The Alteration Profiles of m^6A-Tagged circRNAs in the Peri-Infarct Cortex After Cerebral Ischemia in Mice

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INTRODUCTION

Cerebral infarction causes transcriptional and post-transcriptional alterations including RNA expression levels and epigenetic modifications, resulting in a series of complicated post-stroke pathophysiological processes, such as glutamate excitotoxicity, oxidative stress, apoptosis, nerve regeneration, and angiogenesis (Mehta et al., 2007).
Studies of RNA modification are known as epigenetic transcriptomics. Recent studies have indicated that epigenetic changes occur during the pathophysiological course of diverse diseases as regulators of RNAs (Niu et al., 2013). At present, more than 170 classes of RNA modifications have been identified, among which N6-methyladenosine (m6A) is the most abundant and common modification in Eukaryotes, particularly in the mammalian brains (Dominissini et al., 2012). The m6A modification refers to the methylation of the sixth nitrogen atom in adenosine, which is catalyzed by methyltransferase complexes, such as methyltransferase-like (METTL) 3 and 14 (METTL3 and METTL14), and Wilms’ tumor 1-associated protein (WTAP); eliminated by demethylases, such as fat mass and obesity-associated protein (FTO) and alkylation repair homolog protein 5 (ALKBH5); and recognized by m6A readers, such as YTH domain family (YTHDF) 1, 2, and 3 (YTHDF1, YTHDF2, and YTHDF3) (Shi et al., 2019). Thus, the m6A modification is a dynamic and reversible process. The majority of m6A modifications occur near the 3′UTR and the mRNA stop codon, and regulate various molecular functions in mRNAs related to transcription, alternative splicing, nuclear export, translation, stability, and degradation (Niu et al., 2013). It has been researched that m6A-related enzymes play a critical role in ischemic diseases. FTO has been reported to promote angiogenesis and reduce myocardial fibrosis after myocardial ischemia, however, in the brain, it is predominantly expressed in neurons, whereas downregulation of FTO and ALKBH5 after stroke can induce secondary brain injury by destroying neurons (Chokkalla et al., 2019; Mathiyalagan et al., 2019; Xu et al., 2020). METTL3, METTL14, and YTHDF1 have been shown to regulate axon regeneration, neuronal apoptosis, neurogenesis, and inflammation after cerebral ischemia (Weng et al., 2018; Si et al., 2020; Zhang et al., 2020), which indicates that the m6A modification may be involved in a series of post-stroke molecular events.

Non-coding RNAs, especially circRNAs, show tissue specificity during development and are highly expressed in the brain (Rybak-Wolf et al., 2015). Studies have shown that circRNAs are specifically enriched in the subcellular compartments of neurons, including dendrites, axons, and

| TABLE 1 | The primer sequence of the four differentially m6A-modified circRNAs and m6A-related enzymes. |

| Bidirectional primer sequence                  |
|-----------------------------------------------|
| mnu_circRNA_27268 F:5′ CTTTGTCAGCAAGGAGCATG3′ R:5′ TTTGACAACCGACGTGACCTAT3′ |
| mnu_circRNA_29864 F:5′ GACCTCCCGGGCTGCTG3′ R:5′ GTGGAAGGCTATTGACAT3′ |
| mnu_circRNA_32905 F:5′ CATGGTCTAAAGATGCTTGT3′ R:5′ GTGACCCAGAACCACAAAG3′ |
| mnu_circRNA_20673 F:5′ AACAAAGAAGTTCAGACGTGAGA3′ R:5′ CGTTTTTCTGAGGATGGTGTTAC3′ |
| METTL3 F:5′ GGGCACTTGGATTTAAGGAACC3′ R:5′ CTTAGGGCCGCTAGAGGTAGG3′ |
| METTL14 F:5′ GAGCTGAGAGTGCGGAAGC3′ R:5′ GCAGATGTATCATAGGAAGCCC3′ |
| FTO F:5′ TCCATGCTGGATGACCTCAATG3′ R:5′ GCCAACTGACAGCGTTCTAAG3′ |
| ALKBH5 F:5′ GCCACCGGCGAGTTAAC3′ R:5′ GCCAACGTGACGTCCTAAG3′ |
| YTHDF1 F:5′ AACCAGGGCCATAGGAATACC3′ R:5′ TCCACTTTTCCCCCATGAG3′ |

**FIGURE 1 |** Histopathology of cerebral infarction. (A) TTC staining displayed the infarcted areas in white, and the normal areas in red, (n = 3). (B) H&E staining presented the destruction of morphology in the infarct lateral cortex compared with the Contralateral cortex (n = 3).
synapses (Rybak-Wolf et al., 2015; You et al., 2015; Shigeoka et al., 2016). Therefore, tracking changes in circRNAs and their m^6^A modification levels may provide new insights into the pathogenesis of cerebral ischemia from the perspective of epigenetics, thereby improving intervention and prognosis. CircRNAs can regulate secondary brain injury and repair by binding with proteins and acting as "sponges" for miRNA (Wu et al., 2019; Yang et al., 2020). However, the upstream regulatory mechanism for circRNAs remains unknown. Recently, a study focused on the differences in m^6^A modifications of mRNAs lncRNAs after transient cerebral ischemia (Chokkalla et al., 2019), but the m^6^A modification of circRNA is not yet known. Therefore, in this study, we screened the entire genome for m^6^A-modified circRNAs around the site of cerebral infarction to explore the potential significance of m^6^A-modified circRNAs in stroke.

