-120038640-	**hsa_circ_0024963**	**chr12:939168-990955+**	**NM_001184985**	**WNK1**
chr11:23261835-23271205+	**hsa_circ_0120688**	**chr2:61749745-61764803-**	**ENST00000404992.2**	**XPO1**
chr4:108486454-108508433+	**hsa_circ_0012539**	**chr1:52927184-53018762-**	**NM_001009881**	**ZCCHC**
chr11:32283981-32297161+	**hsa_circ_0118668**	**chr2:202780266-202790202-**	**None**	**None**
chr9:69408311-69432615+	**hsa_circ_0035568**	**chr15:60720627-60748993-**	**NM_024611**	**NARG2**

2. In the "Library preparation" section of the Materials and Methods, the Authors conducted rRNA removal from total RNA. How was the efficacy of this procedure checked, were there any controls?

Response: Thank you very much for your professional comments. In order to examine the removal efficiency of rRNA, after removing rRNA according to the instructions of the Kits, we performed the residual determination of 28S and 18S of rRNA in the samples with ribosomal RNA removed and the samples without ribosomal RNA removed by RT-qPCR. The primer sequences and results are shown in the following Table 2 and Figure 1. These results suggested that rRNA had been effectively removed.

Table 2 The primer sequences of 18S and 28S

28S	Forward	CGGGTAAACGGCGGGAGTAAC
	Reverse	TAGGTAGGGACAGTGGGAATCTCG
18S	Forward	CTCAACACGGGAAACCTCAC
	Reverse	CGCTCCACCAACTAAGAACG

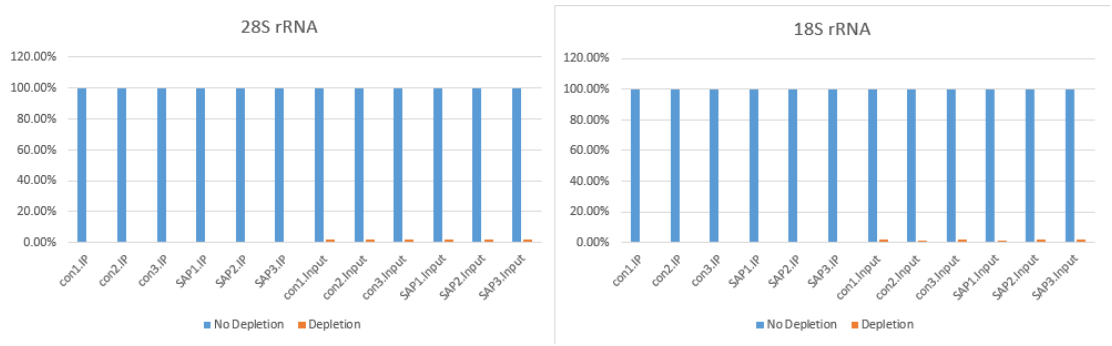

Figure 1 **The residual determination of 28S and 18S of rRNA in the samples with ribosomal RNA removed and the samples without ribosomal RNA removed by RT-qPCR.**

3. In order to have more robust evidence for the claims in this study, the top 10 upregulated and top 10 downregulated circRNAs according to the level of m6A need to be validated by qPCR experiments.

Response: Thank you for this critical and constructive comment. We completely agree with your viewpoint. This experiment to validate the selected m^{6}A-modified circRNAs is our next plan. Actually, the validation of m^{6}A circRNAs must be done by m^{6}A-modified RNA immunoprecipitation PCR (MeRIP-PCR). At present, there are some difficulties to perform this experiment at present due to the limited time. For example, there are about two months to buy the anti-m^{6}A RNA antibody and the relevant kits. In the future, we will carry out this experiment and explore their specific mechanisms in SAP further both in vivo and vitro experiments. Thank you for your professional comment again.

4. The expression level of ALKBH5 and FTO was measured only though Western blots. In order to further support these results, the corresponding mRNA levels of these molecules need to be examined. On that note, statements such as "These results indicated that ALKBH5 may play a key role in the dynamic process of m6A in SAP" are better avoided.

Response: Thank you for this constructive comment. We completely agree with your viewpoint that "the corresponding mRNA levels of these molecules need to be examined". We have examined the expression of ALKBH5 and FTO in mRNA levels. The results were shown in Figure 8A. The corresponding revisions are highlighted in red in the revised manuscript (Page 17, Line 1-10; Page 21, Line 7-12)

Regarding the statement that "These results indicated that ALKBH5 may play a key role in the dynamic process of m6A in SAP", we have corrected the statement. The corresponding revisions are highlighted in red in the revised manuscript (Page 21, Line 11-12)

5. In the Discussion, it is stated that "… m6A circRNAs function in SAP though other mechanisms, such as […] and interaction with RNA-binding proteins". However, the interaction with RNA-binding proteins (RBPs) was not examined in this study. Should the Authors decide to include this claim, at least an in silico prediction of RBP-binding sites needs to be conducted.

Response: Thank you for your valuable comment. We now realize that the statement regarding the interaction with RNA-binding proteins is unprecise and we have deleted this claim. In our next plan, we will perform RNA pull down to explore their interactions with RNA-binding proteins.

• Minor issues:

1. Throughout the text, the 6 in N6-methyladenosine (m6A) should be corrected to an exponent 6, as it is the appropriate name structure.

Response: Thank you for your careful comments. We are sorry for our carelessness in this detail and we have corrected this error.

2. In the Introduction, the Authors state that "…we determined the expression of demethyltransferase to deduce the possible mechanism…". This period is very vague and the molecules which are implied should be explicitly mentioned.

Response: Thank you for your professional comments. We completely agree with your viewpoints and we have stated these molecules clearly. The corresponding revisions are highlighted in red in the revised manuscript (Page 13, Line 4-6).

3. All abbreviated words (wk, bps etc.) should be replaced with the standard word, since they are not obvious to every reader.

Response: Thank you for your careful comments. We now realize that the abbreviated words (wk, bps etc.) should use the standard word and we have corrected them. The corresponding revisions are highlighted in red in the revised manuscript (Page 13, Line 9; Page 19, Line 14)

4. In the Materials and Methods, Authors include an amylase and lipase measurement. The specific "commercial kits" used for the quantification should be mentioned. Moreover, the reason for conducting this assay is not clear and is explained only in the Discussion; it needs to be more evident earlier in the manuscript.

Response: Thank you for your careful comments. We apologize that these information described is unclear. (1) The "commercial kits" are Lipase Assay kits and Amylase Assay kit that purchased from Nanjing Jiancheng Bioengineering Institute. We have corrected them. (2) Considering that the levels of serum lipase and amylase are as one of the diagnostic criteria of

acute pancreatitis [1], we determine their concentrations in serum. Finally, the levels of lipase and amylase are as one of the criteria to confirm the models. We have explained this assay. The corresponding revisions are highlighted in red in the revised manuscript (Page 14, Line 4-6; Page 17, Line27-28; Page 18, Line 1)

[1] Crockett SD, Wani S, Gardner TB, Falck-Ytter Y, Barkun AN. American Gastroenterological Association Institute Guideline on Initial Management of Acute Pancreatitis. *Gastroenterology* 2018; 154(4): 1096-1101 [PMID: 29409760 DOI: 10.1053/j.gastro.2018.01.032]

5. In the "Distribution of m6A sites in SAP and control groups" of the Results, it is mentioned that "circRNAs can be generated from any region of the genome, and all result in a great diversity of lengths". This statement is vague and can be confusing for the reader. In addition, the relevance of this claim to the genomic distribution of m6A circRNAs that is mentioned immediately after is unclear.

Response: Thank you for your professional comments. We are sorry for the unclear statements and have corrected the related statements. **The corresponding revisions are highlighted in red in the revised manuscript (Page 19, Line 2-5)**

6. The limitations of this study should be clearly stated in the Discussion.

Response: Thank you for your professional comment. We now realize that the limitations of our study are required. We have discussed the limitations of our study. The corresponding revisions are highlighted in red in the revised manuscript (Page 25, Line 3-9)

Reviewer #2:

Scientific Quality: Grade B (Very good)

Language Quality: Grade A (Priority publishing)

Conclusion: Accept (General priority)

Specific Comments to Authors: This is correctly conducted, well-designed, interesting animal study regarding genome-wide map of N6-methyladenosine circular RNAs identified in severe acute pancreatitis. Manuscript should be published.

Response: Thank you for reviewing our manuscript. We were pleased to read this conclusion that "This is correctly conducted, well-designed, interesting animal study regarding genome-wide map of N6-methyladenosine circular RNAs identified in severe acute pancreatitis. Manuscript should be published".

The revised manuscript that was highlighted the corresponding revised parts is below.

Genome-wide map of N^6-methyladenosine circular RNAs identified in mice model of severe acute pancreatitis

Running title: Genome-wide m^6A circRNAs in SAP

Jun Wu, Xiao-Hui Yuan, Wen Jiang, Yi-Chen Lu, Qi-Lin Huang, Yi Yang, Hua-Ji Qie, Jiang-Tao Liu, Hong-Yu Sun and Li-Jun Tang

Jun Wu, Xiao-Hui Yuan, Wen Jiang, Yi-Chen Lu, Qi-Lin Huang, Yi Yang, Hua-Ji Qie, Jiang-Tao Liu, Hong-Yu Sun and Li-Jun Tang, Department of General Surgery & Pancreatic Injury and Repair Key Laboratory of Sichuan Province, The General Hospital of Western Theater Command, Chengdu, 610083 China.

Jun Wu, Xiao-Hui Yuan, Wen Jiang, Yi-Chen Lu, Hua-Ji Qie, Jiang-Tao Liu, Hong-Yu Sun and Li-Jun Tang, College of Medicine, Southwest Jiaotong University, Chengdu, 610036 China

Hong-Yu Sun, Laboratory of Basic Medicine, The General Hospital of Western Theater Command, Chengdu, 610036 China

ORCID number: Jun Wu (0000-0003-1555-8026), Xiao-hui Yuan (0000-0002-6462-2672), Wen Jiang (0000-0001-6823-6122), Yi-chen, Lu (0000-0002-4020-4847), Qi-lin Huang (0000-0002-0819-4829), Yi Yang (0000-0002-0189-6246), Hua-ji Qie (0000-0002-6648-7339), Jiang-tao Liu (0000-0002-5993-8103), Hong-yu Sun (0000-0002-8587-0499); Li-jun Tang (0000-0001-6000-9515)

Author contributions: Jun Wu and Xiaohui Yuan contributed equally to this

work. Lijun Tang and Hongyu Sun participated in the study conception and design. Jun Wu and Xiaohui Yuan participated in the writing of the main manuscript. Jun Wu, Xiaohui Yuan, Wen Jiang and Yichen, Lu participated in the performance of the experiments. Qilin Huang and Yi Yang participated in statistical data analysis, and interpretation. Huaji Qie and Jiangtao Liu participated in preparing all the figures. Lijun Tang and Hongyu Sun participated in the revision of the manuscript and final approval.

