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Research on the correlation of changes in plasma lncRNA MEG3 with change in inflammatory factors and prognosis in patients with traumatic brain injury

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Abstract. – OBJECTIVE: To study the correlation between the plasma long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3) and the levels of inflammatory cytokines in patients with traumatic brain injury (TBI) and to evaluate its prognosis to screen new biological targets for the diagnosis and treatment of TBI.

PATIENTS AND METHODS: 40 patients with TBI (TBI group) and 40 healthy people (control group) were collected and venous blood was drawn. The plasma MEG3 in subjects was quantitatively analyzed via quantitative Polymerase Chain Reaction (qPCR). Moreover, the levels of inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-8] in plasma in each group were detected via enzyme-linked immunosorbent assay (ELISA). Finally, the correlation analysis was performed for the MEG3 expression level and inflammatory cytokine levels in patients with TBI. Patients were divided into high-expression MEG3 group and low-expression MEG3 group, high-level inflammatory cytokine group and low-level inflammatory cytokine group according to the median, followed by prognosis evaluation. The MEG3 expression level in TBI group was significantly decreased compared to that in control group, and the levels of inflammatory cytokines in plasma, including TNF-α, IL-1β, IL-6, and IL-8, were significantly higher than in control group.

RESULTS: The results of the correlation analysis showed that the expression level of plasma MEG3 had a significantly negative correlation with the level of each inflammatory cytokine. The prognostic analysis revealed that the prognosis of patients with high MEG3 expression level and low inflammatory cytokine levels was good, while it was poor in patients with low MEG3 expression level and high inflammatory cytokine levels; the difference was significant. In patients with TBI, the expression level of plasma MEG3 is decreased, while the inflammatory cytokine levels are increased, and there is a significantly negative correlation between the two items.

CONCLUSIONS: The prognosis of patients with high MEG3 expression level and low inflammatory cytokine levels is good so MEG3 and inflammatory cytokines can be used as biomarkers for diagnosis and treatment of TBI, improving the prognosis of patients.

Key Words:
Long non-coding RNA MEG3, Inflammatory cytokines, Traumatic brain injury, Prognosis.

Introduction

Brain trauma is also known as traumatic brain injury (TBI) or intracranial injury, which refers to the brain dysfunction caused by head impact, bump, and injury under violence such as skull fracture, electric shock, lightning stroke, head/neck wounds, and motion impact. Its clinical manifestations are cerebral concussion, subdural hematoma, auditory, visual abnormalities, confusion, coma, etc. Moreover, it is one important factor leading to death and disability worldwide[1]. According to statistics, about 52,000 people die of TBI every year and 2,500,000 people are disabled due to TBI. TBI also leads to permanent disability, so it is a serious global public health problem[2]. TBI can be divided into primary brain injury and secondary brain injury according to the occurrence order of brain tissue injury, the latter of which is the root cause of disability and death of patients. At present, TBI can be treated with psychological and behavioral guidance, physical therapy and surgery

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based on the degree of injury; however, searching the potential biological targets for the diagnosis and treatment of TBI is of great significance, since the pathogenesis of TBI is complex. Indeed, it often involves the accumulation of reactive oxygen species, energy metabolism disorders, inflammatory response, etc.[3]

Long non-coding RNA (lncRNA) is a kind of RNA transcript with a length of 200 nt that does not encode the protein. Researches have shown that lncRNA plays an important role in cancer occurrence, cell proliferation, apoptosis, and inflammatory response[4]. The maternally expressed gene 3 (MEG3) is a kind of lncRNA encoded by the maternally imprinted gene. Moreover, some studies[5,6] have shown that the expression of MEG3 is down-regulated in a variety of human tumor cells such as meningiomas, gastric carcinoma, gliomas. MEG3 is a tumor suppressor gene that can act as the tumor suppressor in a variety of cell lines such as HCT116 and U87 MG cells[7]. However, there has been no report on the expression level and biological function of MEG3 in TBI. Inflammatory cytokines are produced by the central nervous system (CNS) and the immune system in the inflammatory response of the body, among which interleukin and tumor necrosis factors are common ones[8]. In cerebral trauma, the body can produce a large number of inflammatory factors and reaction media, thereby participating in the occurrence and development of TBI. Studies have shown that the levels of interleukin-1β (IL-1β) and IL-6 in plasma and cerebrospinal fluid of TBI patients are significantly increased, being positively correlated with the degree of trauma[9]. Furthemore, the level of tumor necrosis factor-α (TNF-α) in cerebrospinal fluid of patients with secondary TBI is higher than in plasma and it is positively correlated with the neurological dysfunction[10]. Therefore, the levels of inflammatory cytokines can be used as the biological targets for TBI diagnosis and treatment, improving the prognosis of patients. This study aimed to investigate the correlation between the plasma lncRNA MEG3 and the levels of inflammatory cytokines in TBI patients, and to evaluate its prognosis to screen reliable biomarkers for the diagnosis and treatment of TBI.

