Original Paper

An Intersectional Study of LncRNAs and mRNAs Reveals the Potential Therapeutic Targets of Buyang Huanwu Decoction in Experimental Intracerebral Hemorrhage

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Key Words

Intracerebral hemorrhage • Long non-coding RNA (lncRNA) • Messenger RNA (mRNA) • Expression profile • Intersectional study

Abstract

Background/Aims: Both experimental and clinical studies have revealed satisfactory effects of the traditional Chinese formula Buyang Huanwu decoction (BYHWD) in improving post-intracerebral hemorrhage (ICH) neurological deficiencies. However, the multifaceted mechanisms of BYHWD in ICH treatment are not comprehensively understood. The present study explored various therapeutic targets of BYHWD by using lncRNA and mRNA transcriptomics. **Methods:** lncRNA and mRNA microarrays were used to identify differentially expressed genes. ICH-induced upregulated genes (ICH vs sham) and BYHWD-induced downregulated genes (BYHWD vs ICH) were first identified. The intersection between these 2 sets was determined to identify ICH-induced highly expressed genes that were reversed by BYHWD. Then, the genes downregulated after ICH and the genes upregulated after BYHWD treatment were used to generate another set of intersections. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses were subsequently performed to determine relative biological functions and signaling transduction pathways according to genes within the intersections. Quantitative real-time PCR was used to validate changes in gene expression observed using the microarray. Finally, a lncRNA-mRNA co-expression network was established to identify links among the genes within the intersections. **Results:** A total of 18 differentially expressed lncRNAs and 33 differentially expressed mRNAs were identified using 2 lncRNA arrays (ICH vs sham and BYHWD vs ICH). The altered genes were enriched in the hemoglobin complex, oxygen transport and oxygen transporter and were closely

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associated with pyruvate metabolism. The co-expression network consisted of 53 nodes and 595 connections (308 positive interactions and 287 negative interactions). **Conclusion:** The hemoglobin complex, oxygen transport, oxygen transporter activity and pyruvate metabolism are possible therapeutic targets of BYHWD in ICH treatment. The present study provides the basis and direction for future investigations to explore the mechanisms by which BYHWD protects against long-term neurological deficiencies after ICH.

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Introduction

Intracerebral hemorrhage (ICH) remains the most lethal and least treatable subtype of stroke worldwide, resulting in a shocking 40% 30-day mortality and 80% disability rate [1–3]. However, advancements in medical and surgical interventions in the last 10 years have failed to produce dramatic improvements in outcomes after ICH, resulting in an unbearable burden [4–6]. Thus, milestones in ICH therapy, particularly for post-ICH neurological deficiencies, are eagerly anticipated.

Traditional Chinese medicine (TCM) has been employed to treat stroke in China for thousands of years [7]. TCM specifically shows satisfactory effectiveness for stroke survivors suffering from long-term motor dysfunctions, swallowing difficulties, cognitive disorders and dysarthria, which are difficult to resolve using Western medicine [8, 9]. Thus, TCM has been considered an important therapeutic alternative to Western medicine for treating stroke since the last century [10, 11]. TCM formula is a holistic system, and specific treatments are usually composed of several medicines containing hundreds of components. These components are mixed together to interact with one other to affect an unknown number of cellular targets, concurrently generating systematic effects [12, 13]. The multi-targeted effects of TCM have aroused growing interest in exploring the related mechanisms.

Buyang Huanwu decoction (BYHWD) is the most frequently prescribed formula for stroke treatment (40.32%) [14]. Evidence-based investigations have found that BYHWD improves neurological deficiencies in both experimental studies and clinical trials [15–17]. Unfortunately, present reports tend to focus on a single target or pathway, and none of these studies has been able to reveal multifaceted regulatory mechanisms of BYHWD after ICH.

Approximately 99% of mammalian RNA transcripts are noncoding RNAs (ncRNAs), and the majority of these are long noncoding RNAs (lncRNAs, > 200 nucleotides) [18]. lncRNAs are generally not translated into proteins but actively participate in many essential biological processes by controlling gene expression at the epigenetic, transcriptional and post-transcriptional levels [19]. Therefore, investigations from the perspective of interactions between lncRNAs and mRNAs may capture a more comprehensive regulatory

Fig. 1. Flowchart of the study. Differentially expressed lncRNAs and mRNAs from 2 intersections (ICH vs Sham and BYHWD vs ICH). GO and KEGG analyses were used to explore closely related biological roles and signaling pathways. qRT-PCR was used to validate the changes in genes based on the lncRNA microarrays. The co-expression network was constructed to explore potential interactions between genes.

network. Previous studies have found that calycosin (an important active component of BYHWD) exhibits cell proliferation-inhibiting and apoptosis-inducing effects through lncRNA HOTAIR [20]. Another active component of BYHWD, quercetin, is found to be involved in apoptosis promotion via the upregulation of lncRNA MALAT1 to inhibit PI3K/AKT signaling [21]. However, these single-compound studies are insufficient to reveal the neuroprotective mechanisms of BYHWD in ICH.