**METHODS AND MATERIALS**

**Cerebral Infarction in Mice**

All experimental procedures were approved by the Institutional Animal Use Committee of the Shenzhen Hospital of Southern Medical University. Animals were raised in a SPF room with free access to food and water, under a controlled temperature (25 ± 2°C) and a 12/12 h day/night cycle. The surgery was performed in accordance with the guidelines for the care and use of laboratory animals. C57BL/6J mice weighing 23–26 g were randomized to three groups: the 3d after MCAO, 7d after MCAO, and sham groups. After anesthetizing the mice with isoflurane, we made a 1 cm incision between the left ear and eye, dissected the temporal muscle, and exposed the left side of the skull under an operating microscope. Next, a small hole was drilled, and a distal middle cerebral artery occlusion (MCAO) was performed with a bipolar electrosurgical knife, leading to a permanent infarction of the local cerebral cortex. And then the bilateral common carotid arteries were immediately occluded for 7 minutes. The sham group underwent the same surgical procedure without the MCAO. During surgery and recovery, the body temperature of the mice were maintained at 37°C.

**TTC Staining**

Three mice were used for histological staining at 24 h after MCAO. Briefly, the mice were killed by cervical dislocation, and brain tissues were isolated and frozen at −20°C in refrigerator for 5 mins. Coronal sections were sliced into 1 mm and maintained at 37°C in 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution (Sigma Aldrich, St. Louis, MO, USA) for 30 min. TTC staining can effectively detect the location of the primary cerebral infarction after MCAO.

**H&E Staining**

One week after cerebral infarction operation, the brain tissue was collected and fixed with 4% paraformaldehyde for 1 week, and then embedded in paraffin. Following alcohol dehydration and xylene dewaxing, coronal sections were stained with hematoxylin.

**FIGURE 2** | Expression of m^6^A related enzymes between MCAO and sham groups. (A) The mRNA expression level of m^6^A writers (METTL3, METTL14, and WTAP), m^6^A erasers (FTO, ALKBH5), and m^6^A readers (YTHDF1, YTHDF3) were changed at 3d or 7d after MCAO compared with the sham, (n = 6/group). (B, C) The protein YTHDF3 were significantly decreased in 3d group, while increased in 7d group, (n = 6/group). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns, not significant.
for 3 min, washed, and then eosin for 2 min. Finally, sections were sealed with neutral gum after being dehydrated.

**m^6^A Immunoprecipitation (MeRIP)**

Total RNA (1–3 μg) and m^6^A spike-in control mixture were added to immunoprecipitation (IP) buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 0.1% NP40; and 40 U/μL RNase inhibitor), incubated with 2 μg anti-m^6^A rabbit polyclonal antibody (Synaptic Systems, 202003) at 4°C for 2 h, washed, resuspended in 20 μL Dynabeads™ M-280 sheep anti-rabbit IgG suspension (Invitrogen, 11203D), and incubated at 4°C for 2 h. The beads were then washed three times with 500 μL of IP buffer and twice with 500 μL of wash buffer [50 mmol/L Tris-HCl, pH 7.4; 50 mmol/L NaCl; 0.1% NP 40; and 40 U/μL RNase inhibitor (Enzymatics, Y9240L)]. The enriched RNA was eluted with 200 μL elution buffer (10 mmol/L Tris-HCl, pH 7.4; 1 mmol/L EDTA; 0.05% SDS; and 40 U proteinase K) and extracted using acid phenol-chloroform and ethanol precipitation. MeRIP assays were performed in three replicates in each group (n = 3).

**Microarray Hybridization of Methylated CircRNAs**

The immunoprecipitate was digested with RNase R to remove linear RNA, amplified, and labeled with super RNA Labeling Kit (Cy3 [for "sup", supernatant] and Cy5 [for "IP"] in accordance with the manufacturer’s instructions, and then purified using the RNeasy Mini Kit. After fragmentation, the circRNAs were hybridized to an m^6^A-circRNA epi-transcriptomic microarray slide (Arraystar) and incubated at 65°C for 17 h in a hybridization oven (Agilent, Santa Clara, CA, USA). The hybridized arrays were washed, fixed, and scanned using an Agilent Scanner G2505C.

