Key miRNAs associated with memory and learning disorder upon exposure to sevoflurane determined by RNA sequencing

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Received October 28, 2019; Accepted April 15, 2020

DOI: 10.3892/mmr.2020.11199

Abstract. The study aimed to identify differentially expressed microRNAs (miRNAs/miRs) and explore the mechanisms governing impaired memory and learning ability in developing brains exposed to sevoflurane. A total of six 7-day-old male ICR mice were randomly assigned into the sevoflurane anesthesia group (treated with 2.4% sevoflurane) or control group (treated with normal saline solution at the same dose). After 14 days, the mice were subjected to a Morris water maze experiment. Then, the animals were sacrificed and hippocampus tissues were isolated. RNAs in hippocampus tissues were sequenced and the differential miRNA expression profiles were identified by a bioinformatics approach. The learning and memory function of mice were significantly affected by sevoflurane exposure. A total of 18 miRNAs were found to be significantly affected by sevoflurane administration. Their target genes clustered into different functional groups, such as 'dephosphorylation', 'vesicle localization' and the 'Wnt signaling pathway'. miR-101b-3p was closely related with 'chromatin binding' and 'protein serine/threonine kinase activity'. The most represented pathways for miRNAs included 'neuroactive ligand-receptor interaction' (miR-1187), 'long-term depression' (miR-425-5p), 'FoxO signaling pathway' (miR-425-5p) and the 'neurotrophin signaling pathway' (miR-467a-3p). miR-467a-3p (degree=89), miR-101b-3p (degree=59), and miR-1187 (degree=51) were the hub nodes in the miRNA regulatory network. The Wnt signaling pathway, miR-467a-3p, miR-1187 and miR-101b-3p may be therapeutic targets for preventing cognitive impairments induced by sevoflurane.

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Key words: sevoflurane, RNA sequencing, differentially expressed microRNA, function enrichment analysis, pathway

Introduction

Sevoflurane, a volatile anesthetic agent, is widely used for pediatric anesthesia in the clinic, and is characterized by rapid onset and offset, and low airway irritation and blood/gas partition coefficient (1). Previous evidence has suggested that long-term exposure to volatile anesthetics have side effects on brain development. It is reported that sevoflurane administration can impair memory processes, spatial memory, and the ability of the hippocampus to learn tasks in human and animal brains (2-4). Thus, concerns regarding the side effects of sevoflurane in children undergoing surgery have been highlighted.

An increasing number of studies have explored the molecular mechanisms that underlie the effects of sevoflurane exposure on the brain (1,4-6). The neurotoxicity of sevoflurane was shown to decrease guanylate kinase concentration in glutamatergic synapses in the development of rat brains (1). Sevoflurane-induced memory impairment is closely related with the suppression of glycogen synthase in the hippocampus (4). In addition, memory impairment following sevoflurane exposure is also reported to occur due to the decreased cytosolic calcium concentration and μ-calpain activity (5). Previous studies suggest that sevoflurane exposure alters the expression of genes involved in cognitive function-related metabolic pathways (6). Another previous study suggested that sevoflurane exposure leads to changes in the expression of receptors and enzymes involved in amyloid β clearance, which contributes to Alzheimer's disease development (7,8). The cognitive dysfunction induced by sevoflurane has been shown to be relieved by mediating the Toll-like receptor 4/myeloid differentiation primary response 88/NF-κB signaling pathway (8).

MicroRNAs (miRNAs/miRs) have been reported to be involved in neuropsychiatric disorders and impairments in cognitive function (9,10). However, the role of miRNAs in sevoflurane-induced neurotoxicity has not been fully clarified. The binding of Wnt ligands to receptors/co-receptors promotes Wnt signaling activation (11). Previous studies have revealed that the WNT signaling pathway is one of the main signaling pathways involved in numerous types of disease, including osteoporosis, cancer and diabetes (12,13). Moreover, it has been identified that miRNAs serve important regulatory roles

in the Wnt signaling pathway (14). However, the association between miRNAs and Wnt signaling in sevoflurane-induced neurotoxicity remains largely unknown. In this paper, miRNA expression in hippocampus samples from newborn mice was characterized by microarray technology. The miRNAs with differential expression were determined, followed by function and pathway enrichment analysis. The aim of this study was to explore the mechanism underlying the effect of sevoflurane on the developing brain and facilitates the discovery of safe anesthetic strategies.

Materials and methods

Animals. A total of four 15-day-old pregnant Institute of Cancer Research (ICR) mice were purchased from Shanghai Sipubikai Laboratory Animal Co., Ltd. The pregnant mice were maintained in an animal house at a temperature of 20±2˚C and humidity of 55±5%, with a 12-h light/dark cycle, and free access to food and water. After parturition, the healthy 7-day-old ICR mice were used for further analysis (15).

