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Original Article

Correlation analysis of miRNA-124, miRNA-210 with brain injury and inflammatory response in patients with craniocerebral injury

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Abstract: Objective: To explore the correlation of serum levels of microRNA (miRNA)-124 and miRNA-210 with brain injury and inflammatory response (IR) in patients with craniocerebral injury (CI) at early stage. Material and methods: Clinical data of 105 patients with CI (case group) admitted to our hospital from January 2018 to January 2020 were retrospectively analyzed. The other 60 non-CI healthy patients underwent physical examination were selected as the healthy group. The serum levels of miRNA-124 and miRNA-210 were detected by real-time fluorescence quantitative polymerase chain reaction (RT-PCR). Results: The levels of serum miRNA-124 and miRNA-210 as well as the inflammatory molecules Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), MEK, and extracellular signal-regulated kinases 1/2 (ERK1/2) in the peripheral blood of the case group were higher than those in the healthy group (P<0.05). Additionally, the serum levels of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), S100B, Tau, macrophage inflammatory protein-1α (MIP-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in the case group were higher than those in the healthy group (P<0.05). The levels of miRNA-124 and miRNA-210 were positively correlated with the serum levels of UCH-L1, GFAP, S100B, Tau, MIP-1α, IL-1β, IL-6, and TNF-α (P<0.05) as well as with the levels of JAK2, STAT3, MEK, and ERK1/2 in the peripheral blood (P<0.05). Conclusion: The elevated levels of serum miRNA-124 and miRNA-210 in patients with CI are closely related to the aggravation of brain injury, overactivation of the IR, and prognosis.

Keywords: Craniocerebral injury, miRNA-124, miRNA-210, brain injury, inflammatory response, correlation

Introduction

Craniocerebral injury (CI) is a common type of severe trauma with the highest incidence, accounting for 9%-21% of systemic trauma, leading to high rates of death and disability [1]. The primary CI caused by external violence and the secondary CI caused by local toxic and side metabolic reactions and inflammatory reactions after injury aggravate the neurological function impairment, with multiple injury factors and complex mechanisms, leading to poor prognosis of most patients with CI [2, 3]. After the occurrence of CI, patients are prone to serious complications such as cerebral infarction, cerebral vasospasm, adrenocortical insufficiency and epilepsy [4]. Some CI patients may suffer persistent functional impairments, such as mood abnormalities, personality changes, and cognitive impairments, even if they get timely treatment.

The inflammatory cytokines such as macrophage inflammatory protein-1α (MIP-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), are involved in the pathophysiological process of post-traumatic CI, affecting the prognosis of patients [5, 6]. MicroRNAs (miRNAs) are small-molecule RNAs that regulate cell proliferation, differentiation, and apoptosis by regulating gene expression, some of which are involved in regulating

nervous system development, signaling, and other functions [7]. Recently, study has found that miRNA-124 is abnormally expressed in peripheral blood and cerebral vascular endothelial cells after cerebral ischemia, and regulates a variety of pathophysiological processes involved in the pathogenesis of age-related ischemic encephalopathy, which functions in regulating autophagy, neuroinflammation, oxidative stress, neuronal excitability, neurological differentiation, etc.; However, it may play a dual role by regulating apoptosis and adversely affect synaptic plasticity and axon growth [8]. MiRNA-210 has been identified as the main miRNA induced by hypoxia. Studies on multiple miR-210 targets suggest that miR-210 not only plays an important role in mitochondrial metabolism, but also participates in angiogenesis, DNA damage response, cell proliferation and apoptosis [9].

In addition, previous studies have also investigated the correlation between serum miRNA-124, miRNA-210 expression levels and inflammatory factors [10, 11]. In the early stage of CI (within 24 h), cerebral ischemia leads to cascade reactions of cerebral ischemia such as mitochondrial dysfunction and imbalance of ion homeostasis, leading to apoptosis of a large number of nerve cells. Inflammation plays a key role in the secondary CI. Inflammation can induce a series of reactions, such as the expression of adhesion molecules and the secretion of inflammatory factors after CI and promote cell apoptosis. In patients with severe CI, due to severe cerebral ischemia and hypoxia, anaerobic metabolism is enhanced, and a large amount of glycolysis products are accumulated, resulting in brain tissue edema and inflammatory infiltration, aggravating the severity of CI and affecting the prognosis of patients. Therefore, it was hypothesized that the expression of serum miRNA-124 and miRNA-210 levels were altered in patients with CI and were closely associated with CI, inflammatory response (IR), and patients' prognosis. For this purpose, this study assessed the correlation of serum miRNA-124 and miRNA-210 levels with IR indicators in patients at early CI stage.

