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Linc00205/Mir-495-3p/Tnfsf15 Axis is Implicated in the Treatment Effect of Hyperbaric Oxygen Therapy on Patients with Post-Stroke Cognitive Impairment

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ABSTRACT

Introduction: We investigated the effects of hyperbaric oxygen (HBO) therapy on post-stroke cognitive impairment (PSCI) and performed the differential profiling of lncRNAs and mRNA.

Methods: The serum levels of different inflammatory factors were detected in PSCI patients with or without HBO therapy. The cognitive functions of patients in different groups were assessed before and after treatment. Differential expression analysis was performed on lncRNAs and mRNAs, followed by functional interaction prediction. The selected candidates were verified by Real-time Quantitative PCR and luciferase reporter assay.

Results: The results of MMSE and MoCA scores showed that patients in both the control (conventional treatment) group and HBO therapy group had significantly higher post-treatment cognitive scores, and in the 6th month after treatment, patients in the HBO group had higher scores than those in the control group. Blood inflammatory factors showed similar results, with the HBO group having higher anti-inflammatory factors IL-4 and IL-10 than the control group, and lower pro-inflammatory factors IL-2, IL-6, TNF-α, IFN-α and IL-17A than the control group. We further identified a competitive endogenous RNA regulator network of LINC00205-hsa-miR-495-3p-TNFSF15 involved in the HBO treatment, and their expression patterns were verified by qRT-PCR.

Conclusion: HBO treatment can improve the cognitive performance of PSCI patients in comparison to conventional treatment scheme. LINC00205/miR-495-3p/TNFSF15 axis may be responsible for the treatment effect of HBO therapy.

Keywords: Bioinformatics analysis; ceRNA; hyperbaric oxygen; oxidative stress; Post-stroke cognitive impairment; TNFSF15

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INTRODUCTION

Stroke is the second leading cause of death worldwide and endangers the quality of life and safety of patients due to impairment of the cognitive function. Post-stroke cognitive impairment (PSCI) refers to a range of syndromes that meet the diagnostic criteria for cognitive impairment within 6 months of the clinical event of stroke (1). The prevalence of PSCI is increasing in an aging population, and PSCI contributes to treatment adherence and compromised self-care ability, placing a significant financial burden on society and families (2). Neuroinflammation due to increased level of inflammatory cytokines is a major cause of neuronal degeneration in ischaemic stroke and adversely affects the cognitive function (3, 4). Understanding the mechanisms underlying PSCI progression has attracted intensified research interest, with the hope to formulate novel intervention strategy to improve the cognitive function of patients with PSCI.

The onset of stroke triggers a series of biochemical chain reactions due to ischaemia and hypoxia, including cerebral circulatory and metabolic disturbances (5). Hyperbaric oxygen therapy (HBO), as an oxygen supplement therapy technique, has the ability to directly and efficiently increase the oxygen supply to brain tissues, minimize cerebral hypoxia and promote neurological recovery (6). HBO therapy has been demonstrated to enhance neuronal survival, preserve the function

Highlight

- **This study investigated the effects of hyperbaric oxygen (HBO) therapy on PSCI.**
- **HBO treatment can improve the cognitive performance of PSCI patients.**
- **Our finding altogether suggest a protective role of miR-495-3p in ischemic conditions.**

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of blood-brain barrier and reduce brain oedema (7). At the molecular levels, HBO therapy improves the metabolism in the central nervous system and attenuates neuroinflammation and apoptosis by enhancing superoxide dismutase activity and promoting the expression of other antioxidant genes, thereby inhibiting neurodegeneration after stroke (8). Under pathogenic neurodegenerative conditions, HBO could promote neurogenesis by orchestrating different signaling processes such as hypoxia-inducible factors and Akt/GSK3β/β-catenin pathway (9, 10). HBO treatment was also found to promote the expression of cytoprotective and angiogenic genes, and repress the inflammatory genes under chronic wound condition (11, 12).

It has been proposed that the long-stranded non-coding RNA (lncRNA), micro RNA (miRNA) and messenger RNA (mRNA) function in the competitive endogenous RNA (ceRNA) network, in which different RNA molecules competitively bind to miRNA to influence its activity and modulate the downstream mRNA targets (13). Since the proposal of ceRNA hypothesis, a growing body of evidence has highlighted the importance of ceRNA networks in regulating the progression of different pathophysiological conditions, including hepatic fibrosis, cancers, and Alzheimer's disease (14, 15). There was evidence that HBO therapy can modulate the expression of non-coding RNAs in the context of ceRNA. For example, HBO treatment was reported to upregulate lncRNA MALAT1 expression in the exosome to suppress miRNA-92a in therapeutic angiogenesis (16). The same ceRNA module (lncRNA MALAT1/miRNA-92a) was also implicated in the therapeutic effect of HBO treatment in a rat model of acute myocardial infarction (17). In neural stem cell, HBO alleviated inflammatory pyroptosis and improved neurogenesis upon oxygen/glucose deprivation by regulating lncRNA-H19/miR-423-5p/NLRP3 Axis (18). In principle, any transcript carrying miRNA interaction element could potentially function as a ceRNA, the ceRNA network represents a widespread post-transcriptional regulation mechanism of gene expression. However, whether ceRNA regulatory module is implicated in the HBO treatment of PSCI remains to be investigated.

In this study, we aim to investigate the effects of HBO therapy on PSCI and profile the expression changes of lncRNAs and mRNAs upon HPO therapy. Patients with PSCI after acute ischaemic stroke were recruited and divided into the control group with conventional treatment and the HBO therapy group. The cognitive recovery of each group were assessed by Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment (MoCA) scores at different time points after treatment. In addition, differential expression analysis was performed on lncRNAs and mRNAs, followed by functional interaction prediction to identify the potential ceRNA axis implicated in the treatment effect of HBO therapy. We also validated the relative expression of LINC00205, hsa-miR-495-3p and TNFSF15 by qRT-PCR.