**Analysis of Microarray Data**

Acquired array images were analyzed using Agilent Feature Extraction software (version 11.0.1.1). Raw intensities of IP (immunoprecipitated, Cy5-labeled) were normalized at a criterion of log2-scaled intensities in spike-in RNA. The percentage of the IP (Cy5-labeled) normalized intensities was considered as m^6^A methylation levels. Differentially m^6^A-modified circRNAs were identified between the two comparison groups by filtering with the fold change and statistical significance (P-value) thresholds. Expression of circRNAs were calculated by the normalized signal value of "IP" plus "sup", the differentially expressed circRNAs were screened at the criterion of with fold change >1.5 and P < 0.05.
Reverse Transcription Quantitative Polymerase Chain Reaction

After collecting samples at 3d and 7d after MCAO, 1 µg of total RNA was reverse transcribed to cDNA. RT-qPCR was performed to determine the mRNA levels of m^6^A-related enzymes (METTL3, METTL14, FTO, WTAP, ALKBH5, YTHDF1, and YTHDF3) by the Applied Biosystems 7300/7500 Real-Time PCR System (Foster City, CA, USA). Additionally, to further confirm the results from the m^6^A immunoprecipitation and microarray, we randomly selected four differentially m^6^A-modified circRNAs (mmu_circRNA_27268, mmu_circRNA_20673, mmu_circRNA_32905, and mmu_circRNA_29864) in samples of 3d after MCAO and detected their level by MeRIP-qPCR analysis. In brief, equal quantity of immunoprecipitated RNA samples were reverse-transcribed to cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, 18080-044), and amplified with gene-specific primers for the circRNAs (Table 1). PCR assays were performed in six replicates in each group (n = 6). All results were analyzed by 2^ΔΔCT^ method.

Western Blots Analysis

Around 50 µg of protein samples extract was resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after denaturation and transferred to PVDF membranes. The membranes were blocked in 5% non-fat milk in tris-buffered saline solution (TBS) for 1.5 h at room temperature and then probed with the primary antibodies: rabbit anti-ALKBH5 (Abcam, ab195377, 1:1,000) and rabbit anti-YTHDF3 (Abcam, ab220161, 1:1,000). After overnight incubation at 4°C, the membranes were washed three times with TBS-T and incubated for 1 h at room temperature with an appropriate secondary antibody: horseradish peroxidase-labeled anti-rabbit IgG (1:5,000). The protein bands were visualized using the enhanced chemiluminescence method, and the intensity of band was quantified using Image J software (Version 1.51j8, NIH, Bethesda, MD, USA).

Function and Pathways Analyses

The functions of differentially m^6^A-modified circRNAs' parent genes and three verified ones' target genes were analyzed by Gene Ontology (GO) analysis (http://www.geneontology.org), and their significant pathways were explored by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (http://www.genome.jp/kegg/) using the DAVID bioinformatics database (https://david.ncifcrf.gov).

Prediction of Network of CircRNA-miRNA-mRNA

Three verified m^6^A-circRNAs were used to predicted miRNAs by Arraystar’s homemade software based on TargetScan and miRanda, and top ten possible miRNAs were screened. Additionally, miRNAs of these ten miRNAs were predicted by miRanda. The network of circRNA-miRNA-mRNA was visualized by Cytoscape.

Statistical Analysis

Our results were analyzed using Statistical Program for Social Sciences (SPSS) software (version 22.0; SPSS, Chicago, IL, USA) and are presented as means ± standard deviations. The student t test was used to detect the significant differences between two groups, one-way ANOVA was used for comparisons between multiple groups, and P < 0.05 was taken as statistically significant.

### TABLE 2 | The top 10 hyper and hypo-methylated circRNAs between 3d vs. sham.

| CircRNA     | m^6^A | Fold change | P value   | Chrom | Type     | Gene     |
|-------------|-------|-------------|-----------|-------|----------|----------|
| mmu_circRNA_27268 | hyper | 2.84197 | 0.0019873 | chr14 | exonic   | Appl1    |
| mmu_circRNA_009696 | hyper | 2.61714 | 0.0065627 | chr19 | intronic | Malat1   |
| mmu_circRNA_017958 | hyper | 2.50106 | 0.0443897 | chr10 | exonic   | Arks1b   |
| mmu_circRNA_000807 | hyper | 2.14106 | 0.0320552 | chr17 | sense overlapping | Rn45s |
| mmu_circRNA_20066 | hyper | 2.12641 | 0.0081174 | chr1 | sense overlapping | DO715306 |
| mmu_circRNA_005252 | hyper | 2.01853 | 0.019242 | chr16 | intronic | Cms1     |
| mmu_circRNA_19450 | hyper | 1.94602 | 0.0100234 | chr7 | sense overlapping | Calm3    |
| mmu_circRNA_43940 | hyper | 1.94234 | 0.0011256 | chr9 | exonic | Arhgap32 |
| mmu_circRNA_007113 | hyper | 1.94061 | 0.0265738 | chr1 | exonic | Rims1    |
| mmu_circRNA_20673 | hyper | 1.9342 | 0.0014401 | chr1 | exonic | Clasp1   |
| mmu_circRNA_24601 | hypo | 2.9504766 | 0.0015035 | chr12 | exonic | Itsn2    |
| mmu_circRNA_38065 | hypo | 2.5043721 | 0.0008175 | chr5 | exonic | Clap69   |
| mmu_circRNA_44763 | hypo | 2.4079717 | 0.0223753 | chr9 | exonic | Dopey1   |
| mmu_circRNA_38624 | hypo | 2.391517 | 0.0020564 | chr5 | exonic | Tbc1d19  |
| mmu_circRNA_38670 | hypo | 2.3893449 | 0.0128245 | chr4 | exonic | Khl2     |
| mmu_circRNA_45982 | hypo | 2.3683147 | 0.0247834 | chrX | exonic | Mtd1     |
| mmu_circRNA_45969 | hypo | 2.3645312 | 0.0431726 | chrX | exonic | Od1      |
| mmu_circRNA_39011 | hypo | 2.3522961 | 0.0412524 | chr5 | exonic | Bmp2k    |
| mmu_circRNA_27887 | hypo | 2.3463249 | 0.0091444 | chr14 | exonic | Mfr1     |
| mmu_circRNA_19997 | hypo | 2.3328161 | 0.0451034 | chr1 | exonic | Mra9     |
### TABLE 3 | The top 10 hyper and hypo-methylated circRNAs between 7d vs. sham.