Approval was obtained from Animal Care and Ethics Committee of Zhejiang Chinese Medical University and all the animal procedures were performed according to the ethical standards.

Experimental groups. A total of six 7-day-old male ICR mice (weight, 250±10 g) were randomly assigned into two groups (n=3/group): The Sevoflurane group and Control group. Mice in the Sevoflurane group were administered with 2.4% sevoflurane for 6 h consecutively between 9:00 am and 3:00 pm according to previously described methods (16). Animals in the Control group were treated with the same dose of normal saline solution via a venous catheter at a rate of 1.0 ml/h. After treatment, all the animals were breast-fed for 14 days followed by a Morris water maze test (17).

Morris water maze. The water maze was comprised of a cylindrical pool (height, 50 cm; diameter, 80 cm) and a platform (diameter, 10 cm). The water surface was 2 cm higher than the platform and water was maintained at 22±0.5˚C. The animals were trained to find the platform and stayed for 2 min in the water maze twice/day for two consecutive days. The pool was divided into four quadrants. Rats were randomly delegated into the four quadrants, and then were placed in the water, facing the wall of the pool. If the mice failed to find the platform within 2 min, mice were placed on the platform for 20 sec. The second trial was conducted after a delay of 5-10 sec. The Morris water maze experiments were monitored by videos recorded on a computer. After training, the time for mice to reach to platform (escape latency) was recorded within 120 sec. Then, a probe trial was conducted after removing platform from the pool, and the mice were placed in a given quadrant and allowed to explore the maze for 120 sec. The cross-platform path length, percentage of the total trial path length that passed through the platform location, duration that the mouse stayed on the platform and the number of times across the platform were recorded.

RNA isolation. After the multiple behavioral tests on each mouse, mice were sacrificed, and hippocampal tissues were isolated. Total RNA was extracted by TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The purity of RNA was detected by a NanoDrop ND-2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and the quality was assessed by an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.).

miRNA profiling with microarray. Total RNA samples with an RNA integrity number >9 were used for microarray analysis. RNA (250 ng) from each sample was dephosphorylated, degenerated and labeled with Cyanine-3-CTP (Cy3) according to the manufacturer's instructions for the Human miRNA Microarray kit (Agilent Technologies, Inc.). After purifying, RNA was hybridized to gene arrays at 55˚C for 20 h and scanned on an Agilent Scanner G2505C system (Agilent Technologies, Inc). The raw data were exported to .txt format by Agilent Feature Extraction (FE) software (version 9.5.3; Agilent Technologies, Inc.) for further analysis.

Data preprocessing and differential expression analysis. The text format data were transformed by Affy package v1.50.0 (18) in R (http://www.bioconductor.org/packages/release/bioc/html/affy.html) and preprocessed by the robust multi-array average method (19,20), including background correction, normalization and expression calculation.

Compared with controls, the miRNAs with differential expression in the Sevoflurane group were assessed by limma v3.26.9 (http://bioconductor.org/packages/release/bioc/html/limma.html) (21). The P-values and fold change (FC) in expression of miRNAs were calculated. $P<0.01$ and $|\log_2 FC|>0.263$ was considered to indicate a statistically significant difference (22,23). Hierarchical clustering of differentially expressed miRNAs was performed by pheatmap package version 1.0.8 (https://cran.r-project.org/web/packages/pheatmap).

Prediction of miRNA target genes. The differentially expressed miRNAs were uploaded to the miRNA-Gene Targets module of miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/miRretsys-self.html) (24,25). Subsequently, the miRNA targets were also predicted by miRanda version 3.0 (http://cbio.mskcc.org/microrna), miRDB version 4.0 (http://mirdb.org/miRDB), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNA22 version 2.0 (https://cm.jefferson.edu/rna22), RNAhybrid version 2.12 (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) and TargetScan version 7.0 (http://www.targetscan.org/) databases.

In order to explore the biological functions of differentially expressed miRNAs, the predicted target genes were subjected to Gene Ontology (GO) (26) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (27) using clusterProfiler version 2.4.3 (https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) (28). $P<0.05$ was set as the threshold value.

miRNA-mRNA regulatory network construction. The predicted miRNA-target pairs were identified, and the miRNA-target regulatory network was constructed by Cytoscape version 2.8 software (29). The topology of the miRNA regulatory network was analyzed and the hub nodes with significant degrees were screened out.

Table I. Sevoflurane exposure affects the learning and memory ability in mice.