METHODS

Ethic Statement

The patients in this study were recruited in accordance with the Declaration of Helsinki's ethical principles and Good Clinical Practice guidelines. The usage of human samples was approved by the Ethics Committee of Shidong Hospital Affiliated to University of Shanghai for Science and Technology, with the approved number as 2019-KY007.

Study Participants

A total of 40 patients diagnosed with acute ischemic stroke and PSCI who were admitted to the Department of Neurology, Shidong Hospital Affiliated to University of Shanghai for Science and Technology, from January 2021 to March 2022, were included in the study. 20 of the PSCI patients were treated with conventional strategy (Control group), and 20 PSCI patients were treated with conventional plus hyperbaric oxygen (HBO) therapy. All the included patients have complete medical records of treatment, functional cognitive performance assessment at admission and after discharge at 3 and 6 months.

Treatment

Patients in the control group (PSCI conventional treatment, n=20) received conventional treatment for acute cerebral infarction, including blood pressure and blood sugar control, anti-platelet aggregation therapy, and lipid-lowering plaque stabilization, and donepezil was administrated to improve the cognitive function. The HBO group (n=20) was further supplemented with hyperbaric oxygen therapy apart from conventional treatment within 72 hours of admission. HBO was conducted in the GY3200D1-C6 oxygen chamber (Hongyuan, China). Patients were under the pressure of 2.2 ATA oxygen using a mask, with a steady pressure increase for 15 min. Patients were subjected to stable oxygen inhalation for 60 min with an interval rest of 5 min, followed by a decompression of 20 min. The total duration of HBO treatment was 100 min per day, and a total number of 10 consecutive HBO treatments were conducted during one course of therapy.

Cognitive Function Score

The mini mental state examination (MMSE) and the Montreal cognitive assessment scale (MoCA) were used to assess the cognitive performance of PSCI patients at different time periods of admission, 3 months and 6 months after treatment in each group. The MMSE is a 30-item scale that includes six areas: orientation (3 points for time orientation, 5 points for place orientation), numeracy (5 points), memory (3 points for immediate memory, 3 points for delayed recall), verbal ability (2 points for naming, 3 points for repetition, 1 point for writing), executive ability (4 points), and visuospatial ability (1 point). There are 30 sub-items, of which 1 mark is awarded for each correct item and no marks are awarded for errors or attempts. The threshold for judging standard performance is 17 points for the illiterate group, 20 points for the individuals with primary school education and 26 points for the individuals with secondary school or above education (19). The MoCA scoring system includes 8 items: visuospatial and executive ability (5 points), naming (3 points), memory and attention (6 points), language (3 points), abstraction (2 points), delayed recall (5 points) and orientation (6 points). The total score is 30 points, with one point added to the result for the individuals with an education level less than 12 years. A total score of 26 points or more is considered as normal performance. The higher the score, the better the cognitive function (20).

Serum Sample Collection and ELISA Analysis of Inflammatory Cytokines

On the morning of the next day of admission, 5 mL of venous blood was drawn from all patients after overnight fasting. The whole blood sample was allowed to clot at room temperature for 30 min, followed by the centrifugation at 3000 r/min for 10 min to remove the clot. The resulting supernatant was collected as the serum samples. The levels of inflammatory indicators, including tumor necrosis factor α (TNF-α), interleukin-2, IL-4, IL-6, IL-10, IL-17A and interferon-α (IFN-α) were determined in the serum samples using ELISA Kits following the manufacturer's instructions.

RNA Extraction and Quality Control for Sequencing

Total RNA extraction was performed using the PAXgene Blood RNA Kit (763134, Qiagen, Germany) according to the standard procedures provided by the manufacturer. The extracted total RNA was quality-checked by electrophoresis in an Agilent 2100 Bioanalyzer (Agilent technologies Santa Clara, US). The extracted RNA samples were further purified by the RNAClean XP Kit (A63987, CA, USA) and RNase-Free DNase Set (79254, Qiagen, GmBH, Germany).

Table 1. The sequences of qRT-PCR primers

Gene		Primer
LINC00205	Forward	5′-TGGGGAGGGGAGACATAAGACA-3′
	Reverse	5′-TTTTACTGTCTCCTGTCTCTGGAGG-3′
TNFSF15	Forward	5′-GGAGAGGCCTGTGTGCAGTT-3′
	Reverse	5′-TAGGAACTCGGTGGCAGAGG-3′
GAPDH	Forward	5′-CGGAGTCAACGGATTTGGTCGTAT-3′
	Reverse	5′-AGCCTTCTCCATGGTGGTGAAGAC-3′
hsa-miR-495-3p	Forward	5′-AACACGCAAACAAACATGGTGC-3′
	Reverse	5′-CAGTGCAGGGTCCGAGGT-3′
U6	Forward	5′-CTCGCTTCGGCAGCACA-3′
	Reverse	5′-AACGCTTCACGAATTTGCGT-3′

Library Construction and Sequencing

RNA samples from 5 control individuals (conventional therapy) and 5 patients with HBO treatment were subjected to RNA-seq analysis. The selection criteria is based on the observation that these individuals showed clinical improvement after treatment, and HPO treatment showed a more significant improvement compared to the conventional treatment of control group. Total RNA was extracted from the plasma samples using the PAXgene Blood RNA Kit (763134, Qiagen, Germany) and quantified using a spectrophotometer. The purified total RNA was subjected to rRNA removal, fragmentation, first strand cDNA synthesis, second strand cDNA synthesis, end repair, 3′ end adenylation, index primer ligation and amplification using TruSeq RNA Library Prep Kit v2 (Illumina, CA, ISA), according to the standard protocols of the library construction of the supplier. The resulted library was size selected on the gel electrophoresis and quality-checked by Agilent 2100 Bioanalyzer. Deep sequencing was performed on the Illumina NovaSeq6000 sequencer in PE150 mode.