| CircRNA               | m^A | Fold change | P value  | Chrom | Type   | Gene         |
|-----------------------|-----|-------------|----------|-------|--------|--------------|
| mmu_circRNA_23150     | hyper | 3.1009042   | 0.0259945 | chr11 | exonic | Psme4        |
| mmu_circRNA_38094     | hyper | 2.7648072   | 0.0337221 | chr5  | exonic | 9330182L06Rik|
| mmu_circRNA_22281     | hyper | 2.5990145   | 0.0182551 | chr10 | exonic | Creb33       |
| mmu_circRNA_25983     | hyper | 2.3819553   | 0.0065519 | chr13 | exonic | Larp4b       |
| mmu_circRNA_28128     | hyper | 2.2970656   | 0.0351191 | chr14 | exonic | Itgb1        |
| mmu_circRNA_28040     | hyper | 2.2407646   | 0.0009723 | chr14 | exonic | UggT2        |
| mmu_circRNA_23280     | hyper | 2.2379241   | 0.0446396 | chr11 | exonic | Cnot6        |
| mmu_circRNA_36627     | hyper | 2.2361166   | 0.0381938 | chr4  | exonic | Orc3         |
| mmu_circRNA_24182     | hyper | 2.1735471   | 0.0422457 | chr11 | exonic | Acty         |
| mmu_circRNA_38487     | hyper | 1.916812    | 0.0307721 | chr5  | exonic | Trmt44       |
| mmu_circRNA_32468     | hypo  | 3.5205166   | 0.03799   | chr19 | exonic | Pcgf5        |
| mmu_circRNA_19150     | hypo  | 3.4371052   | 0.0134725 | chr15 | sense overlapping | Arid2 |
| mmu_circRNA_43936     | hypo  | 2.740639    | 0.0178457 | chr9  | exonic | Pdmd10       |
| mmu_circRNA_19402     | hypo  | 2.5589497   | 0.0254406 | chr5  | sense overlapping | Cdk8 |
| mmu_circRNA_45325     | hypo  | 2.2991652   | 0.0058466 | chrX  | exonic | Sylt5        |
| mmu_circRNA_27622     | hypo  | 2.2918234   | 0.0135477 | chr14 | exonic | Zdhc20       |
| mmu_circRNA_018245    | hypo  | 2.0415875   | 0.0340724 | chr13 | exonic | Nn           |
| mmu_circRNA_19824     | hypo  | 1.981272    | 0.0231978 | chr1  | exonic | AdgrB3       |
| mmu_circRNA_006216    | hypo  | 1.9072746   | 0.0167186 | chr12 | exonic | Dtnb         |
| mmu_circRNA_20158     | hypo  | 1.8690753   | 0.0429307 | chr1  | exonic | Fam126b      |

### TABLE 4 | The top 10 hyper and hypo-methylated circRNAs between 7d vs. 3d.

| CircRNA               | m^A | Fold change | P value  | Chrom | Type   | Gene         |
|-----------------------|-----|-------------|----------|-------|--------|--------------|
| mmu_circRNA_28863     | hyper | 4.0392625   | 0.0031755 | chr15 | exonic | Srebfl2      |
| mmu_circRNA_38904     | hyper | 3.3380276   | 0.0008196 | chr5  | exonic | Uba6         |
| mmu_circRNA_19765     | hyper | 3.0605701   | 0.0096643 | chr1  | exonic | Fam135a      |
| mmu_circRNA_45965     | hyper | 3.0318816   | 0.0066543 | chrX  | exonic | Gia2         |
| mmu_circRNA_33707     | hyper | 2.8852467   | 0.000408  | chr2  | exonic | Scr2a1       |
| mmu_circRNA_42640     | hyper | 2.8462651   | 0.0004994 | chr8  | exonic | Kat6a        |
| mmu_circRNA_30501     | hyper | 2.7141039   | 0.0023387 | chr17 | exonic | Ptdch4       |
| mmu_circRNA_19249     | hyper | 2.7073051   | 0.0215567 | chr2  | sense overlapping | Ola1 |
| mmu_circRNA_19991     | hyper | 2.6706516   | 0.0059689 | chr1  | exonic | Sic9a2       |
| mmu_circRNA_34924     | hyper | 2.67065159  | 0.00596879 | chr1  | exonic | Sic9a2       |
| mmu_circRNA_19150     | hypo  | 3.294896    | 2.653E-05 | chr15 | sense overlapping | Arid2 |
| mmu_circRNA_017958    | hypo  | 2.7671474   | 0.0296888 | chr10 | exonic | Anks1b       |
| mmu_circRNA_43775     | hypo  | 2.736182    | 0.0241969 | chr9  | exonic | Mami2        |
| mmu_circRNA_37703     | hypo  | 2.4422092   | 0.0010981 | chr4  | exonic | Ptp4a2       |
| mmu_circRNA_32488     | hypo  | 2.384711    | 0.0186258 | chr19 | exonic | Pcgf6        |
| mmu_circRNA_30324     | hypo  | 2.3677878   | 0.006817  | chr17 | exonic | Tsc2         |
| mmu_circRNA_43882     | hypo  | 2.3552045   | 0.0095023 | chr9  | sense overlapping | Bmpr |
| mmu_circRNA_28255     | hypo  | 2.3323474   | 0.0390009 | chr15 | exonic | Mtrm12       |
| mmu_circRNA_43940     | hypo  | 2.3177426   | 0.0009251 | chr9  | exonic | Arhgap32     |
| mmu_circRNA_19655     | hypo  | 2.2504896   | 1.558E-05 | chrX  | intronic | XLOC_027457  |
RESULTS