Transcriptomics is a powerful tool for detecting global alterations in RNA expression and, consequently, changes in the corresponding proteins [13, 22]. A transcriptomics study of Radix Astragali (RA) and Radix Angelicae Sinensis (RAS) (monarch and ministerial drugs of BYHWD) for treating osteoporosis found that the genes galectin-9, CCL-2, CCL-7 and CCL-8 are specifically regulated by these drugs [23]. Another transcriptomics study on a TCM formula containing RA and RAS detected 483 treatment-related genes in 63 functional categories and 25 cancer-related pathways. In addition, the most closely related three pathways are apoptosis, cell cycle regulation and the mitogen-activated protein kinase (MAPK) cascade [24]. Therefore, it is rational to believe that transcriptomics analysis of lncRNAs and mRNAs may help shed light on multifaceted mechanisms of TCM formulas by identifying exact therapeutic targets and their interactions [13].

In the present study, ICH-induced upregulated genes (ICH vs sham) and BYHWD-induced downregulated genes (BYHWD vs ICH) were first identified. Then, the intersection between these 2 sets was determined to identify aberrantly highly expressed genes that were reversed by BYHWD. In addition, the genes downregulated after ICH and the genes upregulated after BYHWD treatment were used to generate another set of intersections. Then, the related biological functions and pathways of the differentially expressed genes in the intersections were determined by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Finally, the lncRNA-mRNA co-expression network within the intersections was analyzed (Fig. 1). In this way, the post-ICH BYHWD-generated genetic changes and their related biological functions and signaling pathways were investigated. These findings will provide a more comprehensive perspective and profound understanding of the mechanism of BYHWD against long-term neurological deficiencies after ICH. Moreover, this study provides a relatively accurate direction for further investigations to identify definite mechanisms.

Materials and Methods

Animal Preparation

The Sprague-Dawley (SD) rats used in this study were 8 to 9 weeks of age (male, 180 g–220 g). The SD rats were cared for in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH Publication No. 85-23, revised 1996). The Committee on the Use and Care of Animals of Central South University (CSU) (201403164) approved the animal protocols. The rats were given free access to water and food and were housed at a room temperature of 25 °C on a 12 h light-dark cycle.

BYHWD preparation

The original composition of BYHWD from the “TCM Prescriptions Dictionary” is listed in Table 1. Dried crude herbs were obtained from the Chinese Medicinal Pharmacy of Xiangya Hospital, Changsha, China. Voucher specimens were deposited at the Laboratory of Institute of Integrative Medicine, CSU. The decoction was prepared and subjected to quality control as previously described. Before intragastric administration, the powder was dissolved in distilled water at a concentration of (0.13 g/ml).

ICH induction and animal groups

Animals were randomly assigned to the sham-operated group (short for the sham + vehicle group), ICH group (short for the ICH + vehicle group) and BYHWD group (short for the ICH + BYHWD-treated group) (n=3, per group) (for the randomization of animals, refer to Cheng T [25]). The rats were fixed in a prone

Table 1. Components of the Buyang Huanwu decoction. In the order listed in, herbs were combined in a 60:6:4.5:3:3:3 ratio (dry weight)

Latin name	Family	English name	Chinese name	Part used	Batch number
Radix astragali	Leguminosae	Astragalus mongholicus	Huang qi	Root	091201
Radix paeoniae Rubra	Ranunculaceae	Red peony root	Chi shao	Root	091214
Radix angelicae Sinensis	Umbelliferae	Angelica root	Dang gui	Root	091206
Rhizoma Ligustici Chuan xiong	Umbelliferae	Sichuan lovage	rhizome Chuanxiong	Root	100304
Flos Carthami	Feverfew	Carthamus tinctorius	Hong hua	Flower	100329
Semen persicae	Rosaceae	Peach seed	Tao ren	Seed	091218
Pheretima Aspergillum	Megascolecidae	Earthworm (Lumbricus)	Di long	Whole animal	091222

Table 2. Physiological variables of each experimental group (mean ± SD). Hct: hematocrit; values are expressed as the mean ± SD

	pH	pO ₂ (mmHg)	pCO ₂ (mmHg)	Hct (%)	Glucose (mg/dL)
Sham	7.40 ± 0.05	79.7 ± 4.2	44.3 ± 5.5	45.3 ± 6.7	98.6 ± 6.5
ICH	7.43 ± 0.02	81.7 ± 5.9	42.7 ± 3.2	45.6 ± 4.6	104.6 ± 5.4
ICH+BYHWD	7.42 ± 0.04	83.0 ± 7.1	42.3 ± 4.7	43.0 ± 5.3	107.3 ± 10.8