Supported by the National Natural Science Foundation of China, No. 81772001; the National Clinical Key Subject of China, No. 41792113.

Institutional review board statement: The study was reviewed and approved by the Institutional Ethics Committee (Approval No. A20190252005) at the General Hospital of Western Theater Command (Chengdu, China).

Institutional animal care and use committee statement: The experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the General Hospital of Western Theater Command (Chengdu, China), and were conducted in accordance with the established International Guiding Principles for Animal Research.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: We had submitted the data to the online repository, which can be found at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173298.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Correspondence author: Lijun Tang, MD., Ph.D. MD, PhD, DSc (Med), Professor, General Surgery Center of PLA; The General Hospital of Western Theater Command, Chengdu, Sichuan Province, 610083; China. Tel.: +86 28 8657 0265. E-mail: tanglj2016@163.com

ABSTRACT

BACKGROUND

Severe acute pancreatitis (SAP) is a deadly inflammatory disease with complex pathogenesis and lack of effective therapeutic options. N^6-methyladenosine (m^6A) modification of circular (circ)RNAs plays important roles in physiological and pathological processes. However, the roles of m^6A circRNA in the pathological process of SAP remains unknown.

AIM

To identify transcriptome-wide map of m^6A circRNAs and to determine their biological significance and potential mechanisms in SAP.

METHODS

The SAP in C57BL/6 mice was induced using 4% sodium taurocholate salt.

The transcriptome-wide map of m^{6}A circRNAs were identified by m^{6}A-modified RNA immunoprecipitation sequencing. The biological significance of circRNAs with differentially expressed m^{6}A peaks was evaluated through GO and KEGG analysis. The underlying mechanism of m^{6}A circRNAs in SAP was analyzed by constructing of m^{6}A circRNA-microRNA networks. The expression of demethylases were determined by western blot to deduce the possible mechanism of reversible m^{6}A process in SAP.

RESULTS

Fifty-seven circRNAs with differentially expressed m^{6}A peaks were identified by m^{6}A-modified RNA immunoprecipitation sequencing; of which, 32 were upregulated and 25 downregulated. Functional analysis of these m^{6}A circRNAs in SAP found some important pathways involved in the pathogenesis of SAP, such as protein digestion and regulation of autophagy. In m^{6}A circRNA–miRNA networks, several important miRNAs participated in the occurrence and progression of SAP were found to bind to these m^{6}A circRNAs, such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. Notably, the total m^{6}A level of circRNAs was reduced, while the demethylase alkylation repair homolog 5 was upregulated in SAP.

CONCLUSION

m^{6}A modification of circRNAs may be involved in the pathogenesis of SAP. Our findings may provide novel insights to explore the possible pathogenetic mechanism of SAP and seek new potential therapeutic targets for SAP.

Key Words: Severe acute pancreatitis; Circular RNAs; N^6-methyladenosine; m^{6}A-RIP-seq; Epigenetic analysis

Core Tips: We identified transcriptome-wide map of m^{6}A circular (circ)RNAs and determined their biological significance and potential mechanisms in severe acute pancreatitis (SAP). The main findings were: (1) Function analysis

found that circRNAs with differentially expressed m^{6}A peaks were involved in the key process of SAP. (2) m^{6}A may affect the interplays of circRNAs and microRNAs to participate in pathogenesis of SAP. (3) Demethylase ALKBH5 may play key roles in dynamic process of m^{6}A to downregulate the total m^{6}A level of circRNAs in SAP. We provided novel insights to explore the possible pathophysiological mechanism of SAP and seek new potential therapeutic targets.

INTRODUCTION

Acute pancreatitis (AP) is an pancreatic inflammatory disorder that is associated with substantial morbidity and mortality [1]. Approximately 20% of patients with AP develop into severe AP (SAP) [2]. Due to the extensive pancreatic necrosis, subsequent infection, systemic inflammatory response syndrome and multiple organ failure, the mortality of SAP is up to 30% [2, 3]. Previous studies have suggested that some important pathological mechanisms, including premature trypsinogen activation in the acinar cells and macrophages, mitochondrial dysfunction, pathological calcium signaling, endoplasmic reticulum stress, and impaired autophagy, are involved in the initiation and development of SAP [1]. However, the pathophysiology of SAP is complex and remains unclear, especially the level of gene regulation.

Circular RNAs (circRNAs) were discovered in the 1970s [4] and were identified as single-stranded covalently closed RNA molecules that lack 5′ caps and 3′ tails [5]. In a long time afterwards, they were thought to be the byproducts of splicing [6]. In recent years, as the high-throughput sequencing developed, thousands of circRNAs were found to be expressed in a wide range of mammalian tissues [7, 8], including pancreas [9], and accumulating studies have demonstrated that circRNAs play important roles in the whole process and prognosis of many diseases, including cardiovascular diseases [8], cancer [10], neurodevelopmental processes[11], immune responses and immune diseases [12]. The main mechanisms of circRNAs participated in the initiation

and development of diseases include the following functions [6, 8, 10, 12], interplay with RNA-binding proteins, microRNA (miRNA) sponges, regulating the stability of mRNAs, modulating the transcription of parental gene and the templates for protein synthesis. However, the post-transcription modification of circRNAs remains unclear.

N^6-methyladenosine (m^6A) is the most prevalent internally modification of RNA in eukaryotic cells [13]. In 2017, Zhou *et al* reported that m^6A modification is widespread in circRNAs and m^6A modifications are read and written by the same complexes in circRNAs and mRNAs [14]. The regulatory role of m^6A is mainly performed by three homologous factors, namely so-called "writers", "erasers" and "readers" [13-15]. The writers mainly include methyltransferase-like 3 and 14 proteins (METTL3 and METTL14) and their cofactor WT1-associated protein (WTAP). They form a methyltransferase complex to catalyze the installation of m^6A. The erasers, including alkylation repair homolog 5 (ALKBH5) and fat mass and obesity related protein (FTO), can catalyze the oxidative demethylation of N-alkylated nucleic acid bases. The readers are mainly YT521-B homology (YTH) domain containing proteins family, including YTHDC1, YTHDC2, YTHDF1, YTHDF2 and YTHDF3. They can specifically recognize m^6A and regulate splicing, localization, degradation and translation of RNAs. Recently, it has been found that the m^6A modification of circRNAs plays a key role in innate immunity and tumors though regulating the metabolism and function of circRNAs [15]. In human embryonic stem cells and HeLa cells, m^6A circRNAs display cell-type-specific methylation patterns [14]. In colorectal carcinoma, the m^6A modification can modulate cytoplasmic export of circNSUN2 and stabilize HMGA2, ultimately enhancing the colorectal liver metastasis [16]. However, the roles of m^6A circRNAs in SAP remain unknown.

Here, we investigated the expression profile of m^6A circRNAs in SAP through m^6A-modified RNA immunoprecipitation sequencing (m^6A-RIP-seq). We evaluated the biological significance of circRNAs with differentially

expressed m^{6}A peaks though Gene Ontology (GO) functional annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and explored their underlying mechanism by construction of m^{6}A circRNA–miRNA networks. In addition, we determined the expression of demethyltransferase, ALKBH5 and FTO, to deduce the possible mechanism of reversible m^{6}A process in SAP.

MATERIALS AND METHODS

Animals and preparation of SAP model

Male C57BL/6 mice with the aged 7–8 weeks were provided by Chengdu Dashuo Experimental Animal Technology Co. Ltd. All the mice were housed in ventilated plastic cage system and fed with the same food and water for 7 days to adapt to the environment. The entire research protocol was approved by the Institutional Animal Care and Use Committee at the General Hospital of Western Theater Command.

Before the operation, the mice were randomly divided into control and SAP groups (3 mice per group) and fasted overnight but had free access to water. Isoflurane (5%) was used to anesthetize mice by induction box prior to surgery. Then, the SAP was induced through 4% sodium taurocholate salt that was slowly retrogradely injected into the biliopancreatic duct with a microinfusion pump. All mice were killed 24 h after the establishment of model, and the blood samples and pancreatic tissues were collected for further analysis.

Pancreatic histological analysis

Pancreatic tissue (0.4 cm × 0.4 cm) was fixed in 4% paraformaldehyde solution and dehydrated with ethanol and embedded in paraffin. The samples were cut into sections of 4-μm-thick and stained with hematoxylin and eosin, and examined under light microscopy at 200×. The histopathological score was evaluated with a previously described scoring

system [17]. The scores were averaged for five individual slides from each pancreas.

Amylase and lipase measurement

The concentrations of lipase and amylase in serum were determined using Lipase Assay kit and Amylase Assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions.

RNA isolation and RNA quality control

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the homogenized pancreatic tissues of the control and SAP groups. The concentration of extracted RNA was measured at OD260 and 280 by NanoDrop ND-2000 instrument (Thermo Fisher Scientific, Waltham, MA, USA). We assessed the integrity of RNA using denaturing agarose gel electrophoresis. The OD A260/A280 ratio between 1.8 and 2.0 was set as the RNA purity standard.

Library preparation and m^{6}A-RIP-seq

rRNAs in total RNA were removed using Ribo-Zero rRNA Removal Kits (Illumina, San Diego, CA, USA). The fragmented RNA was incubated with the anti-m^{6}A antibody at 4°C for 2 h in IPP buffer. Then, the mixture was immunoprecipitated by incubation with protein-A beads (Thermo Fisher Scientific) for 2 h at 4°C. The bound RNA was eluted from the beads with m^{6}A (Berry & Associates) in IPP buffer and then extracted with TRIzol reagent (Thermo Fisher Scientific). The immunoprecipitated RNA and input RNA were used to construct the library using NEBNext® Ultra™ RNA Library Prep Kit and double-ended 150-bp sequencing of the m^{6}A-IP and input samples was performed on an Illumina Hiseq sequencer (performed by Cloudseq Biotech Inc., Shanghai, China).