Phases of trial	Index	Sevoflurane group	Control group	P-value
Learning trial	Mean escape latency, sec	119.33±4.04	13.67±2.08	<0.001
Probe trial	Cross-platform path length, mm	71.19±2.45	92.06±2.09	<0.001
	Percentage of total path length in platform	0.62±0.02	0.83±0.03	<0.001
	Platform duration, sec	0.65±0.04	0.83±0.06	0.0028
	Platform entries, number of events	1.33±0.58	2.67±0.58	0.47

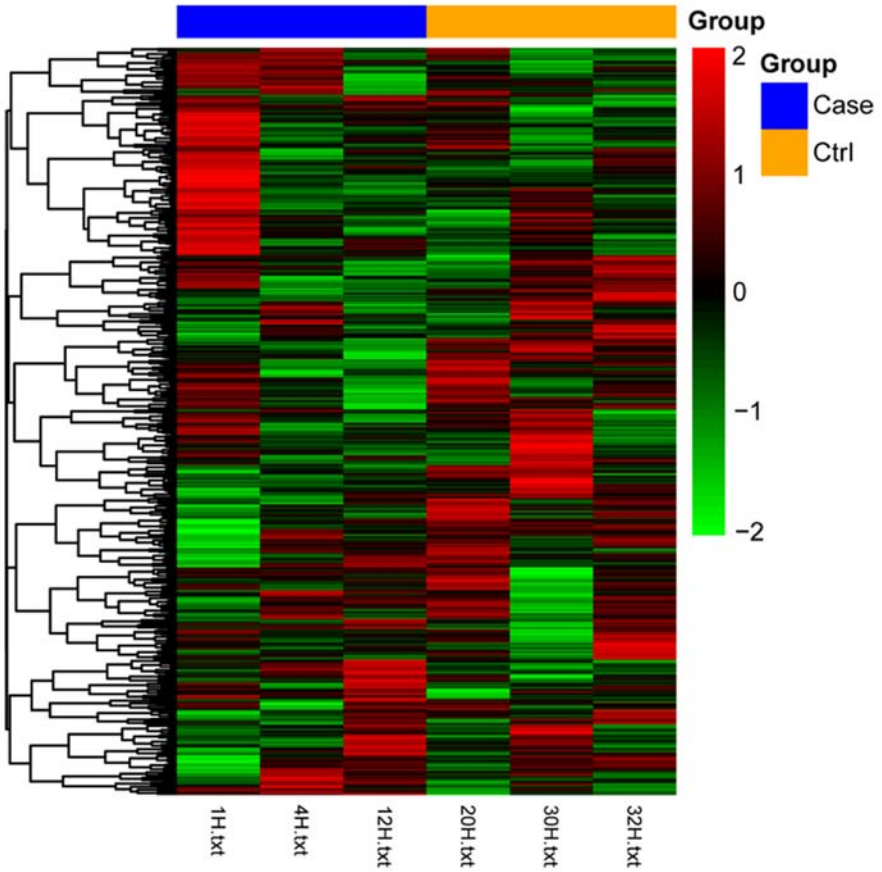

Figure 1. Heatmap of differentially expressed microRNAs in the sevoflurane-exposed group relative to controls. Each column represents samples and each row represents miRNAs. Red represents upregulated miRNAs and green represents downregulated miRNAs. The first four columns represent the level of expression in the sevoflurane group, while the last four columns represent the level of corresponding miRNA expression in the control group.

Statistical analysis. The data are expressed as mean ± SD. Differences between groups were compared using t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Sevoflurane exposure affects learning and memory function in newborn mice. During learning, following exposure to sevoflurane, mice in the Sevoflurane group (119.33±4.04 sec) showed longer mean latency time to reach the platform than those in the Control group (13.67±2.08 sec; P<0.001; Table I). During the probe trial, the cross-platform path length was significantly shorter in sevoflurane-treated mice (71.19±2.45 mm), compared with Controls (92.06±2.09 mm; P<0.001). Similarly, the percentage of the total path length that went through the platform location was significantly lower in sevoflurane-exposed mice (0.62±0.02) compared with the Control group (0.83±0.03; P<0.001). The platform duration in mice exposed to sevoflurane was significantly declined (0.65±0.04 sec) compared with the Control group (0.83±0.06 sec; P=0.0028). In addition, the mean number of times across platform for mice treated with sevoflurane (1.33±0.58) was not significantly lower than the Control group (2.67±0.58; P=0.47).

Data preprocessing. Based on the raw data, expression information for 52,044 miRNAs was available. The expression profiles of 49,880 miRNAs were obtained, followed by data preprocessing. After miRNA overlaps were removed, the mean expression values of 1,247 mature miRNAs were calculated following previously published descriptions (30).