Materials and methods

Baseline data

Clinical data of 105 patients with CI (case group) admitted to our hospital from January 2018 to January 2020 were retrospectively analyzed. The case group consisted 39, 31, and 35 cases of mild, moderate, and severe injury, respectively. There were 72 traffic injuries, 16 fall-related injuries, 12 high falling injuries and 5 others, including 102 cases of closed injury and 3 cases of open injury. The complications: 6 cases of cerebral swelling, 3 cases of traumatic cerebral infarction, 1 case of diffuse axonal injury, and 1 case of acute subdural effusion; Single or combined hemorrhage: 30 cases of subarachnoid hemorrhage, 3 cases of basal ganglia hemorrhage, and 2 cases of ventricular hemorrhage; Fracture condition: 46 cases of skull fracture, 27 cases of simple or combined skull base fracture.

Inclusion criteria: (1) patients diagnosed as CI using CT and MRI, (2) patients with a history of definite craniocerebral trauma, (3) patients with Glasgow Coma Score (GCS) ranging from 3 to 15, (4) patients without shock, (5) patients without other combined site injuries, (6) patients who were admitted for examination within 24 h after injury, (7) patients with no bleeding tendency, and (8) patients with normal cognitive function before illness. Exclusion criteria: (1) patients with a history of previous cerebrovascular disease, craniocerebral surgery, or intracranial tumors; (2) patients with concurrent malignant tumors, intracranial infections, acute and chronic systemic infections, autoimmune disease, hematopoietic system disease; (3) patients with a history of chronic alcohol or drug abuse; (4) patients with pathological CI; (5) patients complicated with Alzheimer's disease; (6) patients with a recent use of antibiotics and anticoagulant drugs; and (7) patients with a history of hypertension, heart disease or diabetes mellitus.

Another 60 non-CI healthy subjects underwent physical examination with matched age and gender were selected as the healthy group. This study was approved by the Ethics Committee of the Second People's Hospital of Dongying (Approval No. 2017-005). All study participants provided written informed consent before participating in the study.

Methods

Levels of miRNA-124 and miRNA-210

RNA extraction and purity detection: First, 2 mL of venous blood was extracted from patients

Table 1. Primer sequences of internal reference β-actin and miRNA-124, miRNA-210

Primer		Primer sequence	Product length
β-actin	Forward	5'-ATGTCACGCACGATTTCC-3'	560 bp
	Reverse	5'-CTGTCCCTGTATGCCTCTG-3'	
miRNA-124	Forward	5'-TTAATGCTAATCGTGACT-3'	325 bp
	Reverse	5'-ACCTGAGAGTAGACCAGA-3'	
miRNA-210	Forward	5'-GCCCATCCTCAAATACAAAGC-3'	310 bp
	Reverse	5'-GGTCCTGAACACAAAATGAGC-3'	

within 24 h after injury, and venous blood was extracted routinely from health subjects after they arrived at the hospital. Blood samples were centrifuged at 3000 r/min at 4°C for 10 min, and the obtained serum was centrifuged again at 3000 r/min at 4°C for 10 min, and then frozen at -80°C in an RNA enzyme tube. The mirVANA extraction kit was used to extract total RNA according to the manufacturer's instructions. Next, 100 μL of mirVANA extraction solution was mixed well with an equal amount of denaturing solution, followed by incubation on ice for 5 min. An equal volume of acid/phenol/chloro/imide was added, mixed, frozen, and centrifuged for 5 min for three cycles. Anhydrous ethanol at 1.25 times of the volume was added and mixed. After the target protein passed through the column, the column was washed with the binding buffer until the baseline value was reached. Samples were added into the wells of a 1 g/mL agarose gel followed by electrophoresis at 70 V for 15 min. The extracted total RNA was diluted with 10 mmol/L Tris-HCl (pH 7.0), and the optical density values at 260 nm and 280 nm were measured using a UV spectrophotometer. A ratio of 1.7 to 2.0 was considered acceptable purity.