Establishment and Validation of the Cerebral Infarction Model

TTC and H&E staining were used to confirm the infarction region of the brain after MCAO in mice (Figures 1A,B). TTC staining showed that the infarcted brain tissue was white, and the non-infarct region was red. Additionally, with H&E staining, the infarct area was pale, and the number of neurons was decreased, whereas the opposite was observed in normal tissue.

The Transcriptional Levels of Methylation-Related Enzymes After Stroke in Mice

To explore the changes in m^6^A methylation on circRNA transcripts, we first determined transcriptional levels of m^6^A-related enzymes in the peri-infarct region of the cerebral cerebral cortex (Figure 2A). We observed that mRNA expression of all enzymes tested was significantly downregulated at 3 days after MCAO compared with the sham group. At 7 days after MCAO, the transcriptional levels of METTL3, METTL4, ALKBH5, and YTHDF3 were decreased, whereas increased in FTO significantly. Interestingly, compared with the 3d group, except for METTL3, METTL14, and ALKBH5, significant increases in transcription of other genes occurred in the 7d group.

ALKBH5 and YTHDF3 Showed Dynamic Alterations in Stroke

We further measured the translation level of m^6^A related enzymes in the area around cerebral cortex infarction (Figures 2B,C). We observed that ALKBH5 and YTHDF3 were significantly decreased at 3 and 7 days after MCAO compared with the sham group. Interestingly, ALKBH5 and YTHDF3 were increased at 7 days in comparison to 3 days after MCAO.

Cerebral Infarction Changed the Level of m^6^A Modification and Expression Profile in CircRNA

The mRNA expression of m^6^A-related enzymes in the peri-infarct cortex was altered, suggesting that cerebral ischemia might change m^6^A modification. We speculated that the methylation modification of circRNAs around cerebral infarction was changed. We performed m^6^A immunoprecipitation and microarray analysis of circRNAs from cortex tissue surrounding the infarcted area after 3 and 7 days of permanent cerebral ischemia in mice. Heatmaps and volcano graphs showed differences in m^6^A methylation in circRNAs between the infarct groups and the sham group (fold change > 1.5, \( P < 0.05, n = 3\) /group) (Figures 3A–F). A total of 12,345 methylated circRNAs were found in our research. In comparison with the sham group, the m^6^A modification was significantly changed in 1,706 circRNAs, including 85 hypermethylated and 1,621 hypomethylated circRNAs at 3d after MCAO.

However, the methylation levels increased in 57 circRNAs and decreased in 66 circRNAs at 7d after MCAO. In general, at 3d after operation, the hypomethylated transcripts

![Conjoint analysis for expression and m^6^A methylation profiles of circRNAs around the infarct cortex in 3d vs. sham (A), 7d vs. sham (B), and 7d vs. 3d (C).](image-url)
reached to 95% in the m⁶A-circRNAs, and only 5% were hypermethylated, while the distribution of these two kinds of transcripts was nearly equal at 7d after MCAO compared to the sham group. Additionally, compared to the 3d group, 2632 circRNAs were hypermethylated, while 186 ones were hypomethylated in the 7d group. The top 10 hyper and hypo-methylated circRNAs with different treatments were listed as Tables 2–4.

As for circRNA expression level, compared with the sham group, numerous circRNAs were differentially expressed, including 163 up-regulated and 289 were down-regulated in the 3d group, while 502 up-regulated and 512 down-regulated in the 7d group. In comparison to the 3d group, 697 circRNAs were significantly increased and 1,012 were decreased in 7 days after MCAO. Besides, we further explored the relation between methylation and expression (Figures 4A–C). We found that in 3d group, 24, 0, 3, and 72 circRNAs were hypomethylated-up expressed, hypermethylated-up expressed, hypermethylated-down expressed, and hypomethylated-down expressed respectively, while turned to 8, 0, 5, 2 ones in 7d group. Compared with the 3d group, there were 63 hypomethylated-up expressed, 14 hypermethylated-up expressed, 448 hypermethylated-down expressed and 3 hypomethylated-down expressed circRNAs in 7d group.