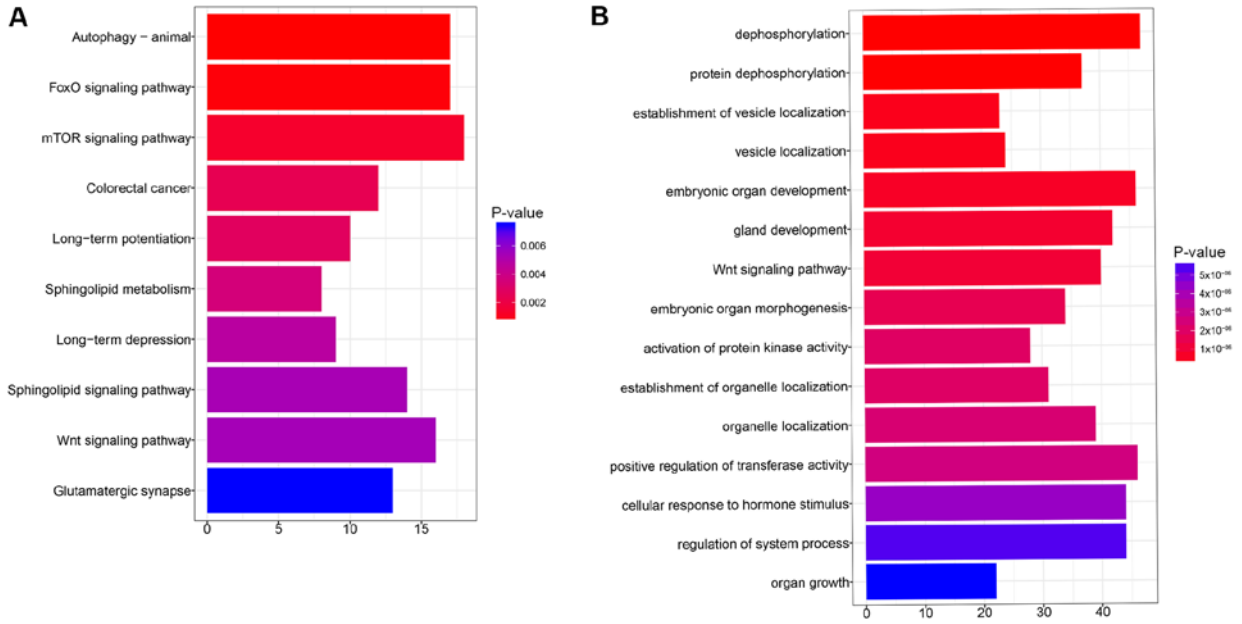

Figure 2. Enrichment analysis of targets associated with sevoflurane. (A) KEGG pathways significantly enriched by gene targets of differentially expressed miRNAs. y-axis, statistically enriched pathway; x-axis, the number of differentially expressed genes in each pathway. (B) GO functions significantly enriched by gene targets of differentially expressed microRNAs. y-axis, statistically enriched GO biological process terms; x-axis, the number of differentially expressed genes in each GO term. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