Screening of DE-mRNAs and DE-lncRNAs

Fragments Per Kilobase of transcript per Million mapped reads (FPKM) value was derived from the sequencing samples using the Bioconductor package in R software. Differential gene expression analysis was performed between two samples using paired t test and Benjamini-Hochberg adjustment for multiple comparisons. The mRNAs and lncRNAs with a p value <0.05 and |log2 fold change (FC)| >2 were considered as the significantly differentially expressed (DE) DE-mRNA and DE-lncRNA. The heatmaps and volcano plots displaying the relative expression levels of DE-mRNA and DE-lncRNA were generated using R package.

CeRNA Network Analysis

DIANA-LncBase v3 database (www.microrna.gr/LncBase) was used for predicting the lncRNA-miRNA interactions of the DE-lncRNAs. The mRNA-miRNA interactions of DE-mRNA were predicted using the predictive target module of the miRWalk3.0 (http://mirwalk.umm.uni-heidelberg.de/). The ceRNA interactions among DE-lncRNA, miRNA, and DE-mRNA were established by linking the shared miRNAs predicted as downstream targets of DE-lncRNAs and upstream regulators of DE-mRNAs. The final list DE-lncRNA-miRNA-DE-mRNA interaction axis was constructed using Cytoscape software (Version 3.7.2, https://cytoscape.org/).

Quantitative Real-time Reverse Transcription PCR (qRT-PCR) assay

The relative expression levels of miRNAs, lncRNAs, and mRNA were quantified by qRT-PCR, using the RNA samples from 40 PSCI patients before treatment (baseline), 20 control subjects after conventional therapy and 20 patients with HBO treatment. 1 μg of total RNA was used for reverse transcription cDNA vias RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific, CA, USA) under the following reaction conditions: 65 ℃ for 5 min, followed by cooling on ice for at least 2 min; 25 ℃ for 10 min and 37 ℃ for 1 h. The reverse transcription was terminated at 70 °C for 10 min. cDNA template was subjected to qRT-PCR analysis using SYBR premix EX TAQ II kit (RR820A, Takara, Dalian, China) with the following cycling conditions: 95 ℃ for 10 min, and 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The relative expression of candidate genes was calculated by $2^{-\Delta\Delta}$CT method and normalization of target gene expression was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The primers used in the study are shown in Table 1.

Dual Luciferase Assay

To study the interaction between lncRNA and miRNA or the interaction between miRNA and mRNA, the sequence containing the wild type interaction sequences or mutated sequences were cloned into the PmirGLO firefly luciferase reporter (Promega, WI, USA). The reporter was transfected into 293T cells with either miRNA mimic or miR-NC using Lipofectamine 3000 reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. 48 hours after the transfection, the relative luciferase activities were measured using Dual-Luciferase Reporter Assay Kit (Promega, WI, USA) on a luminescence microplate reader.

Statistical Analysis

Statistical analysis was performed via Student's t test to compare the difference between two groups using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA). Fisher's exact test was employed to filter the significant Gene ontology term and pathways using R software 3.3.1 (R Development Core Team) in the enrichment analysis. A P value <0.05 (two-tailed) was considered to be statistically significant. The level of significance was set as *p<0.05, **p<0.01, ***p<0.001.

RESULTS

Baseline Characteristics of Patients in the Control and HBO Groups

A total of 40 patients with PSCI (21 males and 19 females) were included in our study. The patients were dividing into two groups: control (conventional treatment) group and HBO therapy group. Based on their medical records upon admission, there were no statistically significant difference in the baseline characteristics of the subjects between two groups, including age, education level, body mass index, alcohol consumption and smoking status (Table 2).

Hyperbaric Oxygen Therapy Improves the Cognitive Performance of PSCI Patients

The assessment of the cognitive performance in the control and HBO group showed that at the admission and discharge, there were no significant difference in the MMSE scores between the two groups. However, HBO group showed significantly higher MOCA scores at

Table 2. Baseline characteristics

Variable	Control group (n=20)	HBO group (n=20)	t/X²	P value
Age (years)	62.15 ± 6.31	61.63 ± 9.42	t=0.205	0.839
Gender, n (%)			0.784	0.376
Male	18 (90.00%)	16 (80.00%)		
Female	2 (10.00%)	4 (20.00%)		
Educational Level, n (%)			1.48	0.687
Illiterate	1 (5%)	2 (10%)		
Primary School	5 (25%)	4 (20%)		
Secondary School or above	14 (70%)	13 (65%)		
Other	0	1 (5%)		
Body mass index (kg/m²)	22.92 ± 1.66	22.54 ± 3.26	0.465	0.645
Smoking, n (%)			0.440	0.507
Yes	8 (40%)	6 (30%)		
No	12 (60%)	14 (70%)		
Drinking alcohol, n (%)			0.902	0.342
Yes	9 (45%)	12 (60%)		
No	11 (55%)	8 (40%)		
Hypertension, n (%)			0.114	0.736
Yes	7 (35%)	6 (30%)		
No	13 (65%)	14 (70%)		
Diabetes Mellitus, n (%)			0.625	0.429
Yes	15 (75%)	17 (85%)		
No	5 (25%)	3 (15%)		

a: t-test was used to compare the differences between groups, and P<0.05 was considered statistically significant.
b: Chi-square test (X2) was used to compare the differences between groups, and P<0.05 was considered statistically significant.
HBO, hyperbaric oxygen

Table 3. Cognitive performance of PSCI patients in the control and HBO groups

	Control group	HBO group	P value[c]
MMSE			
Admission	17.6±3.05*	16.8±3.22	0.547[a]
Discharge	22±2.94	22.4±2.62	0.652[b]
3 month	23.4+2.76	24.8±2.04	0.076[b]
6 month	23.8±2.69*	25.2±1.96	0.15[a]
MOCA			
Admission	14.6±2.28	14±2.47	0.43[b]
Discharge	17.4±3.23*	19.6±2.84	0.015[a]
3 month	19.75±2.65*	22.2±2.48	0.004[a]
6 month	20.4±2.52	23.0±2.20	0.001[b]

*Not normally distributed, [a]Mann-Whitney U test, [b]Independent Samples t-test, [c]between groups.
MMSE, Mini-Mental State Examination; MOCA, Montreal Cognitive Assessment; PSCI, Post-stroke cognitive impairment; HBO, hyperbaric oxygen

discharge. At 3 and 6 months after discharge, there were clear increase of the cognitive scores in both control and HBO groups. In comparison to the control group, MMSE showed an increase in the HBO group although the change was not statistically significant. MOCA scores were significantly higher in the HBO group at 3 and 6 months, indicating that HBO treatment has a beneficial effect on improving the cognitive performance of PSCI patients (Table 3).