position on a stereotactic instrument (Stoelting Co., Chicago, IL, USA) after being deeply anesthetized with pentobarbital (65 mg/kg i.p.). Then, 2.5 µl of 0.5 U collagenase (type VII) in 0.9% sterile saline was slowly injected into the right globus pallidus (site: 1.4 mm posterior and 3.2 mm lateral to the bregma and 5.6 mm ventral to the cortical surface) at an even speed within 2 min by using a 26-gauge needle. After the infusion, the needle was kept in place for 5 min. For the sham-operated group, 2.5 µl of 0.9% sterile saline without type VII collagenase was infused into the same site in the same manner [26, 27]. For the ICH + BYHWD-treated group, intragastric administration of BYHWD (2.05 g/kg) was performed once a day for 21 days. The 4.36 g/kg dose of BYHWD was shown to be effective for mice in our previous study [28], and it was calculated at 2.05 g/kg for rats according to surface area conversion. In addition, this dose was confirmed to be effective by preliminary motor functional analysis in animals with ICH (unpublished data). Equal volumes of distilled water were administered to the sham and ICH groups once daily for 21 days. During the whole process, blood glucose, pH, PaO₂, PaCO₂, arterial blood pressure, and hematocrit levels in the animals were monitored (Table 2). Feedback-controlled heating pads were used to maintain the rectal temperature of animals at approximately 37.5 °C. Animals were sacrificed 21 days after ICH induction. Brain tissues around hematoma were obtained for subsequent analysis.

lncRNA and mRNA microarray

Total RNA was extracted from the right globus pallidus and purified by using an RNeasy Mini Kit (Qiagen, Redwood City, CA, USA). Arraystar Rat lncRNA microarrays (v2.0, containing 13611 lncRNAs and 24626 coding transcripts) were used to detect the expression of lncRNAs and mRNAs in a total of 9 rats (3 groups with 3 replicates). Tissue preparation and microarray hybridization were performed by using an Agilent Gene Expression Hybridization Kit (Agilent Technology, CA, USA). After washing, the arrays were scanned by an Agilent Microarray Scanner and finally analyzed by Agilent Feature Extraction software (version 10.5.1.1). Differentially expressed transcripts (thresholds of ≥ 1.3-fold and p values of < 0.05) were identified by comparing the ICH and sham control groups (ICH vs sham) and the ICH + BYHWD-treated and ICH groups (BYHWD vs ICH). Then, we determined the intersection between the upregulated transcripts in the ICH vs sham groups and the downregulated transcripts in the BYHWD vs ICH groups as well as the intersection between the downregulated transcripts in the ICH vs sham groups and the upregulated transcripts in the BYHWD vs ICH groups in order to identify the mechanism by which BYHWD reverses pathophysiological changes in ICH.

GO and KEGG pathway analyses

GO (<http://www.geneontology.org>) analysis was performed to determine the biological roles based on the molecular functions, biological processes, and cellular components of the aberrantly expressed mRNAs; p < 0.05 and FDR < 0.05 were used as thresholds to define markedly enriched GO terms/pathways. Pathway

analysis (based on KEGG, <http://www.genome.jp/kegg/>) was performed to explore the pathways significantly enriched in differentially expressed genes.

Quantitative real-time PCR validation

The portion of the samples remaining after lncRNA microarray was used for quantitative real-time polymerase chain reaction (qRT-PCR) validation. The method was described in Li Q and Wang J [29]. SuperScript III Reverse Transcriptase (Invitrogen, Grand Island, NY, USA) was used to reverse transcribe total RNA into cDNA according to the instructions of the manufacturer. Quantitative real-time (qRT)-PCR (Arraystar) was performed by using the Applied Biosystems ViiA 7 RT-PCR System and 2× PCR Master Mix. The reaction conditions were as follows: incubation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. The relative expression levels of lncRNAs were calculated by using the $2^{-\Delta\Delta Ct}$ method and were normalized by β -actin levels (R). The primers for each gene are listed in Table 3.

Table 3. Primers designed for qRT-PCR validation of candidate lncRNAs. Tm: temperature. bp: base pair

Gene name	Forward and reverse primer	Tm (°C)	Product length (bp)
β -actin (R)	F:5'CGAGTACAACCTTCTTGACGC3' R:5' ACCCATACCCACCATCACAC3'	60	202
ENSRNOT00000051535	F:5'AAGTCCGCTGTCCATTATCAG3' R:5'AAGTCCGCTGTCCATTATCAG3'	60	141
XR_145869	F:5'GTGCTGACGGAGGATACTGA3' R:5'CGCTTGTGTTTGTGAGGG3'	60	81
XR_347159	F:5'CCTTGCCAGTTGGAGAATG3' R:5'AGGACAAAGACACAGAATCAG3'	60	143
ENSRNOT00000076988	F:5'CTGGAGAAAAGATTTCGTGC3' R:5' ATGTTGGGTAGCGTTGAT3'	60	85
XR_593489	F:5'ATGCGATTTTCGGTGACTAG3' R:5' AGGAGTTGCTGTGGTTTGC3'	60	95

lncRNA and mRNA co-expression network

A co-expression network (CNC network) was constructed with 18 lncRNAs and 33 mRNAs from the intersections based on correlation analysis. The network was constructed by using Cytoscape software (version 2.8.3, The Cytoscape Consortium, San Diego, CA, USA). The 18 lncRNAs were represented by red squares, and 33 mRNAs were represented by blue nodes. Positive and negative relationships were represented by solid lines and dashed lines, respectively.

Statistics

SPSS (version 13.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The results were expressed as the mean $\pm$ SEM. Differences between the “sham-operated group” and the “ICH group” and between the “BYHWD group” and the “ICH group” were analyzed by using Student's t-tests. Spearman's correlation analysis was used to detect the relationship between lncRNAs and mRNAs. A p value of < 0.05 was regarded as statistically significant.