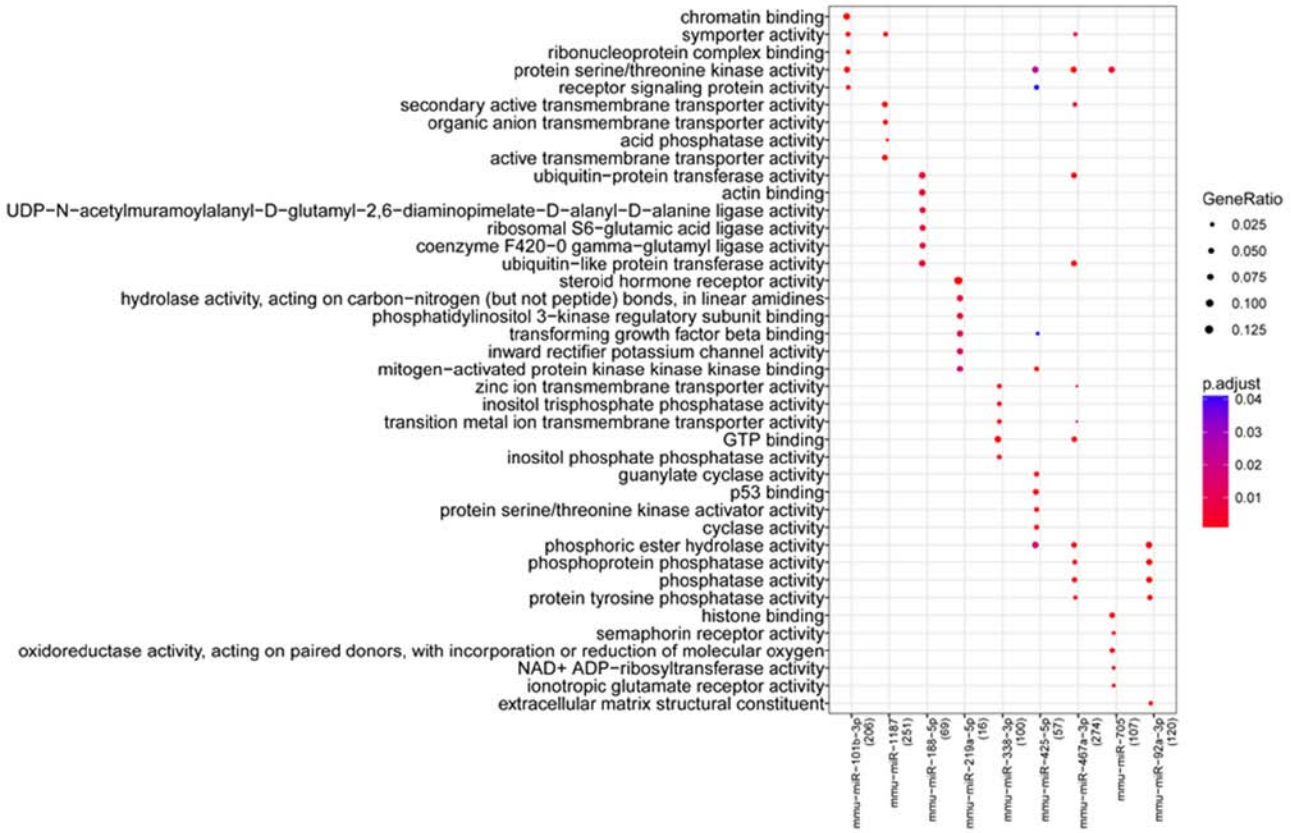

Figure 3. Significant Gene Ontology biological processes closely related with differentially expressed miRs. miR, microRNA.

Differentially expressed miRNAs. With P<0.01 and $|\log_2 FC|>0.263$ (18,19), the expression of 18 miRNAs, including 11 upregulated miRNAs (miR-1897-5p, miR-188-5p, miR-3098-5p, miR-3095-3p, miR-5107-5p, miR-3470a, miR-705, miR-5126, miR-149-3p, miR-1187 and miR-1982-5p) and seven downregulated miRNAs (miR-425-5p, miR-101b-3p, miR-92a-3p, miR-338-3p, miR-467a-3p, miR-219-5p and miR-219-2-3p) were found to be significantly affected by

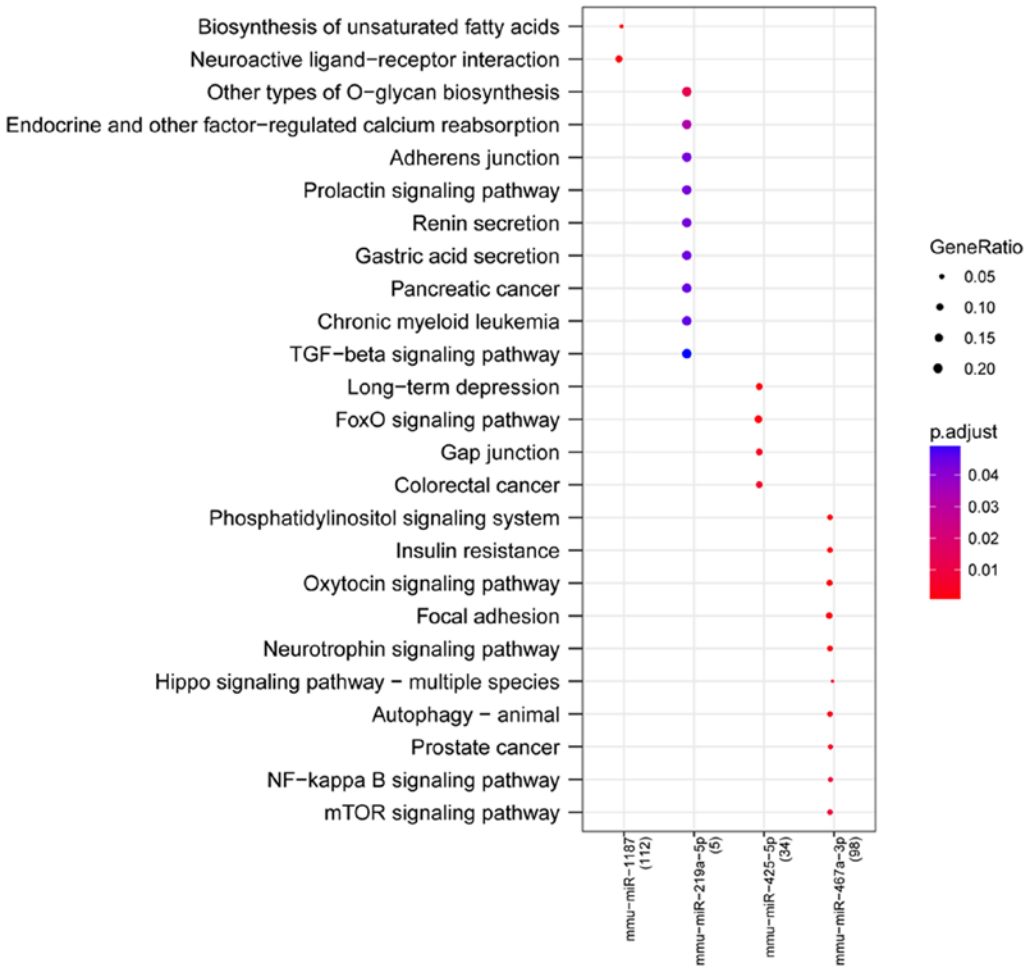

Figure 4. Pathways associated with differentially expressed miRs. miR, microRNA.