Hyperbaric Oxygen Therapy Reduces Inflammation Levels in PSCI Patients

At admission, there was no significant difference in baseline pro-inflammatory and anti-inflammatory factors between patients in the control group and HBO group (Figure 1A and B, p>0.05). We also collected the blood after the treatment before discharge, and the analysis showed that the pro-inflammatory factors including IL-2, IL-6, TNF-α, IFN-α, and

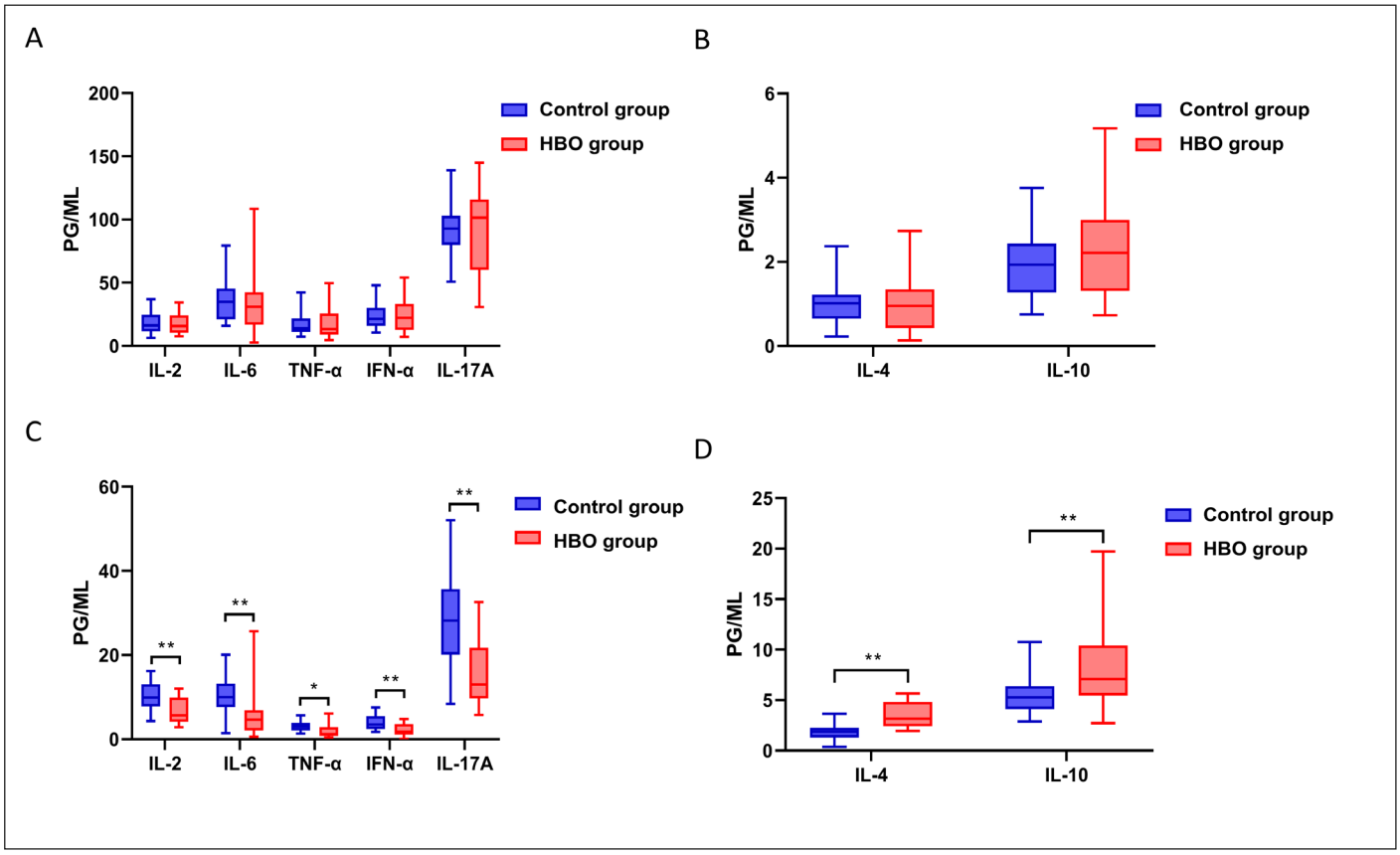

Figure 1. HBO therapy reduces inflammation levels. The levels of pro-inflammatory factors (A) and anti-inflammatory factors (B) of patients in the control group and HBO groups at baseline were determined by ELISA. The levels of pro-inflammatory factors (C) and anti-inflammatory factors (D) in patients in the control and HBO groups after treatment were determined by ELISA. (n=20).