Analysis of MeRIP-Seq data

Paired-end reads were harvested from the Illumina HiSeq 4000 sequencer, and were quality controlled by Q30. To obtain high quality clean reads, 3′ adaptor-trimming and low quality reads were removed by cutadapt software. The clean reads with high quality of the input library were aligned to the mouse reference genome (UCSC MM10) with STAR software. DCC software was used for detecting and identifying circRNAs. The identified circRNAs were annotated using the circBase database and Circ2Traits database. For all samples, raw junction reads were normalized to the number of total mapped reads and log2 transformed. The read alignments on the genome were visualized using the tool IGV (Integrative Genomics Viewer). The adapter-removal reads were aligned to the reference genome using Hisat2 software. The methylated sites in each sample were identified using MACS software. Differentially methylated sites were identified using diffReps software.

GO and KEGG analysis

The parent genes of circRNAs with differential m^6A peaks were selected to analyze their potential biological roles through GO and KEGG pathway analysis. GO analysis included three parts, namely, biological process (BP) analysis, molecular function (MF) analysis, and cell component (CC) analysis [18]. GO analysis was performed by R topGO package. Fisher's exact test in Matlab MCR software was applied to calculate the enrichment of each pathway. The bubble plots and column plots were generated using the ggplot2 in R package (https://ggplot2.tidyverse.org).

Construction of circRNA–miRNA networks

circRNA containing miRNA-binding sites can bind to miRNA response elements competitively, further regulating the target mRNAs [19]. The top 10 upregulated and top 10 downregulated circRNAs according to the level of

m^{6}A were selected to construct circRNA–miRNA networks. The m^{6}A circRNA–miRNA networks were constructed using TargetScan software and miRanda software and the circRNA–miRNA interactions were visualized by Cytoscape.

Western blotting

The whole pancreatic tissues from SAP and control groups were placed in RIPA lysate buffer with protease inhibitor, phosphatase inhibitor and phenylmethylsulfonyl fluoride inside (Total Protein Extraction Kit; Beijing Solarbio Science and Technology Inc.), and the tissues were homogenized with homogenizer. The tissue homogenate was centrifuged at 12 000 g for 30 min at 4°C, and the supernatant was collected. After protein concentration was measured by BCA Protein Assay Kit (Beyotime, China), the supernatant was mixed with loading buffer (Beijing Solarbio Science and Technology), boiled at 100°C for 10 min for protein denaturation, and stored at –80°C after separation. The target proteins were separated by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membrane (0.45 μm, IPVH00010; Millipore, Billerica, MA, USA), blocked in 5% nonfat milk for 1 h at room temperature (22 ± 3°C), and then incubated with primary antibody, FTO (1:1000, D2V1I; Cell Signaling Technology, Danvers, MA, USA), ALKBH5 (1:2000, 16837-1-AP; Proteintech, USA), GAPDH (1:5000, 10494-1-AP; Proteintech) at 4°C overnight. The membranes were washed with Tris-buffered saline with Tween-20 (TBST) (Beijing Solarbio Science and Technology) three times and incubated with secondary antibody (1:10 000, 15015; Proteintech) at room temperature for 1 h. After being washed three times with TBST, the protein bands were visualized by enhanced chemiluminescence (Immobilon Western Chemilum HRP Substrate; Millipore) in a biological imaging system.

Quantitative Real-Time PCR (qPCR)

The total RNA was extracted from SAP and control groups as described above. qPCR was performed using One Step SYBR® PrimeScript™ RT-PCR kit II (Takara Biotechnology Co., Ltd., Dalian, China) and the primers (ALKBH5: forward 5′-GGCGGTCATCATTCTCAGGAAGAC-3′ and reverse 5′-CTGACAGGCGATCTGAAGCATAGC-3′; FTO: forward 5′-CTCACAGCC TCGGTTTAGTTCCAC-3′ and reverse 5′–CGTCGCCATCGTCTGAGTCATT G-3′; GAPDH: forward 5′-GGTGAAGGTCGGTGTGAACG-3′ and reverse 5′-CTCGCTCCTGGAAGATGGTG-3′) were synthesized by Shanghi Sangon Biotech Co., Ltd.. The outcomes were analyzed by means of $2^{-\Delta\Delta CT}$ through normalizing the quantity of GAPDH.

Data analysis

GraphPad Prism 8 (La Jolla, CA, USA) and SPSS 22.0 (Chicago, IL, USA) were used for performing statistical analyses. Student's t test was used for estimating statistically significance between two groups. The results were evaluated through Spearman's correlation coefficient test. All values are shown as mean ± standard error of the mean; $P < 0.05$ was considered statistically significant.

RESULTS

Evaluation of mouse model of SAP

Twenty-four hours after treatment with sodium taurocholate salt, the staining of hematoxylin and eosin on the pancreatic tissues from the SAP group showed typical histopathological changes, including pancreatic lobular edema, extensive acinar cell necrosis, focal expansion of the pancreatic interlobular septum and granulocyte infiltration (Figure 1A). By contrast, under light microscopy, the pancreases from the control group had a complete normal structure. Figure 1B showed the corresponding histopathological scores. At the same time, considering that the levels of serum lipase and amylase are as one of the diagnostic criteria of AP [20], we

determined their concentrations in serum. As a result, the serum lipase and amylase levels in the SAP group were also significantly higher than those in the control group ($P < 0.05$; Figure 1C and 1D). These results confirmed the successful establishment of the SAP mice model.

Overview of m^{6}A circRNAs in SAP

We used m^{6}A-RIP-Seq to investigate expression of m^{6}A circRNAs in pancreatic tissues in the control and SAP groups. We had submitted the data to the online repository, which can be found at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173298. A total of 409 m^{6}A circRNAs were identified in all chromosomes (Figure 2A). Among these, 178 were specifically expressed in the SAP group, 107 in the control group, and 124 were shared in both groups (Figure 2B). m^{6}A level in total circRNAs from the SAP group was lower than that from the control group (Figure 2C). Besides, > 80% of circRNAs contained only one m^{6}A peak in both SAP and control groups (Figure 2D).

Differential m^{6}A modification of circRNAs in SAP

To understand the biological role of m^{6}A modification of circRNAs in SAP, the circRNAs with differentially expressed (DE) m^{6}A peaks were further analyzed. Significant differential expression was defined as fold change > 2 and $P < 0.05$. Compared with the control group, 57 circRNAs with DE m^{6}A peaks were identified; 32 were upregulated and 25 downregulated in the SAP group. Table 1 presents the top 10 methylated m^{6}A sites that were up- and downregulated within circRNAs. Figure 3A shows the m^{6}A circRNAs expression profile in the SAP and control groups though hierarchical cluster analysis. The scatter plot exhibits the variation of DE m^{6}A circRNAs between the SAP and control groups (Figure 3B). The volcano plot depicted DE m^{6}A circRNAs between the two groups (Figure 3C).

Distribution of m^{6}A sites in SAP and control groups

We identified 903 m6A peaks distributed on 781 circRNAs and it is reported that circRNAs can be generated from any region of the genome [21]. Therefore, we firstly analyzed the genomic distribution of m6A and non-m6A circRNAs according to their genomic origins to explore their distribution features. As a results, in non-m^{6}A circRNAs, 45.33% were sense overlapping, 21.15% exonic, 26.71% intronic, 4.94% intergenic and a few antisense; in m^{6}A circRNAs, 42.78% were sense overlapping, 30.32% exonic, 21.27% intronic, 3.42% intergenic and a few antisense (Figure 4A). These results indicated that the majority of m^{6}A and non-m^{6}A circRNAs were commonly encoded by sense overlapping sequences and the number of circRNAs that generated from protein-coding genes in m^{6}A circRNAs was more than those in non-m^{6}A circRNAs.

We further analyzed the distribution of circRNAs with DE m^{6}A peaks. The length of DE m^{6}A circRNAs was mainly enriched in 1–10 000 base pairs (Figure 4B). Although the host genes of m^{6}A circRNAs located in all chromosomes, the dysregulated parts mostly located in chromosomes 4, 9 and 11 (Figure 4C). A previous study reported that most circRNAs that derived from protein-coding genes spanned two or three exons [14]. In this study, the majority of circRNAs from protein-coding genes spanned one or two exons (Figure 4D). Similarly, the majority of m^{6}A circRNAs and non-m^{6}A circRNAs were more commonly encoded by a single or two exons (Figure 4E).

Functional analysis of circRNAs with DE m^{6}A peaks

To explore the function of m^{6}A circRNAs in SAP, GO analysis and KEGG pathway analysis of circRNAs with the DE m^{6}A peaks were performed. Figure 5A presented the top 10 GO terms of circRNAs with upregulated m^{6}A peaks from the three aspects BP, CC and MF. For BP, the most enriched and meaningful GO terms were cellular component organization, macromolecule metabolic process and regulation of developmental process. For CC, the top three terms were focal adhesion, cell–substrate junction and anchoring

junction. For MF, the main represented GO terms were C2H2 zinc finger domain binding and protein binding. The top 10 pathways from KEGG pathway analysis for circRNAs with upregulated m^{6}A peaks were selected and presented in a bubble chart (Figure 5B). Among them, protein digestion and absorption and regulation of autophagy were the major signaling pathways associated with the SAP progression.

The GO terms of circRNAs with downregulated m^{6}A peaks are presented in Figure 5C. For BP, protein-containing complex localization, RNA transport and macromolecule metabolic process were the most enriched and meaningful GO terms. For CC, nucleus, dendrite and dendritic tree were the top three terms. For MF, the main represented GO terms were channel regulator activity, RNA, enzyme and protein binding. As for the KEGG pathway analysis of circRNAs with downregulated m^{6}A peaks, RNA transport was the main pathway (Figure 5D).

Relationship between m^{6}A level and expression of circRNAs in SAP

To explore whether m^{6}A modification could affect the expression of circRNAs, we analyzed the expression level of m^{6}A circRNAs. The expression level of these circRNAs with DE m^{6}A peaks did not have significant differences (fold change < 2 or P > 0.05; Table S1), indicating that m^{6}A modification of circRNAs did not influence expression of circRNAs. To verify this result, we analyzed the cumulative distribution of circRNA expression between the control and SAP groups for m^{6}A and non-m^{6}A circRNAs (Figure 6). This was consistent with the above result.