Results

Identification of mutually expressed lncRNAs and mRNAs among the three groups

In total, 18 differentially expressed lncRNAs were identified from 2 lncRNA arrays (ICH vs sham and BYHWD vs ICH, 3 rats in each group) with a fold-change cut-off of 1.3 ($p < 0.05$). Among them, 10 common lncRNAs were identified from the intersection of 131 upregulated lncRNAs (ICH vs sham) and 164 downregulated lncRNAs (BYHWD vs ICH) (Fig. 2A, Table 4). Another 8 common lncRNAs were identified from the intersection of 494 downregulated lncRNAs (ICH vs sham) and 133 upregulated lncRNAs (BYHWD vs ICH) (Fig. 2A, Table 4).

Additionally, 33 differentially expressed mRNAs were identified from 2 lncRNA arrays (ICH vs sham and BYHWD vs ICH, 3 rats in each group) with a fold-change cut-off of 1.3 ($p < 0.05$). The intersection of 367 upregulated mRNAs (ICH vs sham) and 175 downregulated mRNAs (BYHWD vs ICH) led to the identification of 22 common mRNAs (Fig. 2B, Table 5). The intersection of 459 downregulated mRNAs (ICH vs sham) and 260 upregulated mRNAs (BYHWD vs ICH) led to the identification of 11 common mRNAs (Fig. 2 B, Table 5).

Fig. 2. Hierarchical cluster heat map showing differentially expressed lncRNAs (A) and mRNAs (B) from 2 intersections (ICH vs Sham and BYHWD vs ICH). Each of the three adjacent columns represents samples from the sham-operated group, ICH group and BYHWD group in order. Each row represents genes with lncRNAs (A) and mRNAs (B) labeled in the right-most column. A bar representing expression level is shown in the upper right-most corner. Red indicates relatively high expression; green indicates relatively low expression; and black indicates no change.

GO and KEGG pathway analysis

In total, 33 mutually expressed mRNAs were subjected to GO analyses (<http://www.geneontology.org>). The results showed that the hemoglobin complex (ontology: cellular component, GO: 0005833), oxygen transport (ontology: biological process, GO: 0015671) and oxygen transporter activity (ontology: molecular function, GO: 0005344) were the top 3 GO terms (Fig. 3). In addition, KEGG pathway analysis identified pyruvate metabolism (pathway ID: rno00620) (Fig. 4).

Validation of the Microarray Data Using qRT-PCR

Five differentially expressed lncRNAs (ENSRNOT00000051535, ENSRNOT00000076988, XR_145869, XR_347159 and XR_593489) were randomly selected for analysis using RT-qPCR to validate the expression changes in lncRNAs detected by RNA-seq. The results of the two methods revealed similar expression patterns of the lncRNAs. Both RNA-seq and RT-qPCR showed that the expression of ENSRNOT00000051535, ENSRNOT00000076988, XR_347159 and XR_593489 was upregulated after ICH but downregulated after BYHWD treatment and that XR_145869 expression decreased after ICH but increased after BYHWD treatment (Fig. 5). RT-qPCR validation indicated good reliability and reproducibility of the lncRNA changes determined by RNA-seq.

lncRNA-mRNA co-expression network

We constructed a lncRNA and mRNA co-expression network based on the 18 differentially expressed lncRNAs and 33 interacting mRNAs. There were 53 nodes and 595 connections (308 positive interactions

Fig. 3. Gene ontology of differentially expressed mRNAs. The top 10 GO terms associated with the differentially expressed mRNAs. The differentially expressed mRNAs for GO analysis were obtained by comparing the sham, ICH and BYHWD groups.

Table 4. 18 differentially expressed lncRNAs identified from 2 lncRNA arrays (ICH vs sham, BYHWD vs ICH). Seq name: sequence name. P value: P value calculated by using unpaired t-tests. Fold change: the absolute ratio (no log scale) of the normalized intensities between two groups (ICH vs sham; BYHWD vs ICH). Chr: chromosome number from which the lncRNA is transcribed. Str: the strand of chromosome from which the lncRNA is transcribed; “+” represents the sense strand of the chromosome, and “-” represents the antisense strand of the chromosome. Relationship: intergenic, there are no coding transcripts within 30 kb of the lncRNA; intronic antisense, RNA molecules that are transcribed from the antisense strand without sharing overlapping exons; and natural antisense, RNA molecules transcribed from the antisense strand and overlapping in part with well-defined spliced sense or intronless sense RNAs. Sham 1 to 3, ICH 1 to 3 and BYHWD 1 to 3: normalized intensity of each sample (log2 transformed)