RNA reverse transcription: Reverse transcription was performed according to the manufacturer's instructions, with β-actin as an internal reference. The primer sequences were listed in **Table 1**. First, 3 μL of each sample was used for reverse transcription. The PCR system contained 10 μL 2× TaqMan PCR premix, 7.67 μL water, 1 μL 20× miRNA specific primers, and 1.33 μL cDNA. The PCR conditions comprised 50 cycles of denaturation at 95°C for 10 min, 95°C for 15 s, and annealing at 60°C for 1 min, and the reaction was terminated at 4°C.

Calculation of miRNA-124 and miRNA-210 levels: After electrophoresis, the optical densities of miRNA-124 and miRNA-210 (ΔCt) were determined using an imaging system with β-actin as an internal reference. $2^{-\Delta Ct}$ was used to calculate the relative level.

Expression of inflammatory signaling related molecules in the peripheral blood

First, 2 mL of peripheral venous blood was collected within 24 h after injury, and venous blood was extracted routinely from health subjects after they arrived at the hospital. RNA was isolated using a whole blood RNA extraction kit and cDNA synthesis kit. The RNA was reverse-transcribed into cDNA and amplified by PCR to calculate the levels of Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), MEK, and extracellular signal-regulated kinases 1/2 (ERK1/2).

Indicators of CI

Venous blood (5 mL) was extracted from the patients within 24 h after injury, and extracted routinely from health subjects after they arrived at the hospital. Blood samples were centrifuged at 3000 r/min for 10 min. The levels of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), S100B, and Tau were determined by the enzyme-linked immunosorbent assay (ELISA). ELISA kits were purchased from R&D Companies, Inc. (Houston, TX, USA), and the kit instructions were strictly followed [12]. Higher levels of CI indicators indicate more severe injury.

Indicators of inflammation factors

The levels of MIP-1α, IL-1β, IL-6, and TNF-α in serum were determined by ELISA according to the instructions (R&D Companies, Inc., Houston, TX, USA) [13].

Evaluation criteria

(1) Serum levels of miRNA-124 and miRNA-210 levels as well as those of JAK2, STAT3, MEK, and ERK1/2 were compared between the case group and healthy group. (2) The levels of CI indicators, UCH-L1, GFAP, S100B, and Tau were assessed in the two groups. (3) The indices of serum inflammation factors, MIP-1α, IL-1β, IL-6, IL-8, and TNF-α were determined in two

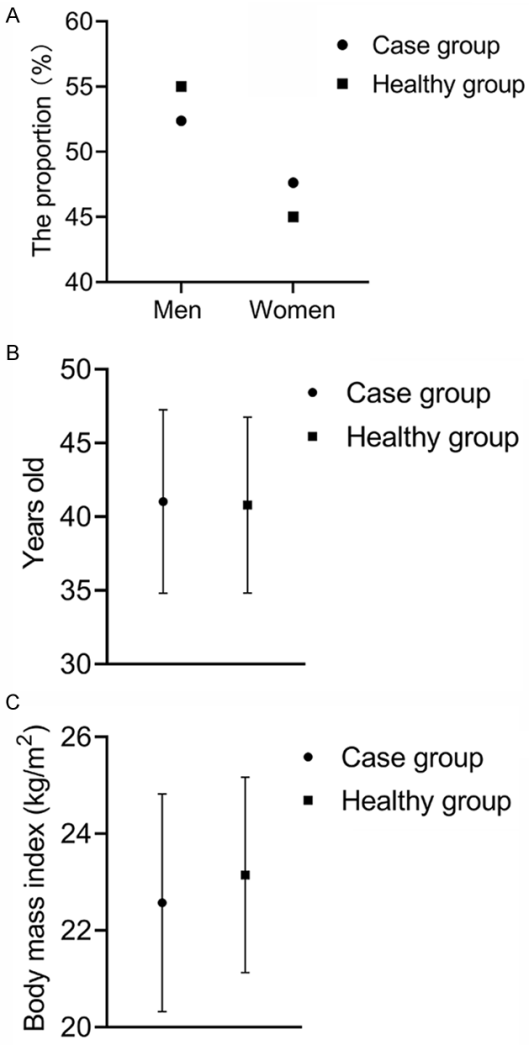

Figure 1. Comparison of baseline data between the two groups. No statistically significant difference was observed in terms of (A) gender, (B) age, and (C) body mass index (BMI) between the two groups.

groups. (4) The correlation between the levels of miRNA-124 and miRNA-210 and those of JAK2, STAT3, MEK, and ERK1/2 was analyzed. (5) The correlation between the levels of miRNA-124 and miRNA-210 and those of CI indicators was examined. (6) The correlations between the levels of miRNA-124, miRNA-210 and those of the serum inflammation factors were analyzed. (7) The prognosis of each patient was followed up and assessed using the Glasgow Outcome Scale-Extended (GOS-E) as shown below: good recovery (5 points), mild disability (4 points), severe disability (3 points), vegetative survival (2 points), and death (1 point). The levels of miRNA-124 and miRNA-210 were determined in patients with poor prognosis (1-3 points) as well as good prognosis (4-5 points), and the correlation between miRNA-124 and miRNA-210 levels and prognosis of patients was analyzed.