Construction of m^{6}A circRNA–miRNA networks in SAP

Given the importance of circRNA–miRNA interaction [22] and to further explore the underlying mechanism of these circRNAs with DE m^{6}A peaks, the top 10 upregulated and top 10 downregulated circRNAs according to the level of m^{6}A were selected to construct circRNA–miRNA networks. In this

network map, several important miRNAs participated in the occurrence and development of SAP were found to bind to these m^{6}A circRNAs (Figure 7), such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. These data suggest that these circRNAs with DE m^{6}A peaks might play a role in the pathological process of SAP.

Expression of demethyltransferase in SAP

Given that the total m^{6}A level of circRNAs was reduced in SAP and to explore how the m^{6}A level was regulated in SAP, we detected the protein and mRNA expression of two demethyltransferases (ALKBH5 and FTO). FTO was reduced at the level of protein, but ALKBH5 was increased in SAP at both the level of mRNA and protein (Figure 8). These results indicated that ALKBH5 might be related to the dynamic process of m^{6}A in SAP.

DISCUSSION

In the present study, we identified transcriptome-wide map of m^{6}A circRNAs and determined their biological significance and potential mechanisms for the first time in SAP. The main findings are as follow: (1) We identified 57 circRNAs with DE m^{6}A peaks and found these DE m^{6}A circRNAs were involved in the key process of SAP by GO and KEGG analysis, such as protein digestion and regulation of autophagy. (2) In m^{6}A circRNA-miRNA networks, several important miRNAs participated in the initiation and development of SAP were found to bind to these m^{6}A circRNAs potentially, suggesting that m^{6}A may affect the interplays with miRNAs. (3) The total m^{6}A level was reduced in SAP, and the demethylase ALKBH5 was found to be upregulated in SAP, indicating that ALKBH5 may be related to dynamic process of m^{6}A in SAP. These results suggested that m^{6}A modification on circRNAs may be involved in the pathophysiology of SAP, which may provide novel insights to explore the possible pathophysiological mechanism of SAP and seek new potential therapeutic targets.

To find effective therapeutic targets for SAP, many studies have explored the underlying molecular mechanisms of SAP. Our previous study found that many circRNAs are expressed in mice with SAP [9] and these circRNAs play an important role in the pathogenetic mechanism of SAP [9, 23]. In recent years, m^{6}A modification of circRNAs was found to be widespread [14] and gained widespread attention in epigenetics. Several important studies have investigated the roles of m^{6}A modification in circRNA metabolism and found that m^{6}A circRNAs play key roles in some diseases [16, 24-28]. In circRNA metabolism, m^{6}A modifications can regulate its translation through recognition by YTHDF3 and eIF4G2, and this progress of translation can be enhanced by METTL3/14 and inhibited by FTO [24, 25]. In addition, m^{6}A circRNAs associate with YTHDF2 in an HRSP12-dependent manner and are selectively downregulated by RNase P/MRP [26]. In innate immunity, Chen *et al* found that unmodified circRNA adjuvant induces antigen-specific T and B cell responses, but m^{6}A modification could abrogate circRNA immunity though YTHDF2-mediated suppression [27]. In male germ cells, the back splicing tends to occur mainly at m^{6}A-enriched sites, which are usually located around the start and stop codons in linear mRNAs, resulting in about half of circRNAs containing large open reading frames. This potential mechanism could ensure long-lasting and stable protein production for specific physiological processes when lacking the corresponding linear mRNAs [28]. These findings showed the important roles of m^{6}A in circRNAs during disease progress. Therefore, it is essential to explore the roles of m^{6}A circRNAs in SAP.

In the present study, the function analysis of DE m^{6}A circRNAs in SAP found that two important pathways were involved in the pathogenesis of SAP, including protein digestion and regulation of autophagy. As an important pathological cellular event, the activation of premature trypsinogen can result in acinar cell necrosis [1]. Many pancreatic injury factors, such as trauma, obstruction of the pancreatic duct and alcohol, can initiate the fusion of

lysosomes with zymogen in acinar cells, leading to the activation of trypsinogen through cathepsin B to trypsin. Once trypsin is released, it can cause self-digestion in and outside the acinar cells, and the release of cathepsin B can cause necroptosis. As a cytoprotective mechanism, autophagy can process and recycle various aged, defective or damaged cytoplasmic contents [29]. Selective macroautophagy is a biological process during which specific damaged organelles and misfolded proteins are processed and recycled. Autophagy is accomplished via a series of steps, which start with the enucleation of cytoplasmic inclusions in the open double membrane formed by the endoplasmic reticulum (ER), Golgi apparatus and plasma membrane [30]. Knocking out *ATG7* genes (which are important to form autophagosome) and *LAMP* genes could lead to pancreatitis with extensive inflammation in mice [29, 31]. Importantly, impaired autophagy leads to trypsinogen activation, ER stress and mitochondrial dysfunction. These events can together make acinar cells become more susceptible to other insults and cellular death [1]. In addition, RNA transport is enriched in GO terms of note, and Chen *et al* found that m^{6}A modification can modulate the export of circNSUN2 to the cytoplasm [16], suggesting that m^{6}A modification regulates transport of circRNAs in SAP. These results were consist with the hypothesis that m^{6}A modification of circRNAs participated in the progression of SAP.

m^{6}A modification of mRNA can influence its expression by regulating transcription, splicing and degradation [32]. In circRNAs, Zhou *et al* and Su *et al* reported that m^{6}A levels are correlated with expression levels of circRNAs in HeLa cells and a rat model of hypoxia-mediated pulmonary hypertension [14, 33]. However, in SAP, we found m^{6}A modification in circRNAs was not associated with expression of circRNAs, suggesting that m^{6}A circRNAs function in SAP though other mechanisms, such asmiRNA sponges. It is worth mentioning that more direct evidence is currently needed to support that m^{6}A can affect circRNA expression.

miRNA sponges are an important function of circRNAs. Cytoplasmic circRNAs can prevent miRNAs from binding to target mRNAs by competitive binding to miRNA response elements, further playing a key role in diseases [8, 34]. For instance, in lung squamous cell carcinoma, circTP63 can competitively bind to miR-873-3p and prevent miR-873-3p from decreasing the level of FOXM1, which upregulates CENPA and CENPB, and finally facilitates cell cycle progression [35]. In SAP, circHIPK3 can enhance pyroptosis via regulating the miR-193a-5p/GSDMD axis in acinar cells, ultimately aggravating this disease [36]. In our previous study, we found that circZFP644 could sponge miR-21-3p, thereby participating in the pathogenesis of SAP [9]. Recently, Su *et al* found that m^{6}A modification of circRNAs could influence the interactions between circRNAs and miRNAs [33]. Therefore, analysis of m^{6}A circRNA–miRNA networks was performed in this study. Several important miRNAs participated in the pathological process of SAP were found to bind to these m^{6}A circRNAs, such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. For example, in caerulein-stimulated AR42J cells, expression of miR-92b-3p was decreased, while overexpression of miR-92b-3p could downregulate the expression of TRAF3 and inhibit the MKK3-p38 pathway, attenuating inflammatory response and autophagy [37]. These results suggest that m^{6}A modification of circRNAs functions by influencing the interactions between circRNAs and miRNAs.

m^{6}A modification is a reversible process that occurs by methyltransferase complex consisting of METTL3, METTL14 and WTAP, and is "erased" by ALKBH5 and FTO [13, 15]. In pancreatic cancer, ALKBH5 could regulate the post-transcriptional activation of PER1 through m^{6}A abolishment, thereby inhibiting the cancer [38]. In hepatocellular carcinoma, ALKBH5 could attenuate expression of LYPD1 by an m6A-dependent manner and act as a tumor suppressor [39]. Overall, these studies have suggested that ALKBH5 plays an essential role in m^{6}A modification. In this study, we found that expression level of ALKBH5 was upregulated in SAP. Consistent with this

result, total m^{6}A level of circRNAs in SAP was reduced, indicating that ALKBH5 may play a role in the dynamic process of m^{6}A in SAP.

However, there are still limitations in our study. Firstly, further in vivo and vitro experiments are needed to further explore the m^{6}A circRNA-mediated precise regulatory mechanisms in SAP. Secondly, the clinical significance and the results should be investigated in SAP patients. Additionally, the precise mechanism of ALKBH5 in m^{6}A circRNAs during SAP needs to be studied. Actually, these are in our next plans to explore the roles of m^{6}A circRNAs in SAP.

In conclusion, our study identified the transcriptome-wide profiling of m^{6}A circRNAs in SAP and predicted their biological significance and possible potential mechanisms, providing new insights to explore the possible pathophysiological mechanism of SAP and seek new potential therapeutic targets.

Article Highlights

Research background

Severe acute pancreatitis (SAP) is a lethal inflammatory disease with mortality up to 30%. But the genetic pathological mechanism of SAP remains unclear and SAP is still lack of effective therapeutic options. N^6-methyladenosine (m^{6}A) modification of circular (circ)RNAs plays a key role in many diseases and physiological processes through regulating the metabolism and function of circRNAs. However, the role of m^{6}A circRNA in SAP has been unexplored yet.

Research motivation

The pathophysiology of SAP at the level of gene regulation is complex and remains unclear. circRNAs are found to participate in many physiological processes and play key roles in pathological processes during SAP. m^{6}A modification can affect the "fate" of m^{6}A modified circRNAs, thereby participating in the regulation of diseases. Therefore, we want to explore

whether the m^{6}A modification of circRNAs is related to the pathophysiological mechanism of SAP, and determine their biological significance and potential mechanisms.

Research objective

The present study aims to determine the transcriptome-wide map of m^{6}A circRNAs and explore their biological significance and its possible mechanisms in SAP.

Research methods

The SAP C57BL/6 mice model was induced by retrograde injection of 4% sodium taurocholate salt. m^{6}A-modified RNA immunoprecipitation sequencing was used to determine the transcriptome-wide map of m^{6}A circRNAs. The biological significance of circRNAs with differentially expressed m^{6}A peaks was identified by GO and KEGG analysis. m^{6}A circRNA-microRNA networks was constructed to explore the underlying mechanism of m^{6}A circRNAs in SAP. The expression of demethylases were measured by western blot and RT-qPCR. H&E staining and measurement of serum lipase and amylase were performed to assess the establishment of SAP mice model.