Seq name	P value		Fold change		Relation		Chr	Str	Relationship	Sham 1	Sham 2	Sham 3	ICH 1	ICH 2	ICH 3	BYHWD 1	BYHWD 2	BYHWD 3
	ICH vs Sham	BYHWD vs ICH	ICH vs Sham	BYHWD vs ICH	ICH vs Sham	BYHWD vs ICH												
XR_593021	0.030	0.013	1.378	1.338	up	down	Chr6	+	intergenic	4.01	3.58	3.84	4.39	4.25	4.17	3.83	3.74	3.99
ENSRNOT00000051535	0.0002	0.039	1.541	1.662	up	down	Chr14	-	intergenic	5.19	5.13	5.06	5.87	5.79	5.78	4.69	4.87	5.49
uc304+	0.012	0.024	1.390	1.351	up	down	Chr1	+	intergenic	5.14	5.41	5.40	5.90	5.77	5.70	5.21	5.30	5.56
XR_347118	0.011	0.045	1.64	1.34	up	down	Chr6	-	intergenic	4.75	4.88	5.10	5.52	5.48	5.87	5.19	5.07	5.34
XR_362144	0.042	0.004	1.400	1.315	up	down	Chr20	+	intergenic	5.20	5.65	5.70	5.91	6.00	6.01	5.55	5.68	5.56
XR_344582	0.045	0.014	2.004	1.646	up	down	Chr2	-	Intronic antisense	3.53	4.48	4.51	5.08	5.02	5.43	4.59	4.22	4.57
XR_593489	0.004	0.035	1.677	1.680	up	down	Chr7	+	intergenic	8.10	8.19	8.01	8.77	8.68	9.07	8.38	8.21	7.69
XR_590843	0.008	0.024	1.560	1.490	up	down	Chr12	+	intergenic	4.94	5.03	4.97	5.44	5.87	5.56	4.95	5.25	4.95
XR_347159	0.005	0.042	1.39	2.66	up	down	Chr6	-	intergenic	7.23	7.47	7.34	7.92	7.77	7.77	7.06	6.69	5.48
ENSRNOT00000076988	0.046	0.024	1.41	1.54	up	down	ChrX	+	intergenic	6.88	6.74	6.65	7.13	7.05	7.57	6.58	6.78	6.53
XR_601070	0.023	0.006	1.41	1.80	down	up	Chr5	+	natural antisense	6.18	6.20	6.06	5.76	5.38	5.60	6.51	6.64	6.64
XR_145869	0.019	0.016	1.56	1.99	down	up	Chr3	-	intergenic	9.72	9.82	10.01	9.47	8.97	9.20	10.33	9.80	10.48
XR_358600	0.020	0.000	1.49	1.41	down	up	Chr12	-	intergenic	5.81	6.10	5.60	5.36	5.24	5.19	5.74	5.74	5.79
XR_596425	0.041	0.009	1.60	1.51	down	up	Chr16	+	intergenic	6.02	5.28	5.48	5.02	4.90	4.82	5.45	5.35	5.73
XR_598484	0.002	0.044	2.10	1.80	down	up	Chr19	+	natural antisense	5.28	5.14	5.07	4.13	4.61	3.70	4.81	4.95	5.24
NR_027877	0.038	0.000	1.31	1.39	down	up	Chr1	+	exon sense-overlapping	5.09	5.10	4.71	4.60	4.55	4.58	5.07	5.00	5.13
XR_590556	0.007	0.024	1.39	1.51	down	up	Chr1	-	intergenic	6.72	6.64	6.60	6.22	6.31	6.02	7.06	6.61	6.65
XR_589953	0.007	0.044	2.22	1.70	down	up	Chr1	-	intergenic	4.11	4.64	4.82	3.40	3.50	3.22	3.85	3.93	4.64

Table 5. 33 differentially expressed mRNAs identified from 2 lncRNA arrays (ICH vs sham, BYHWD vs ICH). Seq name: sequence name. P value: P value calculated by using unpaired t-tests. Fold change: the absolute ratio (no log scale) of normalized intensities between two groups (ICH vs sham; BYHWD vs ICH). Chr: chromosome number from which the mRNA is transcribed. Str: the strand of chromosome from which the mRNA is transcribed; “+” represents the sense strand of the chromosome, and “-” represents the antisense strand of the chromosome. Gene symbol: gene name. Sham 1 to 3, ICH 1 to 3 and BYHWD 1 to 3: normalized intensity of each sample (log2 transformed)