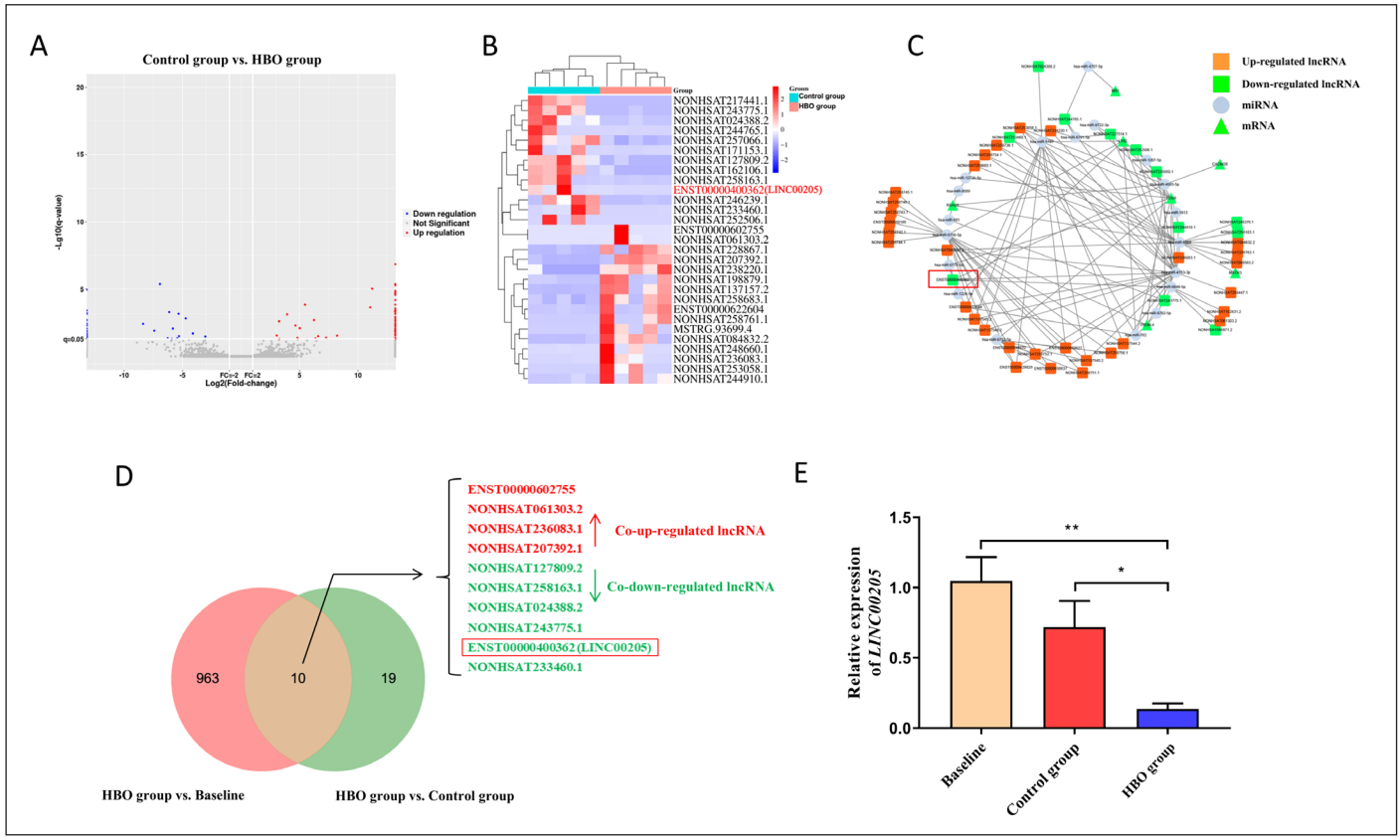

Figure 2. Expression analysis of LncRNA profiles in the control and HBO group. (A) Screening of differential lncRNAs in control and HBO groups (n=5 samples in each group. (B) Heatmaps displaying the DE-LncRNAs between the control and HBO groups. (C) The ceRNA network prediction of the DE-lncRNAs between the control and HBO groups. (D) Intersection of DE-lncRNAs of baseline vs HBO treatment and the control vs HBO group. (E) RT-PCR validation of significantly down-regulated expression of LINC00205 in HBO group (40 PSCI patient samples before treatment (baseline), 20 samples after conventional therapy (control group) and 20 samples after HBO treatment (HBO group)).