Patients and Methods

Patients

40 TBI patients treated in the Department of Cerebral Surgery in our hospital from July 2015 to May 2016 were collected, including 24 males with an average age of 47±21 years old and 16 females with an average age of 52±27 years old. There were 18 cases of car accident, 10 cases of heavy impact, 7 cases of high falling and 5 cases of motion impact. All patients were confirmed via head computed tomography (CT) or magnetic resonance imaging (MRI), and the preoperative Glasgow Coma Score (GCS) was moderate (8-12 points) and the expected survival time was more than 3 months. Moreover, all patients met the criteria of brain trauma operation and received the operative treatment within 24 h after admission. In addition, 40 healthy people were selected as control group, including 20 males with an average age of 49±23 and 20 females with an average age of 51±25 years old. Subjects in control group had no history of brain trauma. There were no significant differences in the age and gender among all subjects ($p>0.05$); all patients and their families signed the informed consent and this investigation was approved by the Ethics Committee of our hospital.

Main Reagents

TRIzol Reagent, Prime Script® RT reagent Kit with gDNA Eraser and SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China); TNF alpha Human enzyme-linked immunosorbent assay (ELISA) Kit, IL-6 Human ELISA Kit, IL-8 Human ELISA Kit, and IL-1 beta Human ELISA Kit (Invitrogen, Carlsbad, CA, USA); primers (Sangon, Shanghai, China), etc.

Methods

Collection of Venous Blood

5 mL venous blood was collected using the ethylenediaminetetraacetic acid (EDTA) anticoagulant tube from TBI patients at 1 h before the operation and centrifuged under low speed and low temperature for 10 min to collect the plasma. After that, the plasma was stored in a refrigerator at –80°C for standby application. The fasting blood was collected from healthy people in the early morning and other operations were the same as above.

Analysis of Plasma LncRNA MEG3 Expression

The total RNA was extracted from the plasma in each group according to the instructions of the kit and identified. 200 ng total RNA was taken and reversely transcribed into cDNA and the quantitative Polymerase Chain Reac-

Table I. Real-time qPCR primers and sequences.

Primer	Sequence (5′-3′)
MEG3-F	CCTTGCATCAGCCAAGCTTCTTG
MEG3-R	ACGGCCCACGTGCCTTTGTG
ACTB-F	GGGAAATCGTGCGTGACATTAAGG
ACTB-R	CAGGAAGGAAGGCTGGAAGAGTG

tion (qPCR) system was prepared and tested in CFX-96 Detection System (Bio-Rad, Hercules, CA, USA). The Ct values collected were treated with cycle threshold method into the relative expression levels, and β-actin was used as the internal reference. The primer sequences are shown in Table I.

Determination of Inflammatory Cytokines in Plasma

The levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) in plasma in each group were detected via ELISA in strict accordance with the instructions of kit.

Correlation Analysis of Plasma MEG3 Expression Level and Inflammatory Cytokine Levels

The correlation of the plasma MEG3 level with the levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) in plasma in 40 patients with TBI was analyzed using the linear regression method.