Seq name	P value		Fold change		Relation		Chr	Str	Gene symbol	Sham 1	Sham 2	Sham 3	ICH 1	ICH 2	ICH 3	BYHWD 1	BYHWD 2	BYHWD 3
	ICH vs Sham	BYHWD vs ICH	ICH vs Sham	BYHWD vs ICH	ICH vs Sham	BYHWD vs ICH												
ENSRNOT00000064182	0.033	0.035	1.31	1.38	up	down	Chr3	-	Slc98a11	5.89	5.51	5.61	6.08	6.00	6.12	5.34	5.61	5.83
NM_001107477	0.000	0.005	2.45	1.95	up	down	Chr1	+	Zfp329	5.74	5.59	5.76	6.94	6.88	7.15	5.79	5.98	6.30
NM_001170604	0.036	0.023	1.44	1.36	up	down	Chr16	-	Il12rb1	3.93	4.38	4.34	4.70	4.62	4.92	4.35	4.13	4.42
NM_012625	0.006	0.018	1.45	1.46	up	down	Chr7	+	Pnch	12.95	13.61	12.96	13.70	13.43	13.56	13.21	13.04	13.79
NM_013414	0.022	0.022	1.57	1.34	up	down	Chr2	-	RbpA	5.88	5.89	5.81	6.68	6.48	6.37	5.95	6.16	6.16
NM_001108665	0.023	0.013	1.85	1.46	up	down	Chr5	-	Myp50	10.08	9.24	9.58	10.53	10.47	10.56	10.12	10.08	9.73
NM_001001012	0.037	0.016	1.55	1.36	up	down	Chr10	+	Ole1405	9.28	8.66	8.86	9.48	9.46	9.76	9.05	9.10	9.22
NM_024402	0.022	0.047	1.40	1.34	up	down	ChrX	-	Alkap4	4.29	4.24	4.55	4.67	4.88	4.98	4.41	4.22	4.64
NM_198776	0.003	0.025	2.29	1.90	up	down	Chr1	-	Hbb-b1	9.29	9.30	9.18	10.64	10.08	10.62	9.69	9.74	9.13
ENSRNOT00000067996	0.000	0.010	2.26	1.77	up	down	Chr8	-	RGD1560775	4.66	4.56	4.47	5.68	5.82	5.74	5.15	5.04	4.58
NM_001135002	0.005	0.036	1.56	1.36	up	down	Chr20	+	Opld2	5.32	5.65	5.39	6.16	5.97	6.15	5.41	5.83	5.73
NM_001001428	0.015	0.023	1.41	1.43	up	down	Chr20	+	Ole1748	7.03	7.16	6.99	7.34	7.69	7.64	7.16	7.10	6.86
NM_001108932	0.008	0.002	1.32	1.32	up	down	Chr20	+	Tymd1	7.11	7.40	7.27	7.66	7.65	7.68	7.33	7.29	7.15
NM_001077666	0.013	0.014	1.33	1.37	up	down	Chr11	+	RGD1308065	8.31	8.21	8.05	8.59	8.50	8.72	8.27	8.21	7.97
NM_001100479	0.005	0.017	1.64	1.49	up	down	Chr13	-	Pcp4	8.00	8.36	8.07	8.74	8.87	8.97	8.29	8.43	8.02
NM_172223	0.004	0.004	1.61	1.72	up	down	Chr3	-	Pump4	9.04	8.98	8.87	9.58	9.53	9.86	8.88	9.02	8.72
NM_012624	0.021	0.040	1.33	1.30	up	down	Chr2	+	Pkr	4.23	4.08	4.23	4.39	4.67	4.71	4.14	4.11	4.37
NM_001111269	0.032	0.049	2.91	2.08	up	down	Chr1	-	LOC609604	8.11	7.87	7.26	9.47	8.31	9.29	8.07	8.13	7.71
NM_00109673	0.012	0.007	1.44	1.46	up	down	Chr9	-	Fat2	8.76	8.61	8.50	9.28	8.96	9.23	8.64	8.67	8.52
NM_001047942	0.033	0.017	1.33	1.37	up	down	Chr2	+	LOC499541	7.25	7.61	7.63	7.96	7.84	7.93	7.26	7.45	7.64
NM_001013053	0.010	0.033	1.57	1.42	up	down	Chr10	-	Hba-a1	9.62	9.62	9.62	10.44	10.37	9.99	9.87	9.79	9.63
ENSRNOT0000004526	0.030	0.005	1.31	1.52	up	down	Chr12	+	Gtd1	5.78	5.43	5.48	5.99	5.86	6.02	5.18	5.53	5.34
NM_053798	0.000	0.045	1.33	1.47	down	up	Chr8	+	Sacm1	9.80	9.85	9.75	9.38	9.42	9.36	10.22	10.04	9.57
NM_001108588	0.33	0.030	2.42	2.10	down	up	Chr3	+	Slc5a12	5.33	4.61	4.69	4.12	3.67	3.01	4.76	4.61	4.66
NM_198780	0.012	0.025	1.46	1.37	up	down	Chr1	+	Poi1	5.17	5.00	4.95	4.43	4.70	4.36	4.84	4.52	5.09
NM_001001281	0.017	0.046	2.06	1.66	down	up	Chr1	-	Ole75	4.53	4.85	4.58	3.88	3.81	3.10	4.25	4.44	4.30
NM_001272262	0.023	0.039	1.56	1.73	down	up	Chr1	-	Tnfr1	7.77	7.16	7.35	6.77	6.83	6.74	7.09	7.65	7.98
ENSRNOT00000041583	0.047	0.044	1.67	1.47	down	up	Chr2	-	AABBG02013.1	4.97	4.17	4.52	3.84	3.63	3.91	4.03	4.61	4.62
NM_001108198	0.041	0.016	1.64	2.71	up	down	Chr9	-	Gucyl1	6.11	6.05	6.12	5.91	5.44	5.30	7.48	7.08	6.41
NM_017300	0.026	0.015	1.57	1.62	down	up	Chr5	-	Baat	6.47	6.25	5.95	5.36	5.61	5.75	6.24	6.50	6.07
NM_001014024	0.019	0.035	1.39	1.33	down	up	Chr1	+	Ora3	8.32	8.22	8.30	7.91	7.93	7.56	8.22	8.12	8.30
NM_001008086	0.006	0.000	1.46	2.14	down	up	Chr20	+	RT1-53	8.07	7.98	7.87	7.26	7.51	7.52	8.62	8.66	8.51
ENSRNOT0000006904	0.008	0.013	1.37	1.70	down	up	Chr2	-	Ipo11	6.36	6.48	6.58	5.89	6.02	6.14	6.78	6.90	6.67

and 287 negative interactions) in this co-expression network. Among these connections, NM_198780 (Gene symbol PCK1) and XR_598484 (Gene symbol: LOC103694317) had the closest relationship, with a PCC of 0.984 (Fig. 6).