Verification of the Change in m⁶A Levels of CircRNAs After Cerebral Infarction

Post-stroke alterations of m⁶A modifications demonstrated by m⁶A immunoprecipitation and microarray analysis were confirmed by MeRIP-qPCR. In comparison with the sham group, the methylation levels of mmu_circRNA_27268 and mmu_circRNA_20673 were significantly up-regulated in the 3d group, while mmu_circRNA_32905 was hypomethylated. Although the difference in methylation of mmu_circRNA_29864 was not statistically significant, there was a downward trend. In general, the validation data was consistent with the m⁶A immunoprecipitation and circRNA microarray analysis (Figure 3G).

Functional Prediction for Parental Genes of Differentially m⁶A-Modified CircRNAs

To understand the significance of the m⁶A modification in the pathology of cerebral ischemia, GO and KEGG analyses were performed to compare the parental genes of differentially m⁶A-modified circRNAs at 3d or 7d after cerebral infarction. The top 10 most significant enrichment scores in biological processes (BP), cellular components (CC), and molecular functions (MF) are displayed in the histograms (Figures 5A–F). With the development of cerebral ischemia, the BP for hypermethylated circRNAs was established for localization in cells and cellular localization at 3 days after ischemia onset, which displayed a transition to meiotic telomere clustering and establishment of the blood-brain barrier at 7 days after MCAO. Analogously, CC showed enrichment of major synapses and cellular junctions at 3d group, which shifted to integral components of the postsynaptic density membrane at 7d group. We observed the involvement of the glutamate receptor, GTPase activator, single-stranded telomeric DNA binding, and phosphotransferase activity in MF. As for the hypomethylated circRNAs, BP, CC, and MF enrichment concentrated mainly on cellular processes, intracellular, and binding at 3 days after MCAO, which changed to protein ubiquitination, ubiquitin ligase complexes, and ubiquitin-like protein ligase activity at 7 days after MCAO.

Pathway Prediction for Parental Genes of Differentially m⁶A-Modified CircRNAs

KEGG analysis demonstrated the most enrichment pathways (Figures 6A–C). Compared with the sham group, various pathways including Hippo, neurotrophin, ubiquitin-mediated proteolysis, and axon guidance signaling were the major enrichment pathways at 3 days after MCAO, then turned into phosphorylated inositol signaling, ubiquitin-mediated proteolysis, and the glutamatergic synapse pathway at 7 days after MCAO. As for differentially m⁶A-modified circRNAs between 7d and 3d groups, the pathways mainly enriched in adherens junction, phosphatidylinositol signaling system, AMPK signaling pathway, and glutamatergic synapse.

The Interaction of CircRNA-miRNA-mRNA

Recent evidences have demonstrated that circular RNAs play a crucial role in regulating the level of miRNA-mediated gene expression by sequestering the miRNAs. Their interaction with disease-associated miRNAs indicates that circular RNAs are important for disease regulation. Ten most significant miRNAs were predicted for three verified m⁶A-circRNAs, and the binding sites were shown in Figure 7. At the same time, we predicted 273 target genes of miRNA and constructed the circRNA-miRNA-mRNA network (Figure 8A). GO analysis indicated that the top ten results of the BP, CC, and MF of target genes were almost related to calcium ion, and the most significant enrichment functions, respectively, were calcium ion transmembrane transport, voltage gated calcium channel complex, and voltage-gated calcium channel activity (Figure 8B). KEGG of target genes revealed the MAPK signaling pathway, focal adhesion, axon guidance, glutamatergic synapse, long-term potentiation, neurotrophin signaling pathway, and PI3K-Akt signaling pathway were mainly enriched pathways (Figure 8C).

DISCUSSION

It’s reported that different pathophysiological processes occur after 3d and 7d of cerebral infarction (F. Zhang et al., 2019), and we took 3d and 7d as time points to explore the dynamic change of m⁶A level after cerebral infarction. Our research suggests that stroke alters the methylation status of circRNAs dynamically. To our knowledge, this is the first study to explore methylation of circRNAs around the cerebral infarct region after MCAO in mice. And we found that the mRNA levels of m⁶A-related enzymes were significantly altered, which were supposed to be a contributing factor of the alteration in methylation of circRNAs. Additionally, we further showed that ALKBH5 and YTHDF3 proteins were changed at different times after stroke. The bioinformatics analysis showed that the differentially

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methylated circRNAs may act in some pathological molecular events after cerebral ischemia.

Notably, the methylation of circRNAs acts as a regulator of other diseases. It has been reported that decreased m^6^A abundance of methylated circRNAs can be observed in senile cataract and hypoxia-induced pulmonary hypertension (Li et al., 2020; Su et al., 2020), while the abundance is increased in some cancers (He et al., 2021). Therefore, the pathophysiological process of diseases may be related to an imbalance in the N6-methylation of adenosine in circRNAs. Recent studies have shown that stroke significantly changes the global level of m^6^A in peri-infarct tissue, and enzymes associated with the m^6^A modification are believed to mediate post-stroke secondary brain damage by participating in apoptosis and inflammation (Chokkalla et al., 2019; Si et al., 2020; Zhang et al., 2020).