Statistical analysis

The data were analyzed using SPSS 25.0 statistical software. Measurement data were expressed as mean ± standard deviation. Normally distributed data were compared using *t* tests, while non-normally distributed data were analyzed using non-parametric Mann-Whitney U tests. One-way analysis of variance was used for multiple group comparison. Correlations were analyzed with Spearman correlation coefficient. $P<0.05$ was used to indicate statistical significance.

Results

Comparison of the baseline data between the two groups

There was no statistically significant difference in terms of gender, age, and body mass index (BMI) between the two groups (all $P>0.05$, **Figure 1**).

Comparison of the levels of miRNA-124, miRNA-210, and peripheral blood inflammatory signaling related molecules between the two groups

Serum levels of miRNA-124, miRNA-210, JAK2, STAT3, MEK, and ERK1/2 in the case group were higher than those in the healthy group ($P<0.05$, **Figure 2**). The expression levels of serum miRNA-124 and miRNA-210 and the expression of inflammation signaling related molecules JAK2, STAT3, MEK and ERK1/2 were lowest in the mild group, followed by the moderate group, and were highest in the severe group ($P<0.05$). With the aggravation of CI, the expression of serum miRNA-124 and miRNA-210 levels and the expression of JAK2, STAT3, MEK and ERK1/2 in peripheral blood were significantly increased (all $P<0.05$, **Table 2**).

Comparison of the serum levels of CI indicators between the two groups

Serum levels of UCH-L1, GFAP, S100B, and Tau in the case group were higher than those in the healthy group (all $P<0.05$, **Figure 3**).