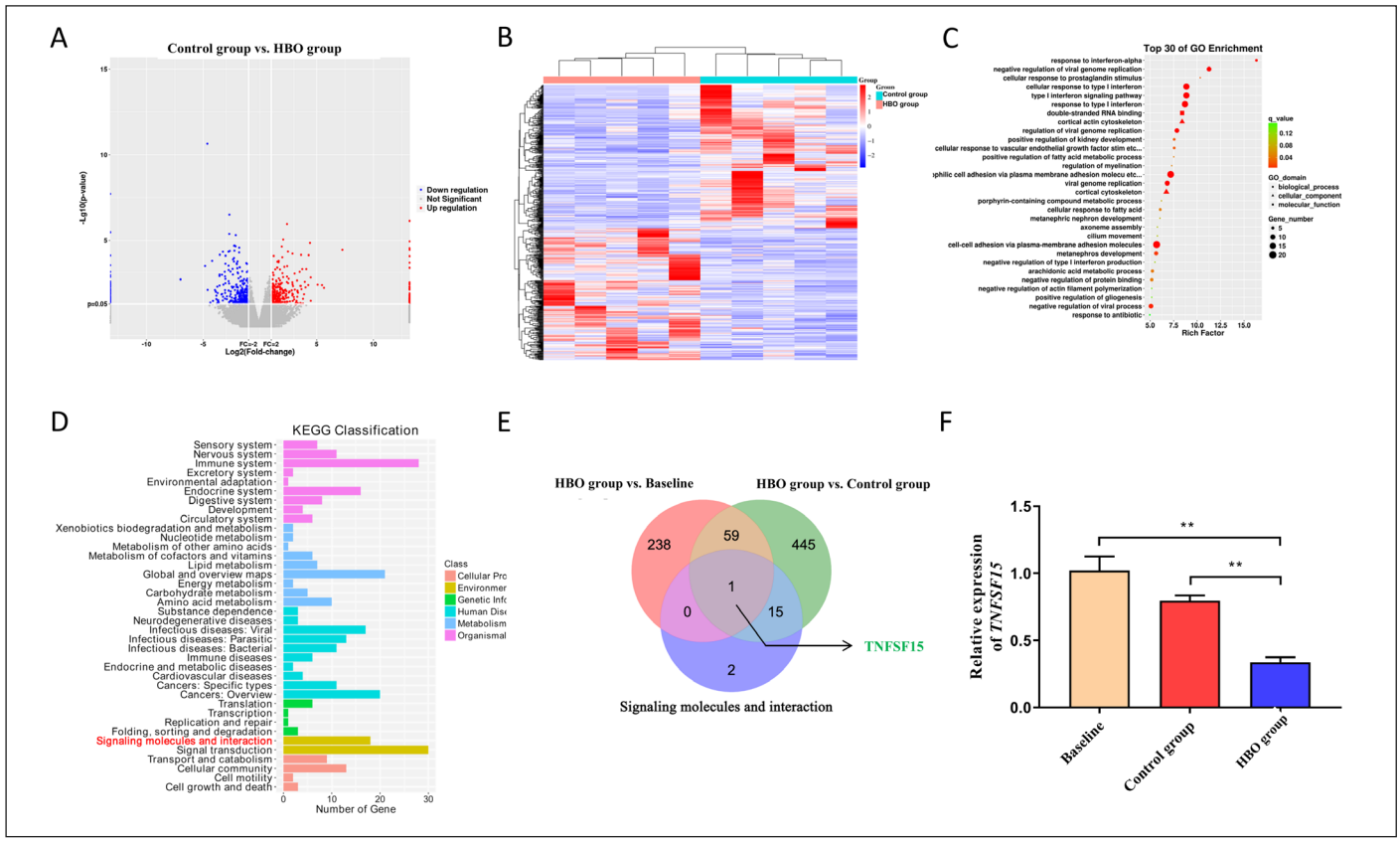

Figure 3. mRNA profiling involved in the control and HBO group. (A) Volcano plots of mRNA expression profiles in control and HBO group. (B) Cluster analysis of differentially expressed mRNAs (DE-mRNAs) in the control and HBO groups. GO enrichment (C) and KEGG enrichment (D) analyses of differentially expressed genes in control group and HBO group. (E) Intersection of DE-mRNAs of baseline vs HBO treatment, the control vs HBO group and the ones involved in signaling transduction. (F) RT-PCR validation of significantly down-regulated expression of mRNA TNFSF15 after HBO treatment (40 PSCI patient samples before treatment (baseline), 20 samples after conventional therapy (control group) and 20 samples after HBO treatment (HBO group)).

IL-17A were significantly lower in the HBO group (Figure 1C, $p < 0.05$), while the anti-inflammatory cytokines (IL-4 and IL-10) were higher in the blood samples of the HBO group when compared to the control group (Figure 1D, $p < 0.05$).

RNA-Seq Demonstrates the Involvement of LncRNAs in HBO Treatment

A growing body of evidence suggests that lncRNAs play important roles in pathological conditions of neurodegeneration(21). To profile the changes of lncRNAs in the therapeutic effect of HBO therapy PSCI, we performed high-throughput sequencing using the total RNA samples from the control group and HBO group before and after treatment (n=5 in each group). The sequencing results detected a total number of 58,425 lncRNAs in all samples, of which 91 were differentially expressed in the HBO group compared to the control group after the treatment (log2FC<-1 or >1 and p<0.05, 58 were up-regulated and 33 were down-regulated) (Figure 2A). 62 lncRNAs with extremely low expression (FPKM value is close to 0) were excluded, and 29 differentially expressed lncRNAs (DE-lncRNAs) were finally obtained (Figure 2B). The ceRNA network of the De-lncRNAs was predicted by databases, and the results implied that these lncRNAs had the potential to regulate different miRNAs and mRNAs network in HBO treatment (Figure 2C).

We also profiled the lncRNA changes in the individuals before (baseline) and after HBO treatment in the HBO group. Among the 973 DE-lncRNAs identified, 10 DE-lncRNAs were shared with the comparison between the control and HBO group after treatment (Figure 2D), suggesting that these 10 De-lncRNAs may be specifically implicated in the HBO treatment. One of these 10 lncRNAs, LINC00205, which showed the most significant down-regulation in the HBO treatment group, was screened for study. qRT-RCR analysis verified that LINC00205 transcript abundance was significantly decreased after HBO treatment when compared to the baseline and the control group (Figure 2E).

Functional Genes Involved in the HBO Treatment of PSCI Patients

To further explore the functional genes regulated by HBO therapy, we analyzed the transcriptome high-throughput sequencing data. The results showed a total of 620 mRNAs were differentially expressed between the control and HBO groups, of which 291 were up-regulated and 329 were down-regulated in the HBO group (Figure 3A). Hierarchical clustering analysis of these differentially expressed genes revealed that the control group and the HBO group were clustered together (Figure 3B). To gain more biological insights into the DE-mRNAs, we next performed gene ontology (GO) and KEGG pathway enrichment analysis. These analyses showed that the differentially expressed genes were enriched in diverse biological processes, cellular composition, molecular functions, and signaling pathways (Figure 3C and 3D), suggesting that HBO therapy exerts its therapeutic effects on cognitive impairment through multiple pathways and multiple mechanisms. In our study, the main focus was the signaling molecules involved in the therapeutic process. We next sought to find the signaling player specifically involved in HBO therapy by finding the intersection of DE-mRNAs among HBO vs control comparison, HBO vs baseline comparison and the DE-mRNAs associated with the signaling molecules and interaction in KEGG analysis. A functional gene (TNFSF15, TNF superfamily member 15) that was significantly down-regulated after HBO treatment was identified (Figure 3E). TNFSF15 was previously reported to be involved in the regulation of NF-κB signalling