Fig. 4. KEGG pathway analysis and detailed network of “pyruvate metabolism”. KEGG pathway analysis identified pyruvate metabolism as the most correlated signaling pathway. Each green box represents a specific gene or key enzyme. Each white box represents a species-specific pathway. Black outlines represent the lack of significant change. The representative significance of each related symbol is shown below. The differentially expressed mRNAs for KEGG pathway analysis were obtained by comparing the sham, ICH and BYHWD groups.

Fig. 5. qRT-PCR validation of 5 randomly selected lncRNAs from the intersection. The results showed that the expression of the lncRNAs ENSRNOT00000051535, ENSRNOT00000076988, XR_347159 and XR_593489 was upregulated after ICH but downregulated after BYHWD treatment, whereas the expression of XR_145869 decreased after ICH but increased after BYHWD treatment. The trends in expression of randomly selected lncRNAs detected by qRT-PCR were similar to those detected by the lncRNA microarray. The remaining portions of the samples from the lncRNA microarray were used for quantitative real-time polymerase chain reaction (qRT-PCR) validation.

Discussion

This study is the first to identify intersections between lncRNA and mRNA expression profiles in experimental ICH treated with BYHWD. Eighteen differentially expressed lncRNAs and 33 differentially expressed mRNA were identified from 2 lncRNA arrays (ICH vs sham and BYHWD vs ICH). The functions of the altered genes from the intersections were

Fig. 6. Co-expression network of lncRNAs and mRNAs from the 2 intersections. Red dots represent lncRNAs, and blue dots represent correlated mRNAs. There were 53 nodes and 595 connections (308 positive interactions and 287 negative interactions) in this co-expression network.

enriched in the hemoglobin complex, oxygen transport and oxygen transporter activity. In addition, these genes were most closely associated with pyruvate metabolism. The genes at the intersections and their related biological functions and pathways might indicate the potential therapeutic targets of BYHWD in ICH treatment.

The hemoglobin complex, oxygen transport and oxygen transporter activity were the top 3 GO terms. All of these molecules and processes involve the same 2 genes, namely, HBA-A1 and HBB-B1. These genes were upregulated after ICH. Hemoglobin (Hb) is a gas transporter with two α chains (hemoglobin alpha [HBA-A1]) and two β chains (hemoglobin beta [HBB-B1]). Each chain contains a heme subunit that binds to and delivers oxygen; four oxygen molecules are transported per molecule of hemoglobin [30, 31]. After ICH, high levels of Hb at lesions usually arise from the following 2 sources: the release of a large fraction of Hb from the hematoma after red blood cell lysis [32, 33] and the expression of the non-erythroid fraction of Hb in neurons and activated macrophages in response to hypoxia inducible factor-1 (HIF-1 α) and erythropoietin (EPO) [34–36]. Heme and iron are also found to significantly increase mRNA levels of HBA and HBB in neurons and glial cells of ipsilateral basal ganglia after ICH [37]. In addition, Hb is a potent cytotoxic chemical [38]. Severe brain edema and high levels of sodium ions are detected 24 h after cerebral infusion of Hb [39]. Other studies have found that Hb and its degradation products, such as iron, carbon monoxide, and biliverdin, participate in oxidative damage and inflammation responses, which result in cell death [32, 40]. Dead cells further trigger a variety of “danger” signals to activate the infiltration of leukocytes and microglia into the cerebral parenchyma, thereby

aggravating inflammatory injury [32, 41].

Previous studies have demonstrated the accumulation of lactate, which results in low pH values in the hematoma and perihematomal regions [42], as also demonstrated by our group (unpublished data). According to the Bohr effect, an acidic environment causes oxygen to dissociate from hemoglobin, leading to increased deoxyhemoglobin and dissociated oxygen. These data support the lack of hypoxia in perihemorrhagic areas [43]. However, excess levels of oxygen usually generate superoxides [44]. When the production of superoxides exceeds the capacity of antioxidant enzymes, oxygen free radicals can inactivate various enzymes, destroy membrane functions, cause DNA damage, and finally result in neuronal death [27, 44, 45]. Hyperoxia exposure in ischemic rats results in significant hippocampal neuronal death and behavioral deficiencies at 7 and 30 days [46]. Hyperoxia is also found to worsen neurological outcomes in a rat model of traumatic brain injury with hemorrhagic shock [47]. Other clinical studies have confirmed that hyperoxia is independently correlated with long-term poor neurological outcomes and in-hospital mortality among patients with brain injury [48, 49]. Therefore, excessive oxygen is a major factor mediating Hb-induced neurotoxicity.