Prognosis Evaluation

According to the medians of plasma MEG expression level and inflammatory cytokine (IL-1β, IL-6, IL-8 and TNF-α) levels, 40 patients with TBI were divided into high-expression MEG3 group (n=20, MEG3 expression level >1.1) and low-expression MEG3 group (n=20, MEG3 expression level <1.1), high-level inflammatory cytokine group (n=20, inflammatory cytokine levels >1.635, 239, 6.0 and 2.1 ng/mL), and low-level inflammatory cytokine group (n=20, inflammatory cytokine levels <1.635, 239, 6.0 and 2.1 ng/mL). Patients were followed up for 1 year after the operation, classified and recorded according to the Glasgow Outcome Scale (GOS) score. Normal: patients can recover to the state before brain trauma; moderate disability: patients have a moderate disability but can live independently; severe disability: patients have a severe disability and cannot live independently; vegetative state: patients are in the vegetative state; death: patients die. The prognosis was evaluated according to the number of patients in the above grade.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA). The investigations were repeated three times and the data were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) and the two-tailed *t*-test were used for intergroup comparison. Correlation analysis was performed using the linear regression method. The difference in prognosis was compared using the rank sum test. $p<0.05$ suggested that the difference was statistically significant.

Results

LncRNA MEG3 Expression was Down-Regulated in Plasma in TBI Patients

The expression levels of MEG3 in TBI patients and healthy people were detected via qPCR. The results showed that the expression level of MEG3 in plasma in TBI group was significantly lower than in control group; in other words, the MEG3 expression was abnormally down-regulated in plasma in TBI patients (Figure 1).

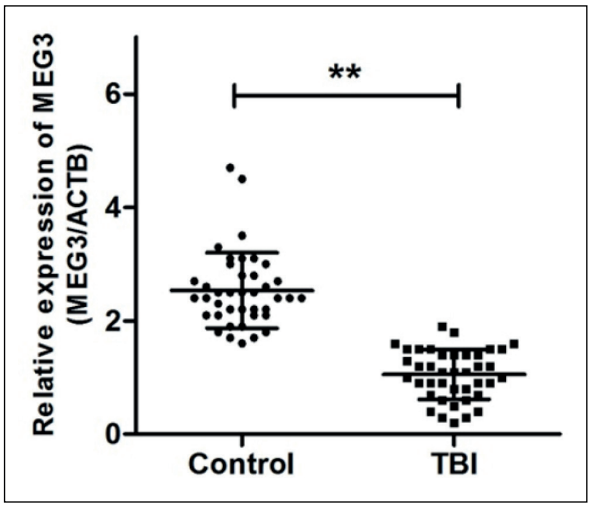

Figure 1. The relative expression levels of MEG3 in plasma in TBI patients and healthy people. The results of Real-time qPCR show that the expression level of MEG3 in plasma in TBI group is significantly lower than that in control group; **$p<0.01$ *vs.* Control.