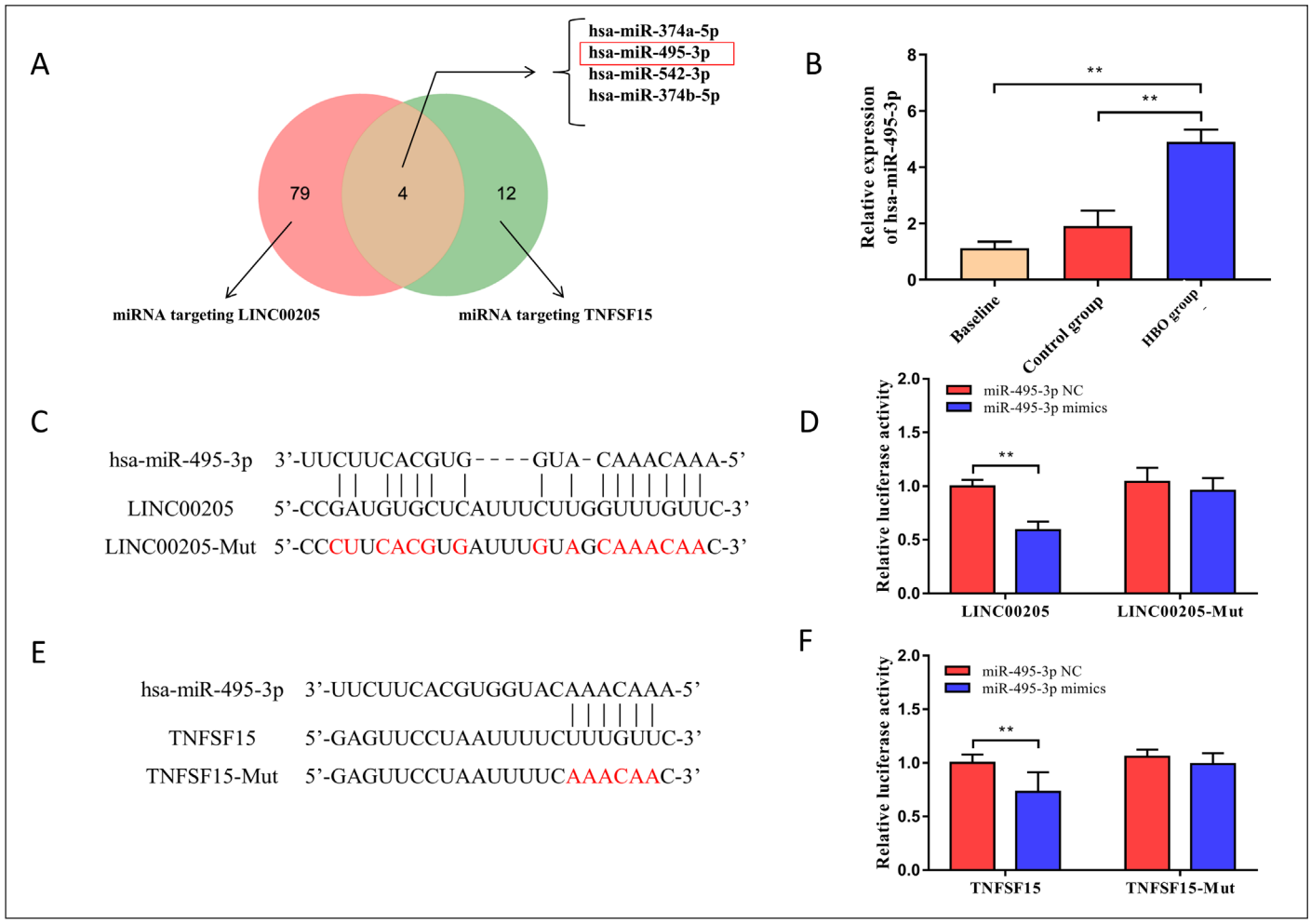

Figure 4. LINC00205 interacts with TNFSF15 and miR-495-3P. (A) Candidate miRNAs were identified through the intersection of LINC00205 targeted miRNAs and the miRNAs targeting TNFSF15. (B) Expression of the miR-495-3p detected by qRT-RCR in 40 PSCI patient samples before treatment (baseline), 20 samples after conventional therapy (control group) and 20 samples after HBO treatment (HBO group). (C) Bioinformatic prediction of potential binding sites for miR-495-3p binding to LINC00205 and (D) in vitro dual luciferase assay to verify the interaction in 293T cells. (E) Bioinformatic prediction of potential binding sites between miR-495-3p and TNFSF15 mRNA. (F) Dual luciferase assay verification of the interaction between miR-495-3p and TNFSF15 mRNA in 293T cells.

pathway(22-24), and it was hypothesized that TNFSF15 may play an important role in the NF-κB-mediated inflammatory response. As quantified by qRT-RCR, TNFSF15 expression was significantly decreased after HBO treatment (Figure 3F).

LINC00205 Interacts with TNFSF15 and miR-495-3P

To establish the interaction network of LINC00205 and TNFSF15 through miRNA, 83 miRNAs were predicted as potential target of LINC00205 and 16 miRNAs were predicted as the upstream regulators of TNFSF15 mRNA. The intersection of the two sets of miRNAs identified 4 miRNAs targeting both LINC00205 and TNFSF15 mRNA (miR-374a-5p, miR-495-3p, miR-542-3p, miR-374b-5p) (Figure 4A). Among the four candidate miRNAs, miR-495-3p expression was most significantly up-regulated in the HBO treatment group, as revealed by qRT-RCR analysis (Figure4B). To validate the targeted interaction relationship between miR-495-3p and LINC00205, the predicted wild type binding sites between miR-495-3p and LINC00205 and the mutated sequences were cloned into a luciferase reporter (Figure 4C). The co-transfection of miR-495-3P mimics significantly inhibited the activity of wilde type LINC00205 reporter, while the inhibition was abrogated when the predicted binding sites were mutated, indicating that miR-495-3P binds to LINC00205 through the predicted interaction sites (Figure 4D). Similarly, the predicted wild type binding sites between miR-495-3p and TNFSF15 mRNA and the mutated sequences were cloned into the luciferase reporter (Figure4E), and the same results were obtained for the validation of the interaction between miR-495-3P and TNFSF15 mRNA (Figure 4F). The above results imply that LINC00205 may function to adsorb miR-495-3P, which in turns regulates TNFSF15 expression.