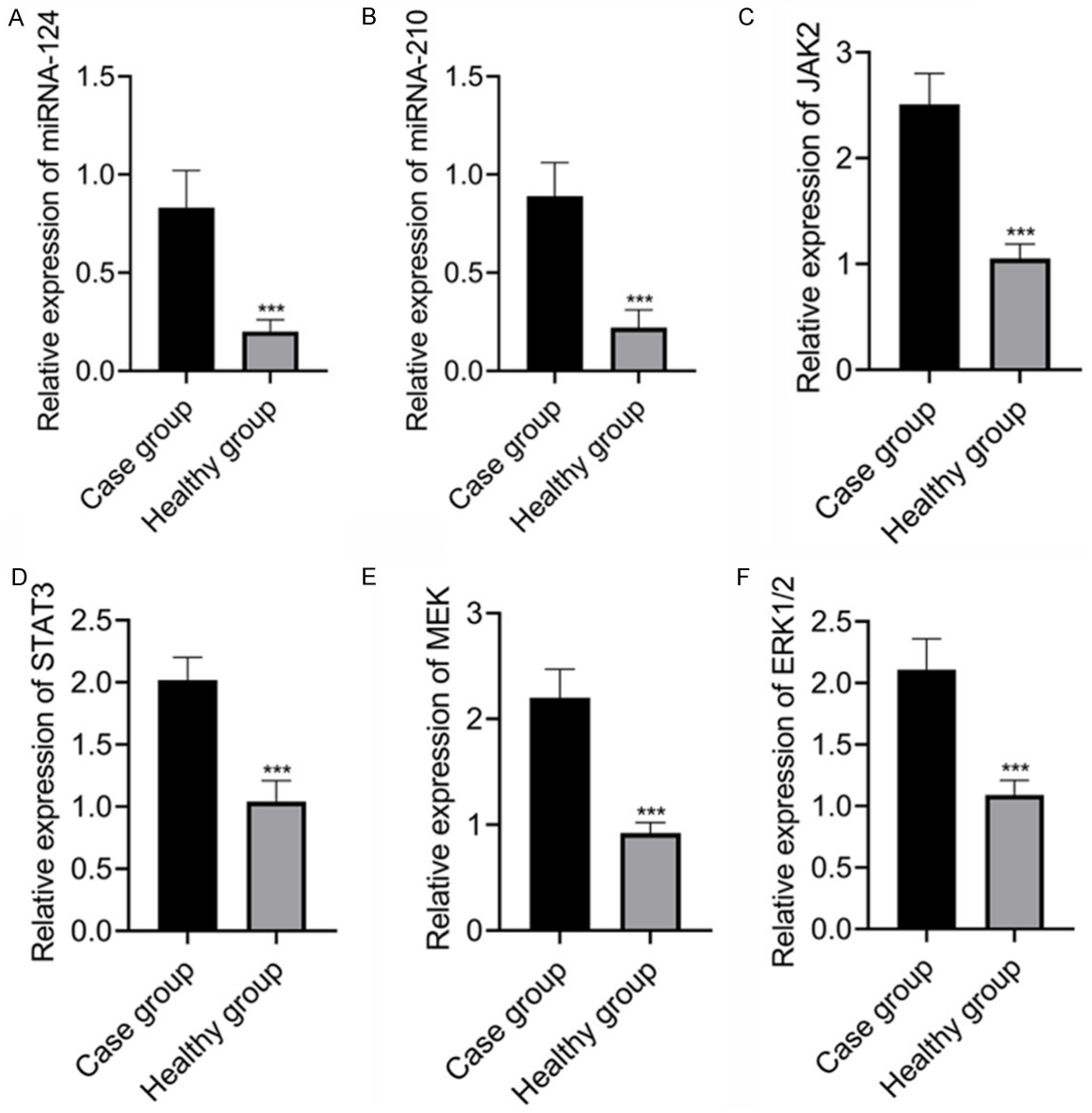

Figure 2. Expression levels of miRNA-124, miRNA-210, and peripheral blood inflammatory signaling related molecules in patients with early craniocerebral injury. Serum levels of (A) miRNA-124, (B) miRNA-210, (C) JAK2, (D) STAT3, (E) MEK, and (F) ERK1/2 in the case group were higher than those in the healthy group. Note: Compared with the case group, $^{***}P<0.001$.

Table 2. Comparison of expression levels of miRNA-124, miRNA-210 and inflammatory signaling related molecules in peripheral blood of patients with mild, moderate and severe craniocerebral injury ($\bar{x}\pm s$)

Group	n	miRNA-124	miRNA-210	JAK2	STAT3	MEK	ERK1/2
Mild	39	0.62±0.18	0.69±0.13	2.03±0.24	1.72±0.15	1.82±0.25	1.65±0.19
Moderate	31	0.86±0.19	0.88±0.19	2.56±0.30	2.02±0.17	2.13±0.26	2.10±0.27
Severe	35	1.05±0.21	1.12±0.20	3.01±0.35	2.35±0.23	2.69±0.31	2.63±0.30
F		46.003	56.445	100.335	106.057	94.327	136.534
P		0.000	0.000	0.000	0.000	0.000	0.000

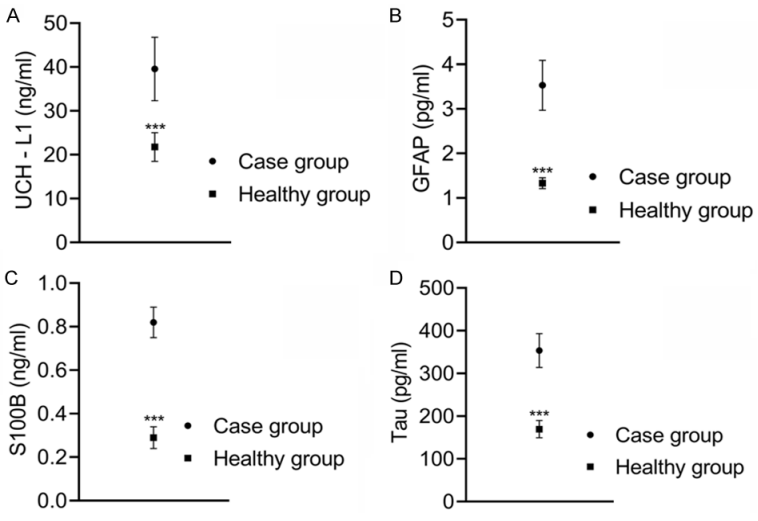

Figure 3. Changes in early craniocerebral injury indicators in patients with craniocerebral injury. Serum levels of (A) UCH-L1, (B) GFAP, (C) S100B, and (D) Tau in the case group were higher than those in the healthy group. Note: Compared with the case group, ***P<0.001.