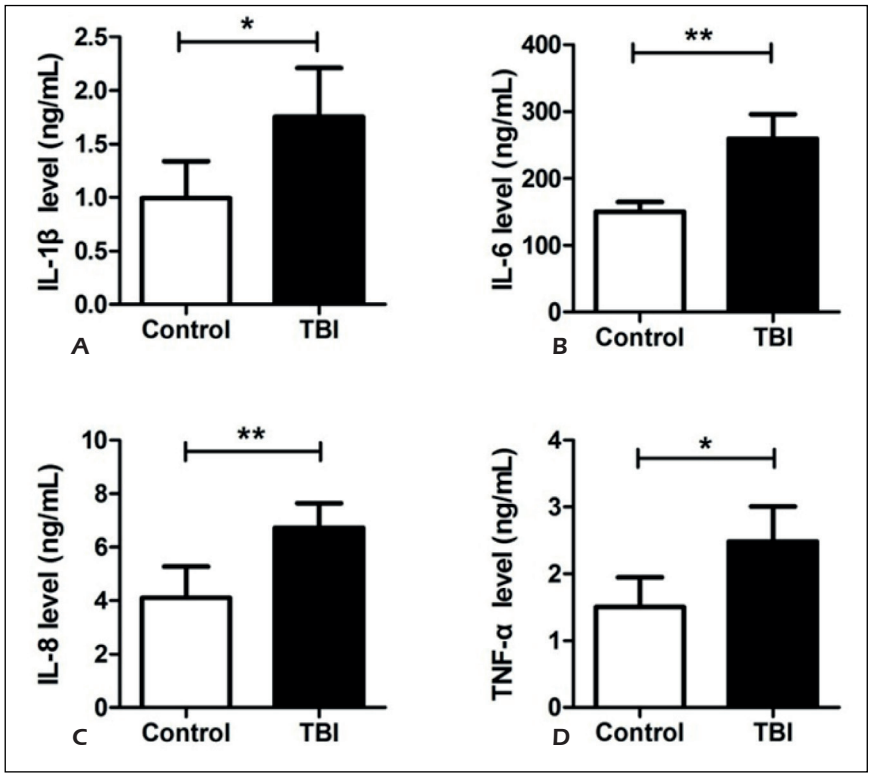

Figure 2. Levels of inflammatory cytokines in plasma in TBI patients and healthy people. **A**, Plasma IL-1β levels; **B**, Plasma IL-6 levels; **C**, Plasma IL-8 levels; **D**, Plasma TNF-α levels. ELISA results show that the levels of IL-1β, IL-6, IL-8, and TNF-α in plasma in TBI group are significantly higher than those in control group. *p<0.05 *vs.* Control; **p<0.01 *vs.* Control.

The Levels of Inflammatory Cytokines in Plasma in TBI Patients were Increased

The levels of inflammatory cytokines in plasma in TBI patients and healthy people were detected via ELISA. As shown in Figure 2 (2A: IL-1β levels in plasma of the two groups; 2B: IL-6 levels in plasma of the two groups; 2C: IL-8 levels in plasma of the two groups; 2D: TNF-α levels in plasma of the two groups), the levels of inflammatory cytokines in TBI group were increased compared to those in control group and the differences were statistically significant.

Correlation Between Plasma MEG3 Expression and Inflammatory Cytokine Levels

The correlation between the plasma MEG3 expression and the inflammatory cytokine levels in TBI patients was analyzed using the linear regression method. As shown in Figure 3 (3A: correlation between plasma MEG3 expression and IL-1β level in TBI patients; 3B: correlation between plasma MEG3 expression and IL-6 level in TBI patients; 3C: correlation between plasma MEG3 expression and IL-8 level in TBI patients; 3D: correlation between plasma MEG3 expression and TNF-α level in TBI patients), the changes in plasma MEG3 expression in TBI patients were negatively correlated with the levels of IL-1β, IL-6, IL-8, and TNF-α (r=-0.4098, -0.3413, -0.5003, and -0.4974), and the differences were statistically significant.

Prognosis Evaluation

The patients in high-expression MEG3 group and low-expression MEG3 group, high-level inflammatory cytokine group and low-level inflammatory cytokine group were followed up for 1 year after operation; the status was recorded, and the prognosis was evaluated. As shown in Table II, the prognosis of patients with high MEG3 expression level and low inflammatory cytokine levels was good, while it was poor in patients with low MEG3 expression level and high inflammatory cytokine levels, and the difference of prognosis was significant (u=8.73, p<0.05).

Discussion

TBI is a kind of cerebral traumatic disease caused by the abrupt acceleration or deceleration in the head, or sudden impact, which may cause secondary injury within several minutes after injury, including the decreased cerebral blood flow, decreased cerebral oxygen supply, and increased