sevoflurane administration. The heatmap of differentially expressed miRNAs, which illustrate that the samples in different groups can be distinguished by the expression profiles of differentially expressed miRNAs, is presented in Fig. 1. Upregulated miRNAs in the sevoflurane group are shown in red, whereas downregulated miRNAs are presented as green.

miRNA-target genes and function enrichment analysis. Among 18 differentially expressed miRNAs, 3 upregulated miRNAs (miR-1187, miR-188-5p and miR-705) and 6 downregulated miRNAs (miR-101b-3p, miR-219a-5p, miR-338-3p, miR-425-5p, miR-467a-3p and miR-92a-3p) were identified to have 1,252 target genes based on the information of seven miRNA related databases. After the overlaps were removed, 1,095 target genes were obtained. Pathway enrichment analysis showed that the target genes were closely involved in the 'FoxO signaling pathway', 'mTOR signaling pathway' and 'Wnt signaling pathway' (Fig. 2A). Target genes clustered into different function groups such as 'dephosphorylation', 'vesicle localization' and the 'Wnt signaling pathway' (Fig. 2B).

miRNA-related functions and pathways. Based on the miRNA-target gene information, the biological function and pathways of the nine miRNAs were assessed by GO and pathway enrichment analysis. Results identified 190 biological process terms and 27 pathways significantly enriched by the nine miRNAs. Results showed that the biological functions and pathways closely related with miRNAs were relatively different. The top five GO function terms (ranked by P-value) of each miRNA are listed in Fig. 3. miR-101b-3p was closely related with 'chromatin binding' and 'protein serine/threonine kinase activity'. miR-1187 was significantly enriched in 'secondary active transmembrane transporter activity', 'organic anion transmembrane transporter activity' and 'active transmembrane transporter activity'. miR-219a-5p was involved in 'steroid hormone receptor activity', 'phosphatidylinositol 3-kinase regulatory subunit binding' and 'transforming growth factor-β binding'. miR-467a-3p and miR-92a-3p were closely related with phosphatase activity.

Pathways that were particularly affected by four miRNAs (miR-1187, miR-219a-5p, miR-425-5p and miR-467a-3p) were screened out by KEGG signaling pathway enrichment analysis. The most significantly affected pathways for miRNAs included 'biosynthesis of unsaturated fatty acids' (miR-1187), 'neuroactive ligand-receptor interaction' (miR-1187), 'other types of O-glycan biosynthesis' (miR-219a-5p), 'long-term depression' (miR-425-5p), 'FoxO signaling pathway' (miR-425-5p), 'phosphatidylinositol signaling system' (miR-467a-3p), 'neurotrophin signaling pathway' (miR-467a-3p), 'Hippo signaling pathway-multiple species' and 'NF-κB signaling pathway' (miR-467a-3p; Fig. 4).