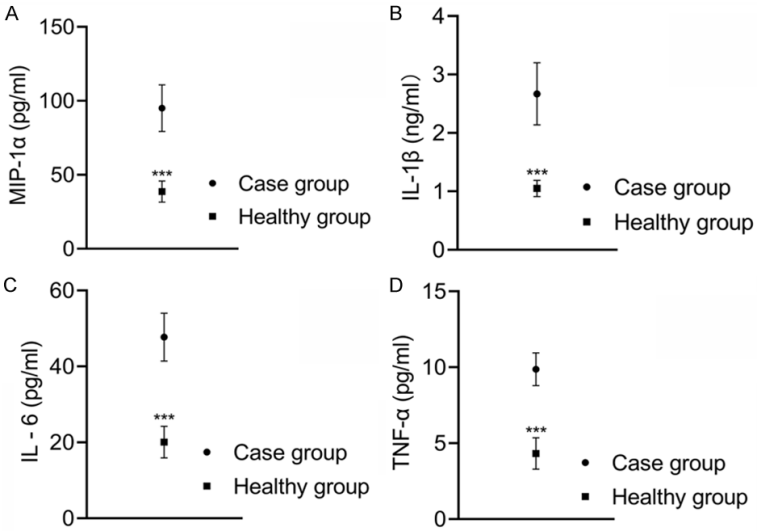

Figure 4. Changes in serum inflammation factors in the early stages of craniocerebral injury. Serum (A) MIP-1α, (B) IL-1β, (C) IL-6, and (D) TNF-α levels in the case group were higher than those in the healthy group. Note: Compared with the case group, ***P<0.001.

Comparison of serum inflammation factors between the two groups

Serum MIP-1α, IL-1β, IL-6, and TNF-α levels in the case group were higher than those in the healthy group (all P<0.05, **Figure 4**).

Correlation between the levels of miRNA-124, miRNA-210 and those of CI indicators

The levels of miRNA-124 and miRNA-210 were positively correlated with those of UCH-L1, GFAP, S100B, and Tau (all P< 0.05, **Table 3**).

Correlation between the levels of miRNA-124, miRNA-210 and serum inflammation factors

The miRNA-124 and miRNA-210 were positively correlated with those of MIP-1α, IL-1β, IL-6, and TNF-α (all P<0.05, **Table 4**).

Correlation of the levels of miRNA-124, miRNA-210 with those of inflammatory signaling related molecules

miRNA-124 and miRNA-210 levels were positively correlated with those of JAK2, STAT3, MEK, and ERK1/2 in the peripheral blood (all P<0.05, **Table 5**).

Correlation between miRNA-124, miRNA-210 levels and patients' prognosis

Among the 105 patients with CI, 82 had good prognosis, while 23 had poor prognosis. The GOS score of the good prognosis subgroup was higher than that of the poor prognosis subgroup, and the levels of miRNA-124 and miRNA-210 in the good prognosis group were lower than those in the poor prognosis subgroup (P< 0.05, **Figure 5**). Correlation analysis revealed that miRNA-124 and miRNA-210 levels were negatively correlated with the GOS scores (r=-0.952, -0.993, P=0.002, 0.000).

Discussion

CI is a common type of trauma, and is associated with the highest rate of disability and mortality. CI is reported to be a common cause of death in people younger than 40 years old in developed countries [14]. Most patients with CI have persistent dysfunction after treatment, which affects their prognosis.

Table 3. Correlation of miRNA-124, miRNA-210 with craniocerebral injury

Variable	UCH-L1		GFAP		S100B		Tau	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
miRNA-124	0.859	0.004	0.971	0.001	0.688	0.008	0.495	0.027
miRNA-210	0.786	0.006	0.950	0.002	0.612	0.010	0.457	0.032

Table 4. Correlation between miRNA-124, miRNA-210 and serum inflammation factors

Variable	MIP-1α		IL-1β		IL-6		TNF-α	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
miRNA-124	0.572	0.012	0.552	0.013	0.979	0.001	0.816	0.003
miRNA-210	0.499	0.036	0.593	0.011	0.902	0.002	0.753	0.005

Table 5. Correlation between miRNA-124, miRNA-210 and expression of inflammatory signaling related molecules in peripheral blood

Variable	JAK2		STAT3		MEK		ERK1/2	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
miRNA-124	0.672	0.009	0.536	0.015	0.469	0.029	0.925	0.002
miRNA-210	0.629	0.011	0.508	0.017	0.431	0.033	0.887	0.003