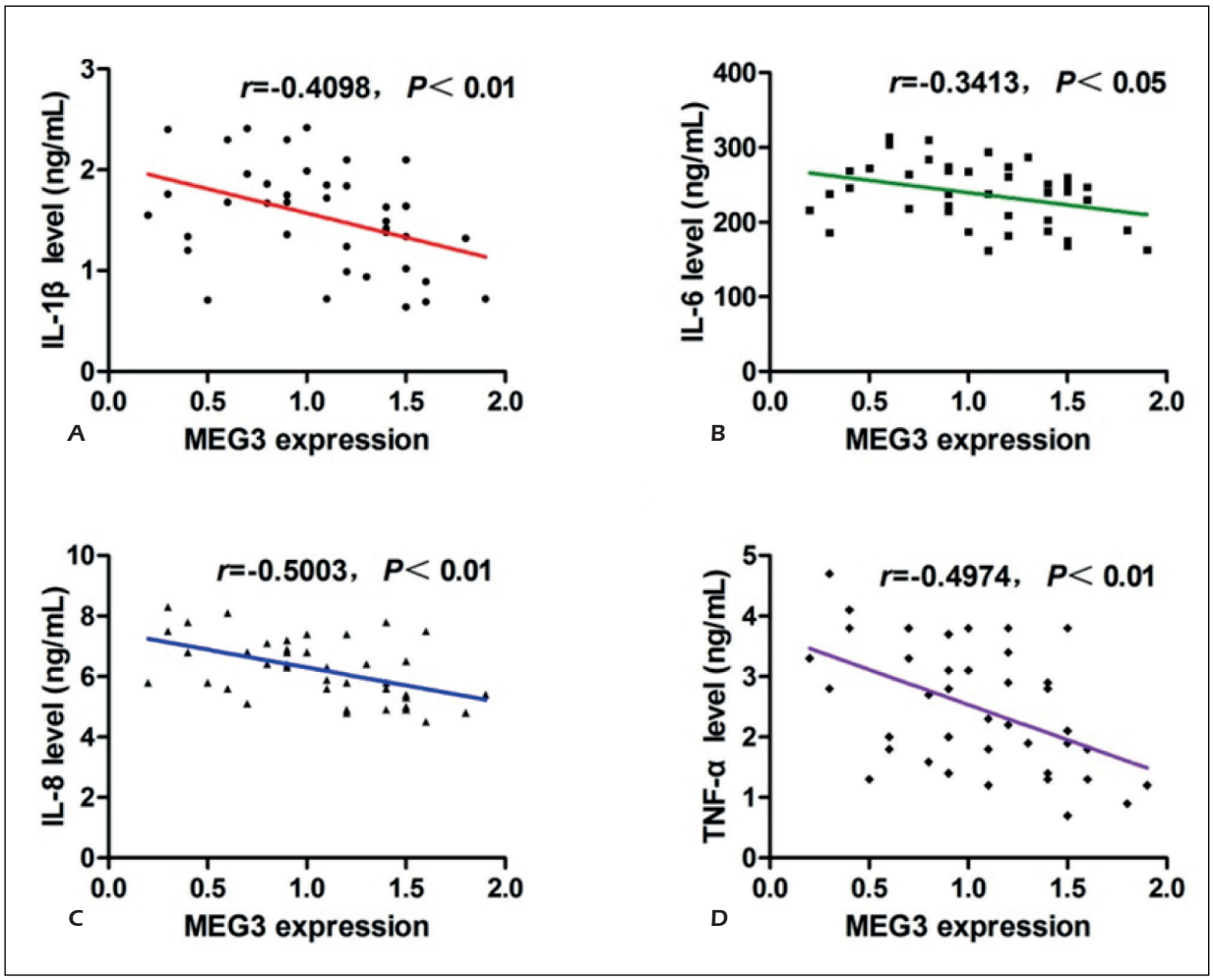

Figure 3. Correlation between plasma MEG3 expression and inflammatory cytokine levels in TBI patients. **A**, Correlation between MEG3 expression and IL-1β level; **B**, Correlation between MEG3 expression and IL-6 level; **C**, Correlation between MEG3 expression and IL-8 level; **D**, Correlation between MEG3 expression and TNF-α level. The correlation analysis shows that the plasma MEG3 expression is negatively correlated with the inflammatory cytokine levels in TBI patients.

intracranial pressure, besides the damage before the injury. Therefore, some studies[11] have shown that the early enteral nutritional support in TBI can accelerate the brain protein synthesis, maintain the body's energy supply, improve the immunity and benefit the prognosis of patients. The pathogenesis of TBI is very complex since there are many physiological processes such as brain tissue edema, calcium overload, neuronal injury, accumulation of reactive oxygen species, apoptosis, and inflammatory response, in the early stage of trauma[12]. Thus, it provides a prerequisite for the aggravation of the disease and the occurrence and development of secondary brain injury. TBI is one of the diseases with the highest rates of mortality and disability in today's society, and the treatment of TBI mainly depends on physical and chemical therapy (such as mild hypothermia therapy) and surgery[13]. Therefore, the screening the biological targets for TBI diagnosis, as well as treatment and prognosis, are of great significance. The inflammatory response plays an important role in the pathogenesis of TBI. When the brain trauma occurs, neutrophils can produce a large number of inflammatory factors such as IL-2, IL-6, IL-8, and TNF-α, which are conducive to the release of inflammatory mediators and can accelerate the patient's inflammatory response, thereby aggravating the disease[14]. IL-6 can induce the expression of vascular endothelial cell adhesion molecule, lead to the vascular inflammation, activate the expression of matrix metalloproteinases (MMPs), degrade the blood-brain barrier, and increase the brain tissue permeability, thus inducing TBI[15]. IL-

Table II. Comparison of prognosis of TBI patients [n (%)].