Research results

In the identified transcriptome-wide map of m^{6}A circRNAs, there were fifty-seven circRNAs with differentially expressed m^{6}A peaks; among which, 32 were upregulated and 25 downregulated. Important pathways in the pathogenetic process during SAP were found by functional analysis of these m^{6}A circRNAs, such as protein digestion and regulation of autophagy. m^{6}A circRNA–miRNA networks showed that several important miRNAs in pathogenesis of SAP were bind to these m^{6}A circRNAs, such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. To be note, the total m^{6}A level of circRNAs was reduced in SAP, accompanied by the upregulated demethylase alkylation repair homolog 5.

Research conclusions

The transcriptome-wide profiling of m^{6}A circRNAs in SAP was identified, and the biological significance and possible potential mechanisms of m^{6}A circRNAs in SAP were predicted, providing new insights into exploring the possible pathophysiological mechanism of SAP and new potential therapeutic targets.

Research perspectives

This present study for the first time identified transcriptome-wide map of m^{6}A circRNAs and determined their biological significance and potential mechanisms. However, the m^{6}A circRNA-mediated precise regulatory mechanisms are need to be explore further in vivo and vitro experiments. What's more, further studies are needed to reveal the precise mechanism of ALKBH5 in m^{6}A circRNAs during SAP. In the future, we will explore them and investigate these m^{6}A circRNAs in SAP patients.

REFERENCES

1 Lee PJ, Papachristou GI. New insights into acute pancreatitis. *Nature reviews Gastroenterology & hepatology* 2019; 16(8): 479-496 [PMID: 31138897 DOI: 10.1038/s41575-019-0158-2]

2 Trikudanathan G, Wolbrink DRJ, van Santvoort HC, Mallery S, Freeman M, Besselink MG. Current Concepts in Severe Acute and Necrotizing Pancreatitis: An Evidence-Based Approach. *Gastroenterology* 2019; 156(7): 1994-2007 e1993 [PMID: 30776347 DOI: 10.1053/j.gastro.2019.01.269]

3 Garg PK, Singh VP. Organ Failure Due to Systemic Injury in Acute Pancreatitis. *Gastroenterology* 2019; 156(7): 2008-2023 [PMID: 30768987]

4 Hsu MT, Coca-Prados M. Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells. *Nature* 1979; 280(5720): 339-340 [PMID: 460409 DOI: 10.1038/280339a0]

5 Meng X, Li X, Zhang P, Wang J, Zhou Y, Chen M. Circular RNA: an emerging key player in RNA world. *Brief Bioinform* 2017; 18(4): 547-557 [PMID:

27255916 DOI: 10.1093/bib/bbw045]

6 Panda AC, Grammatikakis I, Munk R, Gorospe M, Abdelmohsen K. Emerging roles and context of circular RNAs. *Wiley Interdiscip Rev RNA* 2017; 8(2) [PMID: 27612318]

7 Barrett SP, Salzman J. Circular RNAs: analysis, expression and potential functions. *Development* 2016; 143(11): 1838-1847 [PMID: 27246710]

8 Aufiero S, Reckman YJ, Pinto YM, Creemers EE. Circular RNAs open a new chapter in cardiovascular biology. *Nat Rev Cardiol* 2019; 16(8): 503-514 [PMID: 30952956 DOI: 10.1038/s41569-019-0185-2]

9 Yang Y, Ren J, Huang Q, Wu J, Yuan X, Jiang W, Wen Y, Tang L, Sun H. CircRNA Expression Profiles and the Potential Role of CircZFP644 in Mice With Severe Acute Pancreatitis via Sponging miR-21-3p. *Front Genet* 2020; 11: 206 [PMID: 32226441]

10 Meng S, Zhou H, Feng Z, Xu Z, Tang Y, Li P, Wu M. CircRNA: functions and properties of a novel potential biomarker for cancer. *Mol Cancer* 2017; 16(1): 94 [PMID: 28535767]

11 Dube U, Del-Aguila JL, Li Z, Budde JP, Jiang S, Hsu S, Ibanez L, Fernandez MV, Farias F, Norton J, Gentsch J, Wang F, Salloway S, Masters CL, Lee JH, Graff-Radford NR, Chhatwal JP, Bateman RJ, Morris JC, Karch CM, Harari O, Cruchaga C. An atlas of cortical circular RNA expression in Alzheimer disease brains demonstrates clinical and pathological associations. *Nat Neurosci* 2019; 22(11): 1903-1912 [PMID: 31591557]

12 Chen X, Yang T, Wang W, Xi W, Zhang T, Li Q, Yang A, Wang T. Circular RNAs in immune responses and immune diseases. *Theranostics* 2019; 9(2): 588-607 [PMID: 30809295]

13 Shulman Z, Stern-Ginossar N. The RNA modification N(6)-methyladenosine as a novel regulator of the immune system. *Nature immunology* 2020; 21(5): 501-512 [PMID: 32284591 DOI: 10.1038/s41590-020-0650-4]

14 Zhou C, Molinie B, Daneshvar K, Pondick JV, Wang J, Van Wittenberghe

N, Xing Y, Giallourakis CC, Mullen AC. Genome-Wide Maps of m6A circRNAs Identify Widespread and Cell-Type-Specific Methylation Patterns that Are Distinct from mRNAs. *Cell reports* 2017; 20(9): 2262-2276 [PMID: 28854373]

15 Zhang L, Hou C, Chen C, Guo Y, Yuan W, Yin D, Liu J, Sun Z. The role of N(6)-methyladenosine (m(6)A) modification in the regulation of circRNAs. *Mol Cancer* 2020; 19(1): 105 [PMID: 32522202]

16 Chen RX, Chen X, Xia LP, Zhang JX, Pan ZZ, Ma XD, Han K, Chen JW, Judde JG, Deas O, Wang F, Ma NF, Guan X, Yun JP, Wang FW, Xu RH, Dan X. N(6)-methyladenosine modification of circNSUN2 facilitates cytoplasmic export and stabilizes HMGA2 to promote colorectal liver metastasis. *Nature communications* 2019; 10(1): 4695 [PMID: 31619685 PMCID: PMC6795808 DOI: 10.1038/s41467-019-12651-2]

17 Schmidt J, Rattner DW, Lewandrowski K, Compton CC, Mandavilli U, Knoefel WT, Warshaw AL. A better model of acute pancreatitis for evaluating therapy. *Ann Surg* 1992; 215(1): 44-56 [PMID: 1731649]

18 Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000; 25(1): 25-29 [PMID: 10802651]

19 Thomson DW, Dinger ME. Endogenous microRNA sponges: evidence and controversy. *Nat Rev Genet* 2016; 17(5): 272-283 [PMID: 27040487 DOI: 10.1038/nrg.2016.20]

20 Crockett SD, Wani S, Gardner TB, Falck-Ytter Y, Barkun AN. American Gastroenterological Association Institute Guideline on Initial Management of Acute Pancreatitis. *Gastroenterology* 2018; 154(4): 1096-1101 [PMID: 29409760 DOI: 10.1053/j.gastro.2018.01.032]

21 Qu S, Zhong Y, Shang R, Zhang X, Song W, Kjems J, Li H. The emerging landscape of circular RNA in life processes. *RNA Biol* 2017; 14(8): 992-999

[PMID: 27617908]

22 Panda AC. Circular RNAs Act as miRNA Sponges. *Advances in experimental medicine and biology* 2018; 1087: 67-79 [PMID: 30259358 DOI: 10.1007/978-981-13-1426-1_6]

23 Wang B, Wu J, Huang Q, Yuan X, Yang Y, Jiang W, Wen Y, Tang L, Sun H. Comprehensive Analysis of Differentially Expressed lncRNA, circRNA and mRNA and Their ceRNA Networks in Mice With Severe Acute Pancreatitis. *Front Genet* 2021; 12: 625846 [PMID: 33584827]

24 Di Timoteo G, Dattilo D, Centrón-Broco A, Colantoni A, Guarnacci M, Rossi F, Incarnato D, Oliviero S, Fatica A, Morlando M, Bozzoni I. Modulation of circRNA Metabolism by m(6)A Modification. *Cell reports* 2020; 31(6): 107641 [PMID: 32402287 DOI: 10.1016/j.celrep.2020.107641]

25 Yang Y, Fan X, Mao M, Song X, Wu P, Zhang Y, Jin Y, Yang Y, Chen LL, Wang Y, Wong CC, Xiao X, Wang Z. Extensive translation of circular RNAs driven by N(6)-methyladenosine. *Cell research* 2017; 27(5): 626-641 [PMID: 28281539]

26 Park OH, Ha H, Lee Y, Boo SH, Kwon DH, Song HK, Kim YK. Endoribonucleolytic Cleavage of m(6)A-Containing RNAs by RNase P/MRP Complex. *Mol Cell* 2019; 74(3): 494-507 e498 [PMID: 30930054 DOI: 10.1016/j.molcel.2019.02.034]

27 Chen YG, Chen R, Ahmad S, Verma R, Kasturi SP, Amaya L, Broughton JP, Kim J, Cadena C, Pulendran B, Hur S, Chang HY. N6-Methyladenosine Modification Controls Circular RNA Immunity. *Mol Cell* 2019; 76(1): 96-109 e109 [PMID: 31474572]

28 Tang C, Xie Y, Yu T, Liu N, Wang Z, Woolsey RJ, Tang Y, Zhang X, Qin W, Zhang Y, Song G, Zheng W, Wang J, Chen W, Wei X, Xie Z, Klukovich R, Zheng H, Quilici DR, Yan W. m(6)A-dependent biogenesis of circular RNAs in male germ cells. *Cell research* 2020; 30(3): 211-228 [PMID: 32047269]

29 Antonucci L, Fagman JB, Kim JY, Todoric J, Gukovsky I, Mackey M, Ellisman MH, Karin M. Basal autophagy maintains pancreatic acinar cell

homeostasis and protein synthesis and prevents ER stress. *Proceedings of the National Academy of Sciences of the United States of America* 2015; 112(45): E6166-6174 [PMID: 26512112]

30 Gukovskaya AS, Gukovsky I, Algül H, Habtezion A. Autophagy, Inflammation, and Immune Dysfunction in the Pathogenesis of Pancreatitis. *Gastroenterology* 2017; 153(5): 1212-1226 [PMID: 28918190]