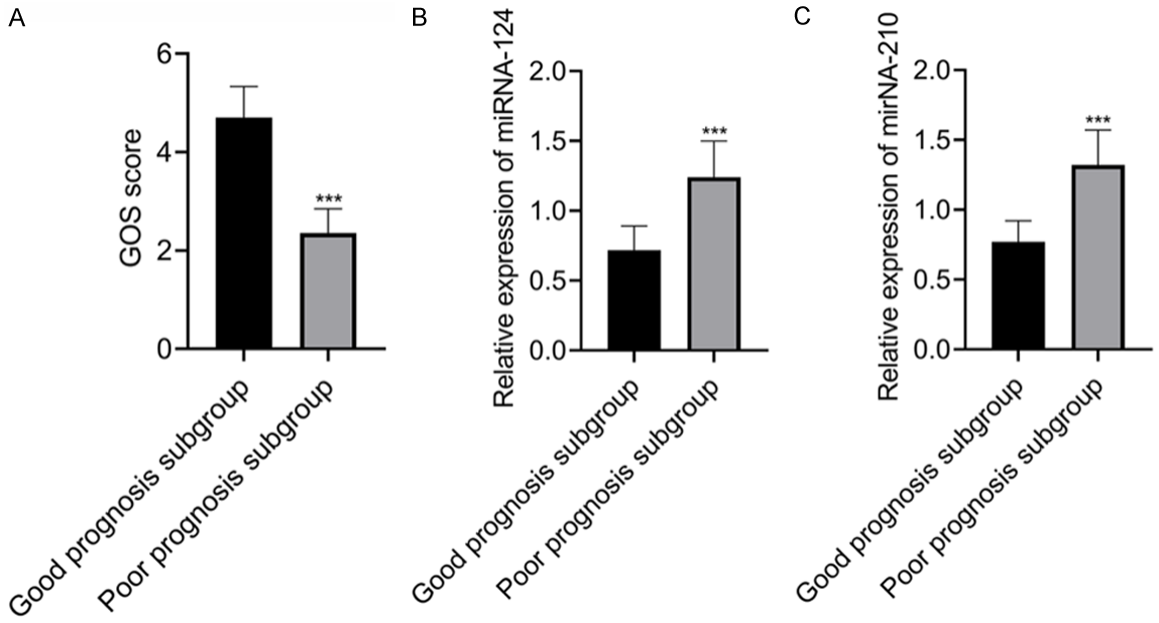

Figure 5. Comparison of miRNA-124 and miRNA-210 expressions in patients with different prognosis. (A) GOS score of the good prognosis subgroup was higher than that of the poor prognosis subgroup, whereas the levels of (B) miRNA-124 and (C) miRNA-210 in the good prognosis group were lower than those in the poor prognosis subgroup. Note: Compared with the poor prognosis subgroup, ***P<0.001.

After the occurrence of CI, the cerebral blood vessels are compressed or stretched, leading to vasospasm and insufficient local blood and oxygen supply. Complications such as cerebral herniation, peripheral organ edema, and hypotension may occur after CI, leading to ischemia and reperfusion, which in turn lead to the changes of miRNAs [15, 16]. miRNA-124 expression was up-regulated in ischemic brain tissues compared with the non-ischemic brain tissues; thus, it is indicative of the early progression of ischemic reperfusion injury [17]. miRNA-

210 induces the migration of endothelial cell to the damaged areas of the vascular basement membrane by regulating the endothelial Notch-l signaling pathway, resulting in the formation of capillary-like structures, improvement of local circulation, promotion of the repair of injured brain tissue, and reduction of ischemic neuronal death [18]. In this study, patients with CI showed increased serum level of miRNA-124 and miRNA-210 compared with the healthy subjects. The reason may be that CI causes abnormal local cerebral blood supply, insufficient oxygen supply and blood supply in some brain tissues, and hypoxic ischemia in cerebrovascular endothelial cells, leading to up-regulation of miRNA-124 and miRNA-210 expressions. With the aggravation of CI, the expression levels of serum miRNA-124 and miRNA-210 were increased significantly. These results suggest that the occurrence of CI can lead to abnormal expression of serum miRNA-124 and miRNA-210, and its expression level may be associated with the occurrence and development of CI. Therefore, miRNA-124 and miRNA-210 have the potential to become biomarkers to evaluate the severity of the disease in patients with CI. The JAK2/STAT3 and MEK/ERK1/2 signaling pathways are closely related to the persistent activation of the CNS IR after the onset of CI, and many miRNAs are regulatory factors of the JAK2/STAT3 pathway [19, 20]. Among these pathways, JAK2 forms a dimer in response to an injury, which in turn recruits and activates STAT3 to regulate inflammatory mediators [21]. MEK participates in the regulation of the IR by activating MAPK and ERK1/2 [22].