Our results also indicated that the transcripts of these enzymes were differentially expressed between the sham and the MCAO groups, which implies that the homeostasis of m^6^A may change after stroke. Interestingly, the transcription level of the demethylase FTO decreased significantly at 3 days after MCAO.

**FIGURE 5** | Function analyses of the parent genes of differentially m^6^A-modified circRNAs. Function analyses of the parent genes of significantly hypermethylated and hypomethylated circRNAs around the infarct cortex in 3d vs. sham (A,B), 7d vs. sham (C,D), and 7d vs. 3d (E,F).
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FIGURE 6 | Pathway analyses of the parent genes of differentially m6A-modified circRNAs. Pathway analyses of the parent genes of significantly hypermethylated and hypomethylated circRNAs around the infarct cortex in 3d vs. sham (A), 7d vs. sham (B), and 7d vs. 3d (C).

compared with the sham group, while it increased significantly at 7d after MCAO compared to the 3d group. This indicates that FTO is decreased in the short term after stroke, which was confirmed by a recent report (Chokkalla et al., 2019). In the brain, FTO and ALKBH5 are predominantly expressed in neurons and increase dynamically during neural development, leading to the proliferation and differentiation of neural stem cells (Li et al., 2017; Chokkalla et al., 2019; Du et al., 2020). Studies have shown the protective role of FTO in proving brain damage and myocardial inflammation by regulating the m6A level (Chokkalla et al., 2019; Dubey et al., 2022). Another study suggested that FTO can increase lipid levels, which leads to a decrease in adenosine, an important factor that promotes neuronal apoptosis. Therefore, FTO deficiency can lead to adenosine accumulation, resulting in neuronal apoptosis (Gao et al., 2020). Although ALKBH5 has been reported to selectively demethylate BCL2 transcripts, which can prevent the degradation of BCL2 mRNA, enhance the expression of the anti-apoptotic BCL2 protein, and inhibit neuronal apoptosis (Xu et al., 2020), the role of ALKBH5 is less to know. Our study showed that ALKBH5 protein decreased in 3d group but increased in 7d group, suggesting that it could be one of the potential targets for treatment of stroke, and increases in demethylase may improve cerebral infarction by changing the m6A modification. Additionally, YTHDF3 also showed the same trend as ALKBH5, and the latest study showed that YTHDF3 and ALKBH5 were involved in synaptic plasticity (Martinez De La Cruz et al., 2021). However, the specific mechanisms require further study.

Recently, research on m6A has focused on the transcription and post-transcriptional processes of mRNAs. Although great progress has been made with mRNAs (Niu et al., 2013), the mechanisms involved in the m6A methylation in non-coding RNAs, especially circRNAs, are still poorly understood. Limited research has shown that the m6A modification...
**FIGURE 7** | The predicted binding sites between three verified differentially m^6A-modified circRNAs and ten miRNAs.
regulates the biogenesis, degradation, stability, translation, and immunogenicity of circRNAs (Chen et al., 2017, 2021; Knuckles and Buhler, 2018; Park et al., 2019; Di Timoteo et al., 2020). CircRNA is produced by selective reverse splicing of primary transcripts, interestingly, the deposition of specific exon regions with m^6^A was suggested to facilitate this process, resulting in the formation of some circRNAs (Di Timoteo et al., 2020). As an m^6^A reading protein, YTHDF2 is known as a degradation tool of mRNAs. YTHDF2 was later found to combine with RNase P/MRP to recognize the degradation of circRNAs with the m^6^A modification (Park et al., 2019). One study showed that circRNAs containing m^6^A motifs were translated in a cap-independent manner, and translation efficiency was related to m^6^A levels levels (Knuckles and Buhler, 2018; Di Timoteo et al., 2020). These pieces of evidence show that the m^6^A modification may act not only on post-transcriptional regulation of mRNAs, but also on circRNAs. Another special regulatory mechanism of m^6^A modification of circRNAs has been demonstrated. Exogenous circRNAs that activate immune responses are thought to be “circFOREIGN”; however, the m6A modification masks them as “circSELF”, which greatly reduces their immunogenicity. These studies have demonstrated the potential functions of m^6^A-modified circRNAs in disease regulation.

MeRIP combined with microarray analysis revealed that the m^6^A modification was altered in a considerable number of circRNAs at 3 and 7 days post-cerebral infarction compared with the sham group. In the early stage after cerebral infarction, the majority of circRNAs were hypomethylated, while the circRNAs were hyper-and hypomethylated to same extent in the later stage. Our results indicated that m^6^A methylation was a dynamic process after stroke. Abnormal methylation of circRNAs
was supposed to affect multiple molecular events that lead to secondary injury after ischemia. Numerous circRNAs, such as circTTC3, circCCDC9, and circTLK, have been found to regulate autophagy, nerve regeneration, axonal plasticity, and the blood-brain barrier after cerebral infarction (Wu et al., 2019, 2020; Chen et al., 2020; Yang et al., 2020, 2021), and correcting their dysregulation improved the brain damage.