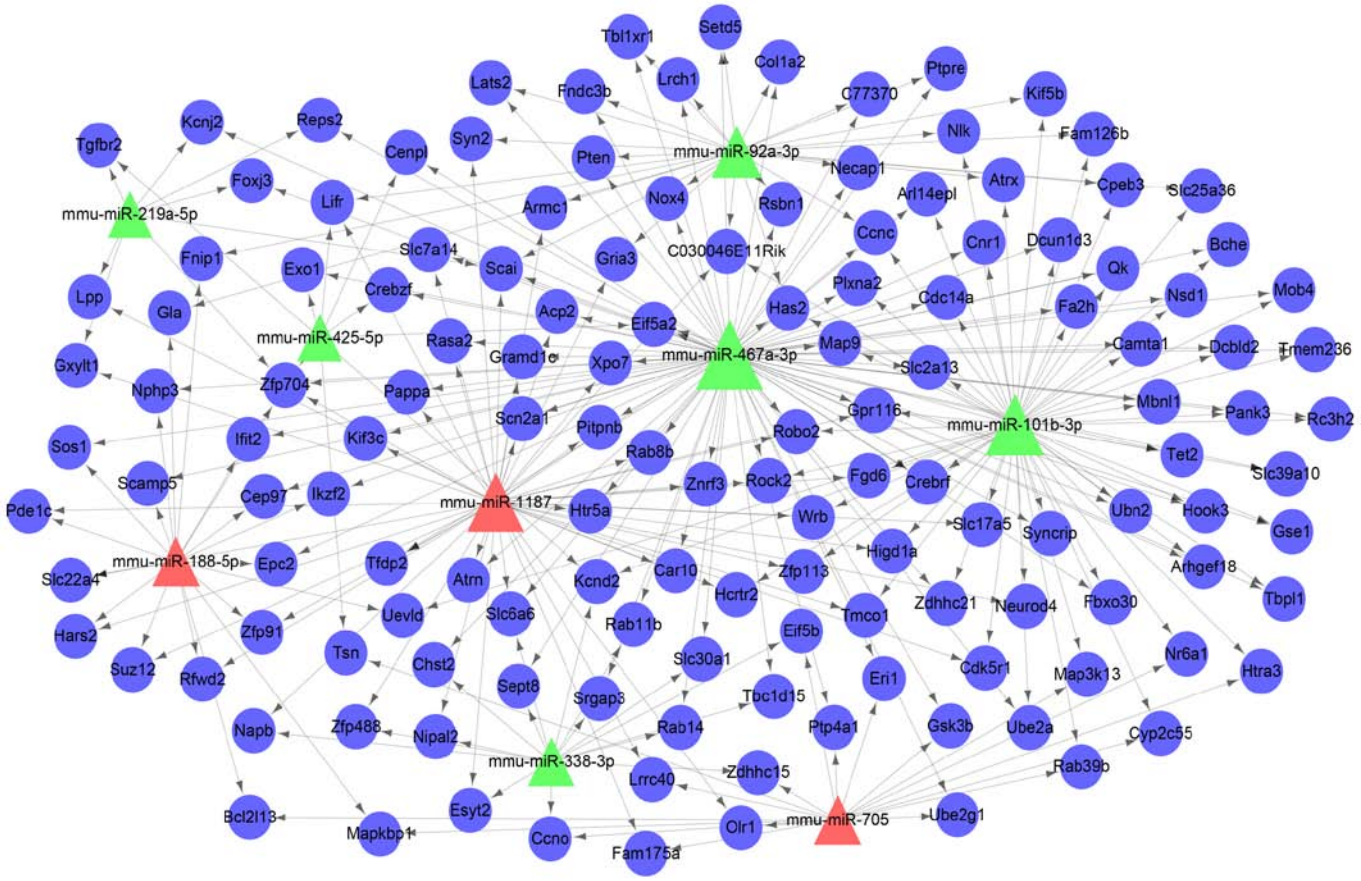

Figure 5. miRNA-target gene regulatory network. Green triangle, downregulated miRNA; red triangle, upregulated miRNA; blue circle, miRNA target genes. The size of the triangle nodes indicates the degree to which miRNAs contribute to the network. miRNA/miR, microRNA.

Table II. Top 10 nodes in the miR regulatory network.

Name	Degree	Regulated
miR-467a-3p	89	Down
miR-101b-3p	59	Down
miR-1187	51	Up
miR-92a-3p	28	Down
miR-188-5p	19	Up
miR-705	18	Up
miR-338-3p	16	Down
miR-425-5p	9	Down
miR-219a-5p	8	Down
Zfp704	5	/

miR, microRNA.

miRNA-target gene regulatory network. A miRNA regulatory network containing the nine miRNAs and 141 genes was constructed (Fig. 5). The top 10 nodes with the highest degrees were listed in Table II. The hub nodes included miR-467a-3p (degree=89), miR-101b-3p (degree=59), miR-1187 (degree=51) and Zfp704 (degree=5).

Discussion

The alterations of the miRNA expression profile in the hippocampus tissues of mice exposed to sevoflurane may provide novel insight to understand the molecular mechanisms of memory and learning impairments resulting from altered development of the brain and allow opportunities to develop new therapeutic management strategies. The aim of this paper was to identify differentially expressed miRNAs in hippocampus tissues between mice exposed to sevoflurane and controls, and elucidate the molecular mechanisms that govern memory impairment in sevoflurane-exposed mice. The results identified 18 differentially expressed miRNAs. After function and pathway enrichment analysis, the target genes of differentially expressed miRNAs were found to be significantly enriched in the 'Wnt signaling pathway'.

A previous study also showed that the dysregulated miRNAs were closely related with Wnt signaling pathways underlying sevoflurane-induced neurotoxicity in the development of mice brains (31), which was consistent with the present results. Ye *et al* (31) predicted the gene targets for miRNAs with TargetScan, miRanda and PicTar. In the present study, the target genes for dysregulated miRNAs were predicted by miRWalk 2.0 combined with miRanda, miRDB, PITA, RNA22, RNAhybrid and TargetScan databases, which enhanced the accuracy of significant pathway identification

and indicated the importance of the Wnt signaling pathway in the impaired cognition resulting from exposure to sevoflurane.