During CI, glial cells and neurons are damaged, resulting in the release of multiple neural molecules from the cells into the blood. UCH-L1, a specific cysteine hydrolase, is usually expressed only in brain tissues and plays an important role in the metabolism of amino acids in neuronal cells [23]. GFAP and S100B are found in glial cells; GFAP is involved in cytoskeleton formation, while S100B is involved in the calcium-dependent regulation of biological processes [24]. Microtubule-associated Tau distributed in neuronal cells is mainly involved in the formation of microtubules and maintains their structural stability [25]. Studies [26] have shown that serum UCH-L1, GFAP, and S100B levels are increased in patients with CI, and higher concentrations are associated with severer CI and poorer prognosis; thus, these factors are potential predictors of the prognosis of CI. In the present study, it was found that patients with CI showed increased serum levels of UCH-L1, GFAP, S100B, and Tau compared with the healthy subjects, similar to the results reported above, indicating that a large number of neuronal molecules are released during the CI.

After the occurrence of CI, IR continues to be activated, releasing numerous inflammatory factors, causing inflammatory injury and further aggravating CI. MIP-1α is a pro-inflammatory factor that can chemotactically attract various inflammatory cells to the site of CI and aggravate the local IR [27]. IL-1β and TNF-α are also pro-inflammatory factors, which can cause an inflammatory cascade reaction and aggravate the inflammatory injury. Moreover, IL-1β and TNF-α have been shown to have toxic effects and directly damage neuronal cells [28]. IL-6 is a multifunctional cytokine that promotes the infiltration of inflammatory cells, increases the adhesion of monocyte-endothelial cells, damages endothelial cells, and disrupts the blood-brain barrier. In a previous study [29], serum levels of IL-1β, IL-6, and TNF-α were significantly increased in patients within 24 h after CI compared with healthy subjects, and the severity of CI was directly related to the increase degree of the serum levels. In the current study, it was found that serum levels of MIP-1α, IL-1β, IL-6, and TNF-α in the case group were higher than those in the healthy group, similar to the results reported previously. These results suggest that the overactivation of the IR during the development of CI stimulates the release of numerous inflammatory factors. miRNA has been confirmed to be involved in regulating the expression of inflammatory cells and mediators. Previous studies [30, 31] have shown that miRNA-124 and miRNA-210 play a role in regulating inflammatory response. In this study, correlation analysis revealed that the expression levels of miRNA-124 and miRNA-210 were highly correlated with the expression levels of serum CI markers UCH-L1, GFAP and IR factors IL-6 and TNF-α as well as the levels of inflammatory signaling molecule ERK1/2, positively correlated with CI markers S100B and Tau and IR factors MIP-1α and IL-1β as well as inflammatory signaling molecules JAK2, STAT3 and MEK, and negatively correlated with the GOS scores. It is

suggested that miRNA-124 and miRNA-210 are closely related to the aggravation of CI and IR in patients with CI. The mechanism may be that after the occurrence of CI, a large number of nerve molecules are released, and the JAK2/STAT3 pathway and MEK/ERK1/2 pathway are activated simultaneously, leading to excessive activation of IR, massive production of inflammatory cytokines such as IL-1β, IL-6 and TNF-α in the body. Inflammation stimulates the overexpression of miRNA-124 and miRNA-210, resulting in negative feedback regulation.

In summary, the expression level of serum miRNA-124 and miRNA-210 is increased in patients with CI at the early stage, and higher expression level indicates severer injury. Their expression is closely correlated with the aggravation of CI, overactivation of the IR, and the prognosis of patients. Early monitoring of miRNA-124 and miRNA-210 can reflect CI and IR degree of patients, and miRNA-124 and miRNA-210 can serve as potential biological markers to evaluate the severity and prognosis of patients with CI. However, there are still some limitations in this study, including patient selection bias, small sample size and lack of representativeness. Multi-center and large-sample size are needed for further study.

Disclosure of conflict of interest

None.

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