31 Mareninova OA, Hermann K, French SW, O'Konski MS, Pandol SJ, Webster P, Erickson AH, Katunuma N, Gorelick FS, Gukovsky I, Gukovskaya AS. Impaired autophagic flux mediates acinar cell vacuole formation and trypsinogen activation in rodent models of acute pancreatitis. *The Journal of clinical investigation* 2009; 119(11): 3340-3355 [PMID: 19805911]

32 Zhu ZM, Huo FC, Pei DS. Function and evolution of RNA N6-methyladenosine modification. *Int J Biol Sci* 2020; 16(11): 1929-1940 [PMID: 32398960]

33 Su H, Wang G, Wu L, Ma X, Ying K, Zhang R. Transcriptome-wide map of m(6)A circRNAs identified in a rat model of hypoxia mediated pulmonary hypertension. *BMC Genomics* 2020; 21(1): 39 [PMID: 31931709]

34 Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell* 2011; 146(3): 353-358 [PMID: 21802130]

35 Cheng Z, Yu C, Cui S, Wang H, Jin H, Wang C, Li B, Qin M, Yang C, He J, Zuo Q, Wang S, Liu J, Ye W, Lv Y, Zhao F, Yao M, Jiang L, Qin W. circTP63 functions as a ceRNA to promote lung squamous cell carcinoma progression by upregulating FOXM1. *Nature communications* 2019; 10(1): 3200 [PMID: 31324812]

36 Wang J, Li X, Liu Y, Peng C, Zhu H, Tu G, Yu X, Li Z. CircHIPK3 Promotes Pyroptosis in Acinar Cells Through Regulation of the miR-193a-5p/GSDMD Axis. *Front Med (Lausanne)* 2020; 7: 88 [PMID: 32318575]

37 Sun H, Tian J, Li J. MiR-92b-3p ameliorates inflammation and autophagy

by targeting TRAF3 and suppressing MKK3-p38 pathway in caerulein-induced AR42J cells. *International immunopharmacology* 2020; 88: 106691 [PMID: 32822908 DOI: 10.1016/j.intimp.2020.106691]

38 Guo X, Li K, Jiang W, Hu Y, Xiao W, Huang Y, Feng Y, Pan Q, Wan R. RNA demethylase ALKBH5 prevents pancreatic cancer progression by posttranscriptional activation of PER1 in an m6A-YTHDF2-dependent manner. *Mol Cancer* 2020; 19(1): 91 [PMID: 32429928]

39 Chen Y, Zhao Y, Chen J, Peng C, Zhang Y, Tong R, Cheng Q, Yang B, Feng X, Lu Y, Xie H, Zhou L, Wu J, Zheng S. ALKBH5 suppresses malignancy of hepatocellular carcinoma via m(6)A-guided epigenetic inhibition of LYPD1. *Mol Cancer* 2020; 19(1): 123 [PMID: 32772918]

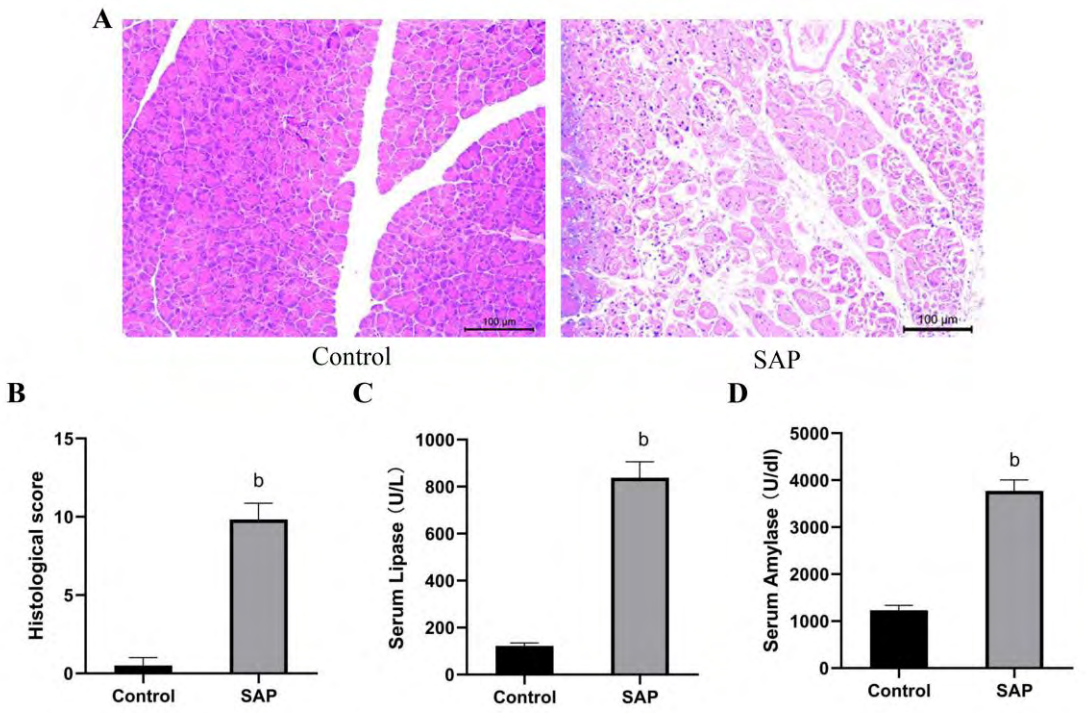

Figure 1 Evaluation of mouse model of severe acute pancreatitis (SAP). (A) Representative images of pancreatic tissues stained with hematoxylin from control (left) and SAP (right) groups (100×). (B) Histological score of pancreatic tissues in control and SAP groups. (C, D) Levels of serum lipase and amylase, respectively. $^{b}P < 0.01$ versus control group, n = 3 per group.

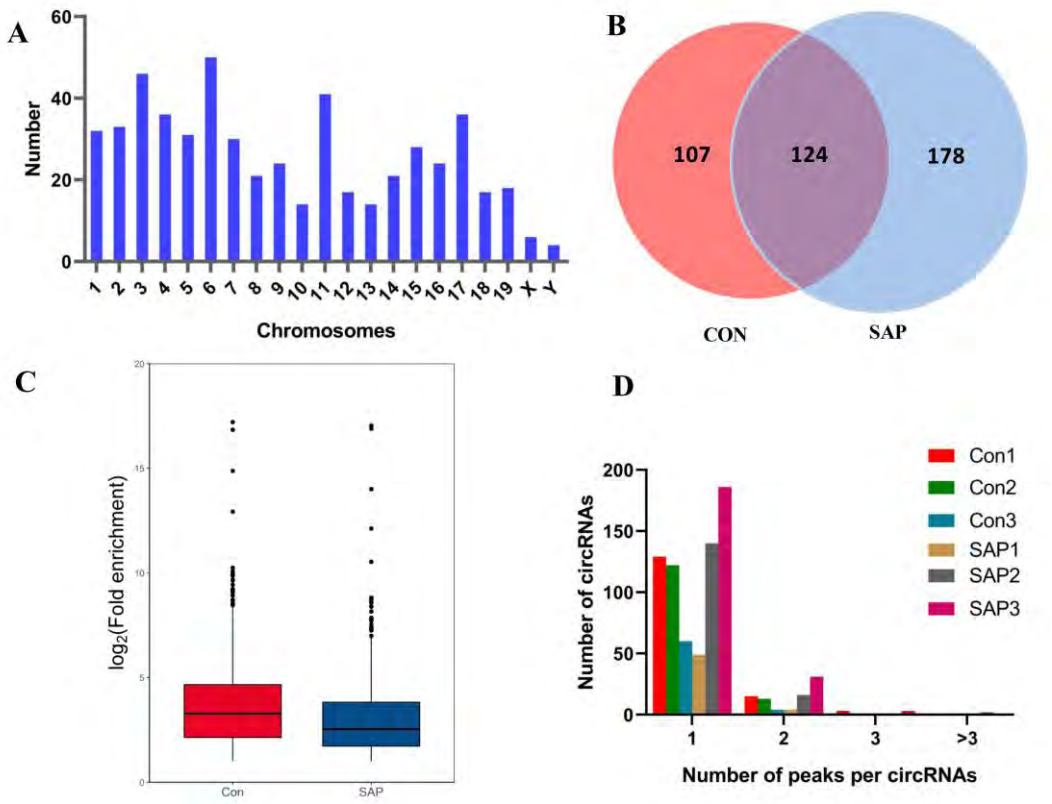

Figure 2 Overview of N^6-methyladenosine (m^{6}A) circular (circ)RNAs in severe acute pancreatitis (SAP). (A) Number of identified m^{6}A circRNAs according to distribution on chromosomes. (B) Venn diagram exhibiting number of common and specific m^{6}A circRNAs between control and SAP groups. (C) Box plot showing level of m^{6}A peaks enrichment in circRNAs in control and SAP groups. (D) Number of circRNAs containing variant numbers of m^{6}A peaks.