DISCUSSION

In recent years, stroke has become the most significant health threat to the elder population. Stroke is a cerebrovascular disease caused by a lack of oxygen and nutrients supply to brain tissues due to the obstruction of cerebral blood flow, and cognitive impairment is one of the common complications following stroke (1). In addition, PSCI can lead to an increase in mortality and impact on the treatment compliance, thereby undermining the rehabilitation of motor and language functions and downgrading the life quality in PSCI patients (25, 26). Timely intervention is critical for the recovery from stroke-induced cognitive impairment.

Pharmacological treatment, rehabilitation and cognitive training have been implemented to improve the cognitive function in PSCI patients (27). Donepezil is a commonly used clinical acetylcholinesterase inhibitor, which can alleviate the oxidative stress after stroke and inhibit acetylcholine degradation to improve cognitive function (28). In recent years, HBO therapy has been implemented to improve cognitive

performance, prevent neurological degeneration and improve quality of life after traumatic brain injury and stroke (29, 30). HBO treatment opens up a new therapeutic avenue for the treatment of neurodegenerative diseases in the elderly, especially for the improvement of cognition and brain metabolism in patients with mild cognitive impairment. However, the molecular mechanisms contributing to the beneficial effect of HBO therapy remains unknown.

In this study, we randomized the recruited patients to exclude the confounding actors including age, gender, education level and living habits and assessed the effects of HBO therapy on the cognitive performance of PSCI patients in an unbiased manner. We observed an improved MMSE and MoCA scores in patients with PSCI in HBO group in comparison to the control group conventionally treated patients, which are consistent with previous studies in post-stroke patients (31, 32). These results suggest that HBO therapy combined with donepezil produces superior cognitive recovery in PSCI patients. Neuroinflammation is a significant detrimental factor contributing to the neurodegeneration after stroke (33). We found that there were reduced levels of pro-inflammatory factors in the serum of the HBO group when compared to the control group, while the levels of anti-inflammatory cytokines increased in the HBO group. Previous studies have reported that HBO therapy is able reduce the oxidative stress, inflammation and neuronal apoptosis, thereby improving functional recovery from stroke, which is consistent with our results (30, 34).

The dysregulation of lncRNAs has been implicated in the progression of ischemic stroke in clinical patients and the mouse model (35-37), suggesting that lncRNAs may serve as novel biomarkers and therapeutic targets for ischemic stroke. To explore the underlying mechanism of HBO, we performed high-throughput sequencing analysis of the RNA samples in the control and HBO groups. We identified LINC00205 as a downregulated lncRNA after HBO therapy, which was verified by qRT-PCR analysis. mRNA profiling identified TNFSF15 as a key downregulated gene in HBO samples. TNFSF15 is implicated in oxidative stress regulation and inflammatory response, and this gene could be induced by tumor necrosis factor (TNF) and IL-1α to activate NF-κB and MAPK signaling pathway and induce apoptosis in endothelial cells (38). In our data, TNFSF15 expression was significantly lower in the HBO group when compared to the samples before treatment or in the control group, and it is hypothesized that the lower levels of pro-inflammatory cytokines in the HBO group may be attributed to reduced TNFSF15 expression. Future work is warranted to validate the role of TNFSF15 in modulating neuroinflammation and cognitive recovery after stroke.

As our study suggested, the decreased TNFSF15 expression was correlated with reduced LINC00205 expression after HBO therapy. Importantly, through the ceRNA network analysis we further identified miR-495-3p as a connecting point between LINC00205 and TNFSF15. We showed that miR-495-3p could interact with both LINC00205 and TNFSF15 mRNA. miR-495-3p expression was heavily upregulated upon HBO therapy, which is in contrast to the downregulation of LINC00205 and TNFSF15. These findings suggest that LINC00205 may serve as a sponging factor to absorb miR-495-3p. The downregulation of LINC00205 after HBO therapy release more activity of miR-495-3p to repress TNFSF15 expression. Interestingly, other studies have demonstrated that miR-495-3p is downregulated in cardiomyocytes after ischemia-reperfusion induction, which can exacerbate inflammatory damages and promote cardiomyocyte injury (39, 40). Therefore, these studies and our finding altogether suggest a protective role of miR-495-3p in ischemic conditions, although the underlying mechanisms may be different in different tissues.

Although we provide a possible function and mechanism of LNC00205/miR-495-3p/TNFSF15 axis in HBO treatment of PSCI patients, there are some limitations in this study. Firstly, the functional role of miR-495-3p/LINC00205/TNFSF15 axis in cognitive recovery and HBO therapy after stroke should be validated in animal model. Next, there are other potential lncRNAs which may also play important roles in the cognitive recovery after stroke, and their functions should also be clarified in the future. Further, the recruitment of a large cohort of patients is required to assess the value of these lncRNAs as potential diagnostic marker for cognitive impairment after stroke.

In summary, we found that HBO therapy could significantly improve the cognitive performance of PSCI patients when compared to the conventional therapy. We also reported LNC00205/miR-495-3p/TNFSF15 axis as a potential regulatory ceRNA module underlying the beneficial effect of HBO therapy. Future work is warranted to further scrutinize the functional role of LNC00205/miR-495-3p/TNFSF15 axis in cognitive recovery after stroke.

Ethics Committee Approval: The usage of human samples was approved by the Ethics Committee of Shidong Hospital Affiliated to University of Shanghai for Science and Technology, with the approved number as 2019-KY007.

Informed Consent: The patients in this study were recruited in accordance with the Declaration of Helsinki's ethical principles and Good Clinical Practice guidelines.

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