However, the upstream regulatory mechanisms of these circRNAs are not well-known. Interestingly, N6-methylation of adenosine alters the abundance of circRNAs by regulating their generation and degradation (Park et al., 2019; Di Timoteo et al., 2020). Therefore, changing the methylation status may correct the level of circRNAs and affect their functions in cerebral ischemia. Our conjoint analysis of circRNAs’ expression and m^6^A methylation profiles screened all circRNAs changed in both methylation and expression levels. We found that the differentially expressed circRNAs mainly shows hypomethylation at 3 days after MCAO. However, the trend of methylation and expression seems to be opposite at 7 days after MCAO. The hypermethylated circRNAs were mainly down-expressed, while the hypomethylated ones were up-expressed. Our study indicated that the methylation level and abundance of circRNAs are not the only relationship. Other research also reported that increasing methylation levels can both increase or decrease the expression of circRNAs (Park et al., 2019; Su et al., 2020; Chen et al., 2021). It will be necessary to investigate the specific mechanisms that alter m^6^A-modified circRNAs in the pathological process after stroke.

CircRNA is derived from the precursor of mRNA. Thus, it can make great significance in pathophysiological process of diseases by regulating its parental gene. To further determine the function of differentially methylated circRNAs around cerebral infarction, we conducted the bioinformatics analysis. Our genomic analysis showed that BP and CC terms were involved in cell localization and intracellular transition at 3 days after MCAO, and shifted to the establishment of the blood-brain barrier and protein ubiquitination at 7 days after MCAO. Moreover, MF term happened the transition of glutamate receptor to phosphotransferase activity. Our results show that the secondary changes after stroke mainly occur from early nerve injury to late brain repair, indicating that differentially methylated circRNAs may regulate these pathological processes. In addition, previous research displayed that cerebral ischemia caused oxidative stress, neurovascular regeneration, and synaptic formation (Mehta et al., 2007). In our study, numerous signaling pathways, including ubiquitin-mediated proteolysis, axon guidance, hippo, neurotrophin, glutamatergic synapse, adherens junction, and phosphatidylinositol signaling, were significantly enriched. These pathways regulate the processes of oxidative stress, nerve regeneration, angiogenesis, synaptic formation, and plasticity after cerebral ischemia (Reichardt, 2006; Ding et al., 2014; Zhao et al., 2016). Glutamate is an excitatory neurotransmitter that mediates excitatory synaptic transmission. During cerebral ischemia and hypoxia, glutamate is released in large quantities, which over-activates glutamate receptors, leads to excessive Ca^{2+} influx, and causes brain damage and neuronal death (Mayor and Tymianski, 2018), a major cause of secondary brain injury. Therefore, understanding the regulation mechanism of m^6^A-modified circRNAs in the glutamate signaling pathway might be helpful for better intervention and prognosis and reduces secondary brain injury after stroke.

CircRNA can act as a sponge of miRNA to reduce the inhibitory effect of miRNA on mRNA to promote the expression of mRNA (Wu et al., 2019). Therefore, we selected the verified circRNAs to predict the network of circRNA-miRNA-mRNA. BP, CC, and MF show the predicted miRNAs mainly involve calcium ion transmembrane transport, voltage-gated calcium channel complex, voltage-gated calcium channel activity. Research shows calcium overload occurs in damaged neurons to mediate mitochondrial swelling injury and neuronal necrosis in the peri-ischemic cortex (Pivovarova and Andrews, 2010). It is reported that target genes cacna1g, tpcn2, panx1, and Fyn are involved in pathophysiological processes such as neuroexcitatory conduction, autophagy, inflammation, and synaptic plasticity in cerebral infarction (Shin et al., 2008; Shestopalov and Slepek, 2014; Guo et al., 2020; Rajani et al., 2021). In addition, 10 miRNAs have been predicted in our circRNA-miRNA-mRNA network. At present, little research has focused on these miRNAs. Interestingly, mmu-mir-3547-5 has been reported to play an important role in anti-atherosclerosis, and atherosclerosis is related to the pathogenesis of cerebral infarction (Zhang et al., 2018). In this network, mir-3547-5p and tpcn2 were the downstream targets of mmu_circRNA 27268 and mmu-mir-3547-5p, respectively. Therefore, we speculated that the mmu_circRNA 27268, mir-3547-5p, and tpcn2 may form a network axis, and participate in the pathological process of cerebral infarction by regulating calcium overload.

**CONCLUSION**

In summary, this study is the first to show changes of m^6^A methylation in circRNAs in cerebral infarction. The bioinformatics analysis predicted the possible functions and related pathways of m^6^A modified-circRNAs in secondary injury and the repair of cerebral infarction. However, understanding how the methylated circRNAs regulate the post-stroke pathophysiological mechanism will require further investigation. Our results provide new insights into the molecular mechanism of ischemia stroke.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196448.
ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Use Committee of the Shenzhen Hospital of Southern Medical University.

AUTHOR CONTRIBUTIONS

FL and LL conceived and designed the study and interpreted experiments. YL performed the experiments. YL, XL, ZC, and WZ analyzed the data. All authors read and approved the final submission.

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