The Wnt signaling pathway is involved in various biological processes, such as cell proliferation, tissue regeneration, stem cell renewal and axon guidance (15,32,33). It is reported that the Wnt signaling pathway is involved with ~3% of differentially expressed transcripts that are prominent in the brain and spinal cord after spinal cord injury (SCI) in lampreys (34). Previous work has suggested an essential role for Wnt signaling in developing and adult brains (35). Blocking Wnt signaling has been shown to inhibit functional recovery following SCI (34), which indicates the significant role of Wnt signaling in neural repair and regeneration of the peripheral nervous system. In addition, synapse degeneration is closely associated with cognitive impairment and deficits in learning and memory. Evidence suggests that synapse degeneration, as an early event in neurodegenerative disease, is linked with Wnt signaling deficiency (36). The Wnt signaling pathway has been proposed as a therapeutic target for neuronal circuit recovery following synapse degeneration. A previous study also showed that reactivation of the Wnt signaling pathway improves neuroblast formation and neural function in the brain after focal cerebral ischemia in mice (37). Additionally, the Wnt signaling cascade is involved in the neuronal differentiation of human non-neural tissue-derived stem cells (35). Wnt signaling pathways are implicated in the differentiation of neural stem cells in human brain development (38). Taken together, these findings indicated a significant role of the Wnt signaling pathway in mediating cognitive disorders in brains exposed to sevoflurane.

The miRNA target gene regulatory network illustrated that miR-467a-3p (degree=89), miR-101b-3p (degree=59), and miR-1187 (degree=51) were hub nodes with multiple connections with target genes, which suggested a regulatory role for these miRNAs. The upregulated miR-188-5p (degree=19) was found to be another significant node in the miRNA regulatory network. A recent study suggested that miR-188-3p was upregulated in sevoflurane-treated mice and involved in sevoflurane-induced cognitive dysfunction (39), which was consistent with the present results. miR-188-5p is an alternative mature body of miR-188 and, to our knowledge, has not yet been reported to be dysregulated following sevoflurane exposure. It has been reported that miRNA-188-3p targeting mouse double minute 2 plays a significant role in sevoflurane-induced apoptosis pathways (39). The key role of miR-188-5p in miRNA-target gene regulatory networks may provide new insight into further gene targets in cognitive impairment induced by sevoflurane.

A recent study showed that overexpression of miR-467a-3p inhibits the neural differentiation of mouse embryonic stem cells (ESCs) (40). Neural stem cells differentiated from ESCs are suggested to be involved in cognition impairment-related diseases in humans (41,42). miR-467a-3p has also previously been found to be involved in the apoptosis of vascular smooth muscle cells (43). The present study showed that miR-467a-3p was differentially expressed in the hippocampus tissues of mice exposed to sevoflurane, which indicated that the neural differentiation and proliferation were dysregulated upon sevoflurane exposure. In addition, the pathway enrichment analysis conducted in this study showed that miR-467a-3p was closely related with the neurotrophin signaling pathway. Neurotrophic factors are implicated in the development and maintenance of the nervous system (44). The dysregulation of neurotrophin signaling has been found to be associated with neurodegeneration in Alzheimer's disease (44). Taken together, these data suggested that miR-467a-3p may play a significant regulatory role in the maintenance of neuron function.

Furthermore, miR-1187 has been found to be a novel miRNA in the inhibition of osteoblast differentiation (45). miR-101b-3p has been found to be enriched in hepatocytes and is markedly upregulated following hepatocyte damage (46). Although evidence of the role of miR-1187 and miR-101b-3p in the regulation of plasticity in the hippocampus is lacking, the present study showed that miR-1187 was closely associated with 'neuroactive ligand-receptor interaction' and miR-101b-3p was closely related with 'receptor signaling protein activity'. The differential expression of miR-1187 and miR-101b-3p may impact neuroactive signaling interactions in brains exposed to sevoflurane.

In conclusion, the present findings suggested that the Wnt signaling pathway is involved in mediating cognitive disorder upon exposure to sevoflurane. miR-467a-3p may play a significant regulatory role in the maintenance of neuron function. miR-1187 and miR-101b-3p may be implicated in the regulation of neuroactive signaling interactions. The miRNAs and their related pathways may be important therapeutic targets to prevent sevoflurane-induced memory and learning disorders.

Acknowledgements

Not applicable.

Funding

This study was supported by Zhejiang Medical and Health Science and Technology Plan (grant no. 2014KYA159), Zhejiang Traditional Chinese Medicine Science and Technology Plan (grant no. 2019ZA047) and Natural Science Foundation of Zhejiang Province (grant no. LY20H150002).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HS and ZL conceived and designed the study; HH and XX acquired the data and analyzed and interpreted the data; TT performed the statistical analysis; HS drafted the manuscript; and ZL revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Approval was obtained from Animal Care and Ethics Committee of Zhejiang Chinese Medical University and all the animal procedures were performed according to the institution's ethical standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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