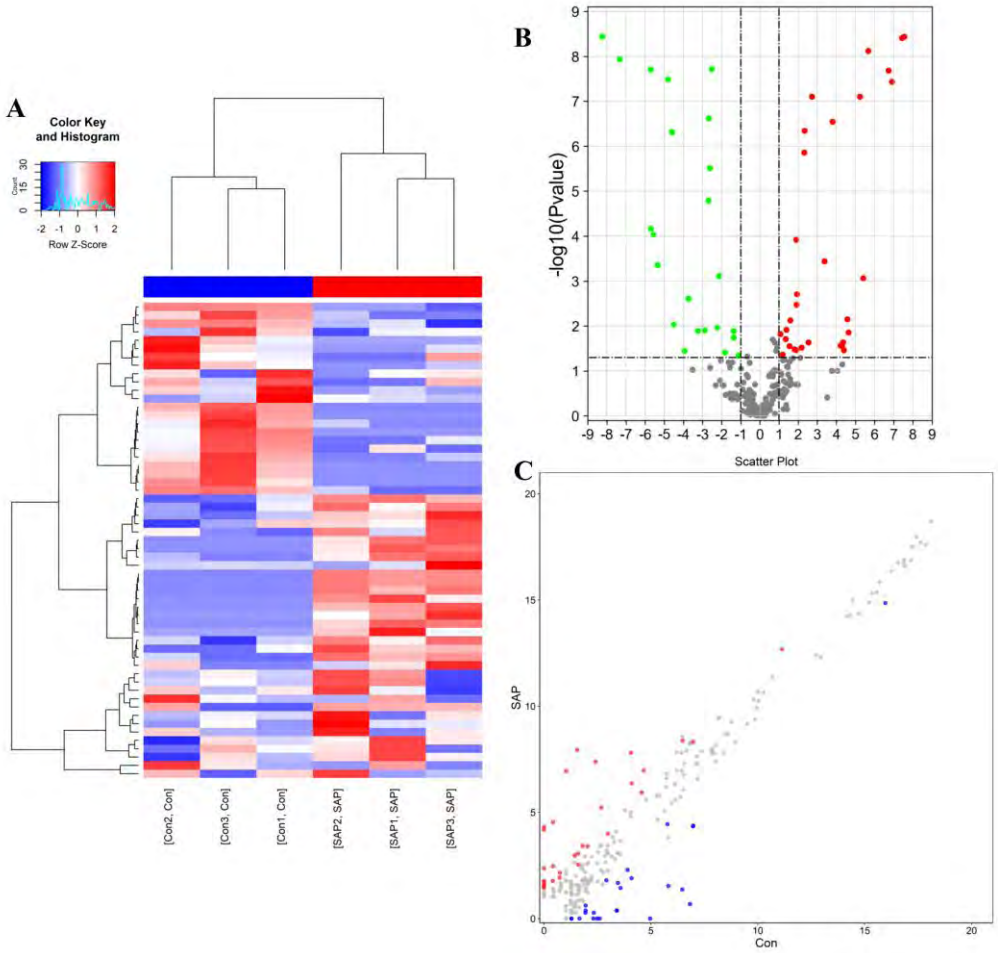

Figure 3 Differential N⁶-methyladenosine (m⁶A) modification of circular (circ)RNAs in severe acute pancreatitis (SAP). (A) Hierarchical clustering graph exhibiting differential m⁶A modification of circRNAs in control and SAP groups. Higher expression is presented in red and lower expression in blue. (B, C) Volcano and scatter plot showing the circRNAs with significant differentially expressed (DE) m⁶A peaks.

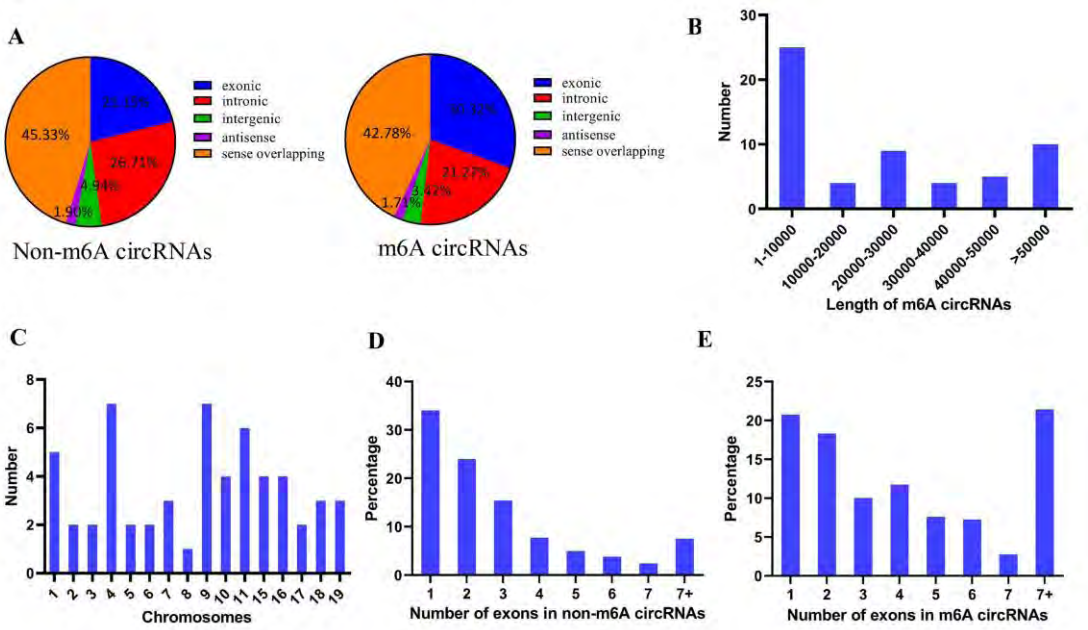

Figure 4 Distribution of N^6-methyladenosine (m^{6}A) sites in severe acute pancreatitis (SAP) and control groups. (A) Distribution of genomic origins of non-m^{6}A circular (circ)RNAs (left) and m^{6}A circRNAs (right). (B) Number of circRNAs with differentially expressed m^{6}A peaks based on the distribution of length. (C) Chromosomal distribution of all differential m^{6}A sites within circRNAs. (D, E) Distribution of non-m^{6}A and m^{6}A circRNAs based on the number of exons in each circRNA.

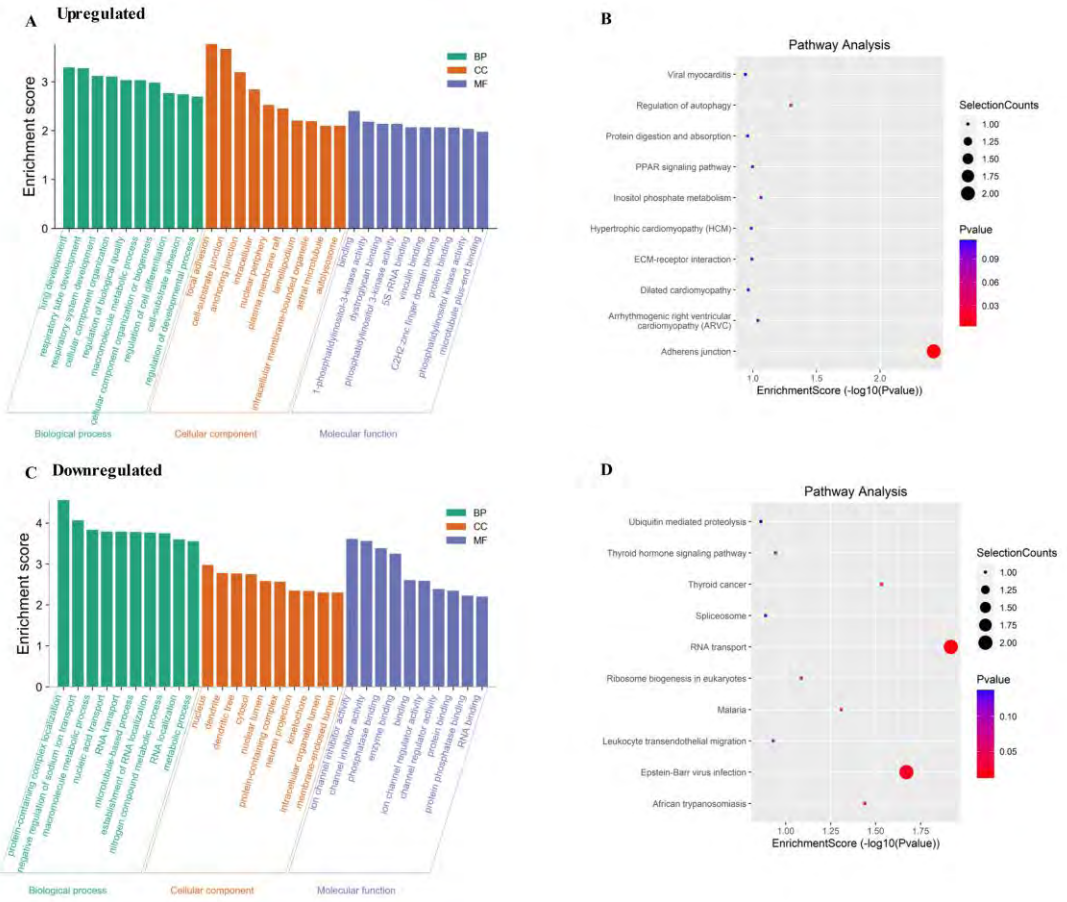

Figure 5 Functional analysis of circular (circ)RNAs with differentially expressed N^6-methyladenosine (m^{6}A) peaks though Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. GO (A) and KEGG (B) analysis of circular (circ)RNAs with upregulated m^{6}A peaks. GO (C) and KEGG (D) analysis of circRNAs with downregulated m^{6}A peaks. GO analysis include biological process (BP) analysis, cellular component (CC) analysis, and molecular function (MF) analysis.

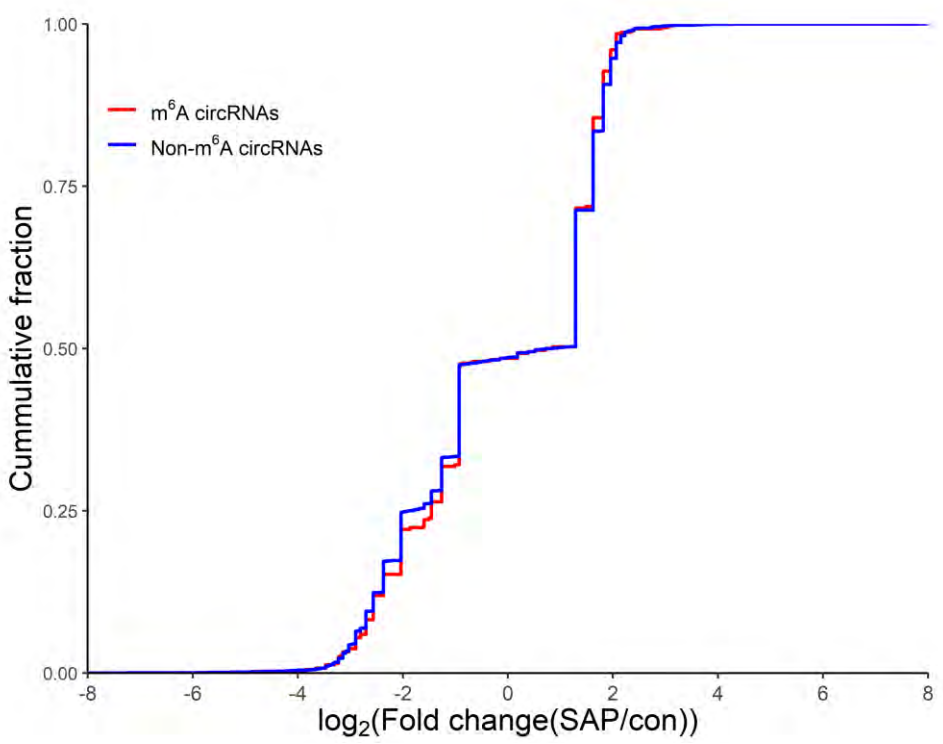

Figure 6 Relationship between N⁶-methyladenosine (m⁶A) level and expression of circular (circ)RNAs in severe acute pancreatitis (SAP). Cumulative distribution of circRNAs expression between control and SAP groups for m⁶A circRNAs (red) and non-m⁶A circRNAs (blue).