Group	No.	Normal	Moderate disability	Severe disability	Vegetative state	Death
High-expression MEG3 group	20	14 (70%)	2 (10%)	2 (10%)	2 (10%)	0 (0%)
Low-expression MEG3 group	20	10 (50%)	5 (25%)	2 (10%)	2 (10%)	1 (5%)
High-level IL-1β group	20	10 (50%)	4 (20%)	4 (20%)	1 (5%)	1 (5%)
Low-level IL-1β group	20	15 (75%)	2 (10%)	1 (5%)	1 (5%)	1 (5%)
High-level IL-6 group	20	11 (55%)	4 (20%)	3 (15%)	1 (5%)	1 (5%)
Low-level IL-6 group	20	14 (70%)	2 (10%)	3 (15%)	1 (5%)	0 (0%)
High-level IL-8 group	20	9 (45%)	6 (30%)	2 (10%)	2 (10%)	1 (5%)
Low-level IL-8 group	20	13 (65%)	3 (15%)	3 (15%)	1 (5%)	0 (0%)
High-level TNF-α group	20	10 (50%)	4 (20%)	4 (20%)	2 (10%)	0 (0%)
Low-level TNF-α group	20	15 (75%)	2 (10%)	1 (5%)	1 (5%)	1 (5%)

8 can activate the neutrophils in the course of TBI, accelerate the inflammatory response, and cause the brain edema[16]. IL-1β is highly expressed in the intercellular space of brain tissues, which can promote the production of β-defensin-4, and aggravate the brain tissue edema[17]. TNF-α can induce the production of IL-6, IL-8, and inflammatory mediators, thereby promoting the brain edema, and it can also activate lymphocytes to infiltrate into the brain[18]. Several studies[9,10] have shown that the levels of inflammatory factors are significantly increased in cerebrospinal fluid or plasma in TBI patients, which is related to the prognosis of patients, severity of disease, and nerve damage, etc.

LncRNA can exert a variety of biological functions in the body. Therefore, it is often used as a biomarker of disease, especially for cancer, to investigate the molecular mechanism of the tumor. In recent years, the roles of lncRNA in nervous system diseases and inflammatory response have been reported. Some studies[19] have shown that lncRNA is abnormally expressed in the hypoxic-ischemic brain damage model of neonatal rats, suggesting that it may be involved in the course of brain injury. A variety of lncRNAs are specifically expressed in the neural stem cells of the ventricle and hippocampal dentate nucleus of mammals and play important roles in the differentiation of stem cells into neurons and in the neuronal repair process[20]. In addition, lncRNA can regulate the production of downstream inflammatory factors by regulating various signaling pathways in the body, playing an indirect role in the inflammatory response[21]. MEG3 is a kind of tumor suppressor gene, which can play a role as the tumor-inhibiting factor; however, its roles in inflammatory response and TBI are rarely reported. This study showed that the MEG3 expression was abnormally down-regulated in plasma in TBI patients, indicating that MEG3 plays a certain role in the pathological process of TBI. Besides, the levels of IL-1β, IL-6, IL-8, and TNF-α in plasma in TBI patients were significantly increased compared to those in normal people, and they had significantly negative correlations with the changes in MEG3 expression, suggesting that MEG3 can play an important role in the course of TBI by regulating the levels of inflammatory cytokines in plasma in TBI patients. To sum up, MEG3 can participate in the inflammatory response process of TBI and regulate the occurrence and development of TBI, but the specific molecular mechanism still remains to be further studied.

Conclusions

We showed that the postoperative prognosis of patients with high MEG3 expression and low inflammatory cytokine levels is good. Therefore, MEG3 and inflammatory cytokines can be used as the biological targets for diagnosis and treatment of TBI, which have great significance in improving the prognosis of patients.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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