Increased Hb levels not only induce oxygen toxicity to kill neurons but also promote the overexpression of nitric oxide synthase (NOS) and nitric oxide (NO) to disrupt the blood-brain barrier (BBB) [50]. Cerebral Hb injection-enhanced BBB permeability is mediated by increased NOS and NO levels through the regulation of tight junction proteins (zonula occludens-1, claudin-5, occludin, and junctional adhesion molecule-1). In addition, brain edema formation due to BBB leakage is one of the most lethal secondary injuries after ICH and is believed to be a strong predictor of adverse functional outcomes [51]. Moreover, endothelial cytotoxicity can become aggravated when the free radical NO and superoxide exist together and act in a synergistic manner [52]. This may lead to further BBB impairment and more severe cerebral edema. Thus, Hb-induced brain injury is partly mediated by exceedingly high levels of oxygen and NO.

After BYHWD administration, as the levels of HBA-A1 and HBB-B1 decreased, the hemoglobin complex level, oxygen transport and oxygen transporter activity decreased as well, followed by the alleviation of a series of impairments induced by Hb and peroxidation. Therefore, it is reasonable to hypothesize that BYHWD reverses Hb-induced oxidative damage after ICH by regulating the HBA-A1 and HBB-B1 genes. A series of data has demonstrated antioxidative functions of BYHWD. Pretreatment with BYHWD greatly reduces the level of reactive oxygen species (ROS) in kidneys of rats after brain death [53]. BYHWD also enhances the activity of superoxide dismutase (SOD) in rats with myocardial ischemia [54]. *In vitro*, BYHWD protects Schwann cells from hydrogen peroxide (H₂O₂) damage [55]. BYHWD also decreases H₂O₂-induced apoptosis in human umbilical vein endothelial cells (HUVECs) [56]. Additionally, pretreatment with BYHWD significantly decreases neuronal NOS activity in the striatum cortex and caudate putamen in rats with permanent focal cerebral ischemia [57]. BYHWD also rescues the myocardium from ischemia by inhibiting the expression of NO and NOS [58]. More importantly, it can be deduced that both oxygen and NO-mediated cytotoxicity result from excess Hb, which is transcribed from the HBA-A1 and HBB-B1 genes. Therefore, in addition to its antioxidative effect, BYHWD is believed to directly control the expression of HBA-A1 and HBB-B1. However, the underlying mechanisms responsible for this effect are still not fully understood. Fortunately, the findings of this study indicate some therapeutic targets of BYHWD and provide interesting directions for future investigations.

KEGG pathway analysis indicated that the genes in the intersection were closely associated with pyruvate metabolism. Phosphoenolpyruvate carboxykinase 1 (PCK1) and pyruvate kinase in liver and red blood cells (PKLR and PK) are involved in this pathway. Our results showed that PCK1 expression decreased after ICH but increased after BYHWD administration, which was opposite to the pattern of PK expression. During the process of pyruvate metabolism, PCK1 catalyzes the generation of phosphoenolpyruvate from oxaloacetate, and PK subsequently catalyzes the transformation of phosphoenolpyruvate to pyruvate. Oxidative stress induced by Hb exerts a dominant inhibitory effect on the endogenous PCK1 gene [59]. PK is abundantly expressed on red blood cells (RBCs). Thus,

high levels of PK levels are released from RBCs in hematomas. Although PK is upregulated after ICH, the reduced levels of PCK1 are unable to catalyze the production of sufficient phosphoenolpyruvate, the substrate of PK. As a result, low levels of pyruvate are generated after ICH. Pyruvate can ameliorate ICH-induced brain injury and protect neurons from Hb-induced apoptosis [60]. Therefore, changes in PCK1/PK facilitate neuroprotection following ICH.

Our study found that BYHWD increased the mRNA level of PCK1. This increase might be associated with the reduction in ROS levels by BYHWD, which ameliorates the effects of oxidative stress on PCK1. In addition, CNC network analysis indicated that PCK1 was positively correlated with lncRNA LOC103694317. These findings imply that BYHWD might regulate PCK1 mRNA expression via LOC103694317. However, it is difficult to interpret the decrease in PK after BYHWD treatment based on the present evidence. According to the CNC network, PK mRNA was negatively correlated with XR_358600 (Gene symbol: LOC102546487, PCC: -0.878). It is possible that BYHWD downregulates PK by modulating LOC102546487. In summary, BYHWD might partly rescue the disruptions in pyruvate metabolism by regulating the mRNA expression of the key enzymes PCK1 and PK.

Conclusion

In conclusion, the present study is the first to demonstrate potential therapeutic targets of BYHWD in ICH treatment at the mRNA and lncRNA levels. These targets are the hemoglobin complex, oxygen transport, oxygen transporter activity and pyruvate metabolism. The findings of the present study provide a basis and direction for future investigations to explore the mechanisms by which BYHWD protects against long-term neurological deficiencies after ICH.

Disclosure Statement

No conflict of interests exists.

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YW, TT and HJC designed the experiments. HJC, TL, PFL, ALY, HJZ, EH and WH performed the experiments. YW, TT, HJC and JKL analyzed the data. TL, PFL, ALY, HJZ and JKL provided insights on the interpretation of the results. HJC wrote the manuscript. YW and TT supervised the whole experimental process and the preparation of the manuscript. All authors read and approved the final manuscript.

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