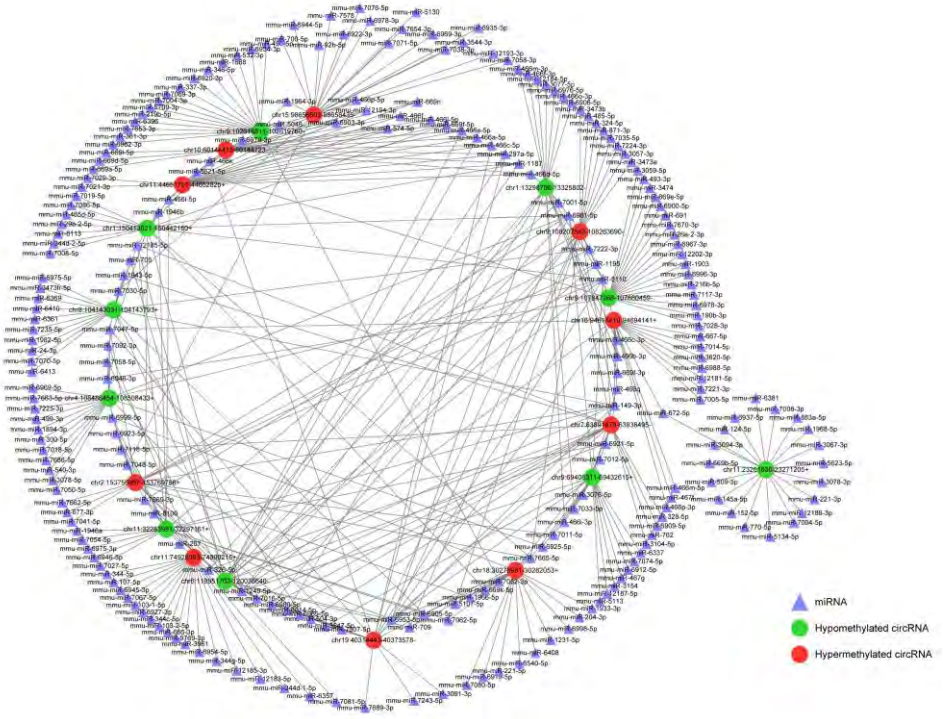

Figure 7 Construction of N⁶-methyladenosine (m⁶A) circular (circ)RNA–miRNA networks in severe acute pancreatitis (SAP). A map showing the

interaction networks of the top 10 upregulated and top 10 downregulated circRNAs according to the level of m^{6}A, and their around 20 target miRNAs with the most stable binding in SAP. Green circles represent hypomethylated circRNAs, red circles represent hypermethylated circRNAs and triangles represent miRNAs, compared with control group.

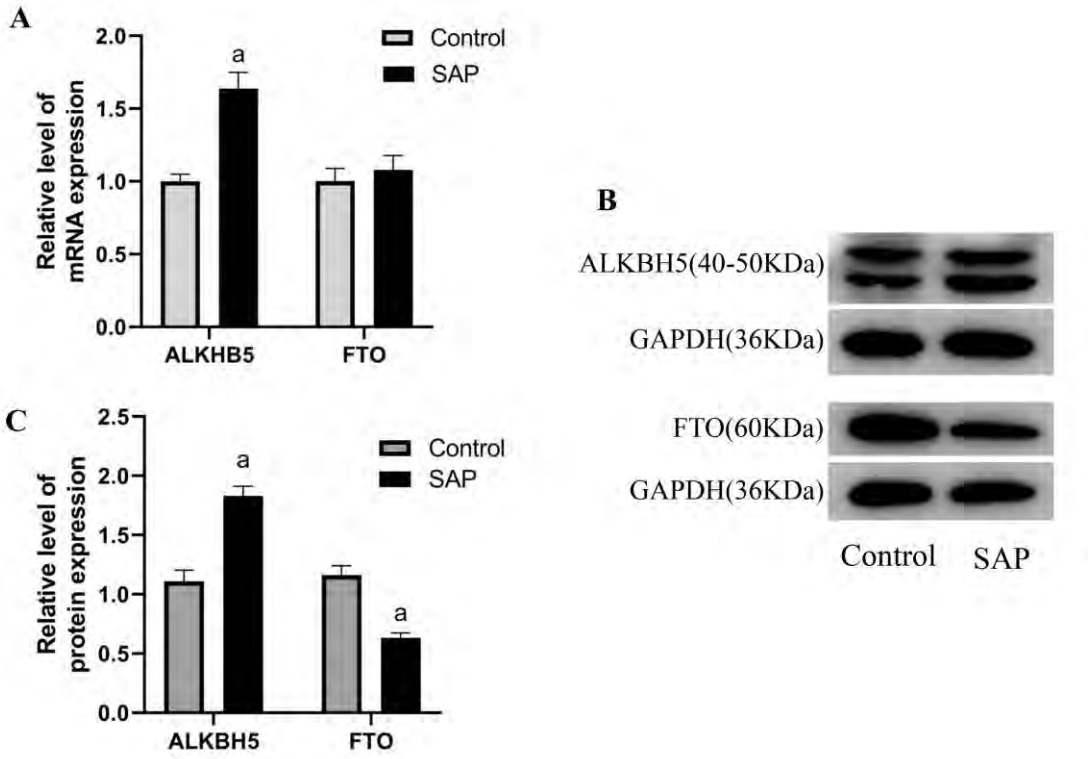

Figure 8 Expression of demethyltransferase in severe acute pancreatitis (SAP). (A) Relative mRNA levels of ALKBH5 and FTO (normalized by the quantity of GAPDH) in each group. (B) Representative images of western blot detected with alkylation repair homolog 5 (ALKBH5), FTO, and GAPDH antibodies in control and SAP groups. (C) Relative protein levels of ALKBH5 and FTO (measured as the ratio of ALKBH5, FTO to GAPDH by band density) in each group. Data are representative of at least three independent experiments. $^aP < 0.05$ versus control group.

Table 1 Top 20 Differently Expressed m^{6}A Peaks compared with control group

	PeakStart	PeakEnd	circRNA	Regulation	Foldchange	P value
chr15	98658229	98658320	chr15:98656602-98658435-	up	187.2	3.67392E-09
chr11	74929241	74929540	chr11:74928993-74990215+	up	172.8	3.92116E-09
chr9	108248361	108248660	chr9:108207543-108263690-	up	120.034482	3.70238E-08
chr2	153763381	153763760	chr2:153756037-153769786+	up	106.330434	2.09239E-08
chr18	30281961	30282053	chr18:30276981-30282053+	up	50.9545454	7.61781E-09
chr19	40346381	40346760	chr19:40314443-40373578-	up	42.4	0.026929988
chr16	94641481	94641740	chr16:94611419-94694141+	up	37.6772727	7.9549E-08
chr10	60144412	60144720	chr10:60144413-60144723-	up	24.9	0.014047401
chr11	44652781	44652825	chr11:44651797-44652825+	up	23.9	0.007108941
chr7	63895821	63896100	chr7:63891679-63938495-	up	21.1	0.034908475
chr9	107852341	107852720	chr9:107847268-107860459-	down	302.6	3.63198E-09
chr8	104143561	104143760	chr8:104143031-104143793+	down	160.728571	1.15908E-08
chr1	150426881	150427260	chr1:150413021-150442180+	down	52.095238	1.98141E-08
chr1	13312381	13312680	chr1:13298706-13325802-	down	51.7	6.86061E-05
chr6	119970581	119970800	chr6:119951703-120038640-	down	47.4	9.37286E-05
chr11	23271132	23271205	chr11:23261835-23271205+	down	40.6	0.000442849
chr4	108499346	108499398	chr4:108486454-108508433+	down	27.79	3.27262E-08
chr9	102619691	102619760	chr9:102618811-102619760-	down	24.25	4.83147E-07
chr11	32296401	32296600	chr11:32283981-32297161+	down	24.1	4.96449E-07
chr9	69414201	69414580	chr9:69408311-69432615+	down	22.6	0.00943617

ROUND 2

Dear Editor,

We would like to thank all the Reviewers for their critical and insightful comments on our manuscript entitled "**Genome-wide map of N6-methyladenosine circular RNAs identified in mice model of severe acute pancreatitis**" by Jun Wu and colleagues, which helped us to improve the quality of the manuscript.

We have now made minor revisions to the manuscript and addressed all the concerns raised. Please find the following point-by-point responses to the Reviewers' critiques and comments below.

Thank you very much for considering our manuscript for publication in *World Journal of Gastroenterology*.

Corresponding author:
Lijun Tang, Professor.
The General Hospital of Western Theater Command (Chengdu Military General Hospital), Sichuan Province, 610083, China. Phone: 0086-028-86571858; Fax: 0086-028-86571251. E-mail: tanglj2016@163.com

Responses to the reviewers:

Reviewer #1:
The Authors have adequately addressed the Reviewers' comments; the appropriate corrections were made, and the revised manuscript is significantly improved. The clarifications that are provided contribute to the coherence and quality of the study and all Figures are useful and comprehensible. Overall, the paper is well-written and contributes to the existing knowledge in its field. I have only one additional concern: some information that the Authors provide in their response to the Reviewers is not incorporated to the text; for example, the Table that shows the results of the

conservation analysis and the paragraph that describes the rRNA removal efficacy examination need to be included in the manuscript.

Response: Thank you for your critical and insightful comments on our manuscript, which improved the quality of our manuscript and increased the significance of our study. We were pleased to read your conclusion that "Overall, the paper is well-written and contributes to the existing knowledge in its field". We completely agree with your viewpoint. We have added the results of the conservation analysis and the description regarding the rRNA removal efficacy examination in the manuscript. The corresponding revisions are highlighted in red in the manuscript (Page8, Line2-3; Page9, Line15-20; Page11, Line23-25; Page14, Line17-24; Page18, Line17-18).