Original article

# Alteration of microRNA expression in cerebrospinal fluid of unconscious patients after traumatic brain injury and a bioinformatic analysis of related single nucleotide polymorphisms

Wen-Dong You, Qi-Lin Tang, Lei Wang, Jin Lei, Jun-Feng Feng, Qing Mao, Guo-Yi Gao[*], Ji-Yao Jiang

*Department of Neurosurgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, 200127, People's Republic of China*

A B S T R A C T

*Purpose:* It is becoming increasingly clear that genetic factors play a role in traumatic brain injury (TBI), whether in modifying clinical outcome after TBI or determining susceptibility to it. MicroRNAs are small RNA molecules involved in various pathophysiological processes by repressing target genes at the post-transcriptional level, and TBI alters microRNA expression levels in the hippocampus and cortex. This study was designed to detect differentially expressed microRNAs in the cerebrospinal fluid (CSF) of TBI patients remaining unconscious two weeks after initial injury and to explore related single nucleotide polymorphisms (SNPs).
*Methods:* We used a microarray platform to detect differential microRNA expression levels in CSF samples from patients with post-traumatic coma compared with samples from controls. A bioinformatic scan was performed covering microRNA gene promoter regions to identify potential functional SNPs.
*Results:* Totally 26 coma patients and 21 controls were included in this study, with similar distribution of age and gender between the two groups. Microarray showed that fourteen microRNAs were differentially expressed, ten at higher and four at lower expression levels in CSF of traumatic coma patients compared with controls ($p < 0.05$). One SNP (rs11851174 allele: C/T) was identified in the motif area of the microRNA hsa-miR-431-3P gene promoter region.
*Conclusion:* The altered microRNA expression levels in CSF after brain injury together with SNP identified within the microRNA gene promoter area provide a new perspective on the mechanism of impaired consciousness after TBI. Further studies are needed to explore the association between the specific microRNAs and their related SNPs with post-traumatic unconsciousness.
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## Introduction

Traumatic brain injury (TBI) is an insult from external physical force, leading to structural and functional impairments of the brain. Post-traumatic disorder of consciousness remains a severe consequence of cognitive dysfunction after severe TBI. It is estimated that over one million people are affected annually by unconsciousness after TBI all over the world, making it a critical public health problem, causing a great social and economic burden, particularly in low- and middle-income countries.[1–5] Severe TBI can result in prolonged disorders of consciousness, and approximately 10%—15% of severe TBI patients are discharged from hospital in a vegetative or minimally conscious state.[5] For a long time, in spite of considerable effort, no effective treatment was available for post-traumatic coma. A recent evidence-based review of current interventions to promote arousal from coma showed that effective treatments are limited except for amantadine use in children.[1] In another study, Giacino et al[5] also found that amantadine accelerated the pace of functional recovery during active treatment in adult patients with post-traumatic disorders of consciousness, but its actual clinical improvement was questioned.[6] The current situation demonstrates the limited knowledge available so far about the complex and multifactorial pathophysiological process of consciousness disorders following TBI. Fortunately, recent evidences from genetic association studies show that genetic factors,

especially single nucleotide polymorphisms (SNPs) (single base mutations), make up the majority of genetic variations in human population and play an important role in various brain diseases including TBI.[7,8] In addition, the patients exposed to a similar extent of physical force have experienced different consequences regarding severity of unconsciousness, indicating variable susceptibility among individuals to impaired consciousness after TBI.

MicroRNAs are small, endogenous, non-coding 21−25-nucleotide RNAs, which play an important role in regulating gene expression and in numerous biological and pathological processes.[9] These small RNAs are receiving increasing attention as potential biomarkers for detecting, identifying, classifying, and treating diseases. Various studies have addressed alterations in microRNA expression levels after experimental TBI. Lei et al[10] reported the alteration in microRNA expression patterns in cerebral cortex of rats after fluid percussion brain injury. Redell et al[11] demonstrated different microRNA expression levels in hippocampus after controlled cortical impact. Based on these findings, we concluded that microRNAs could serve as a potential biomarker for unconsciousness after TBI.

In addition to extracellular signals that can lead to the different expression of microRNAs in various types of TBI, including brain injury, ischemia, and physical stimulation,[12] variations in DNA sequence encoding these molecules can also influence microRNA expression levels. For this reason, we also hypothesized that polymorphisms in microRNA genes might have a role in traumatic coma. However, as far as we know, little information exists that can be used to correlate microRNA gene polymorphisms with post-traumatic coma.

In this study, we employed a microarray platform to detect different expression levels of microRNAs in cerebrospinal fluid (CSF) of coma patients two weeks after TBI, and then conducted a bioinformatic scanning over the promoter regions of these microRNA genes to identify the potential functional SNPs.

## Materials and methods

### Ethics statement

This study was approved by the ethics committee of Renji Hospital, affiliated to School of Medicine, Shanghai Jiao Tong University, Shanghai, China. Written informed consent was obtained from each participant's legally authorized representative.

### TBI patients and controls

Participants in this study were recruited from head injury admissions to Department of Neurosurgery, Renji Hospital, Shanghai, China, from September 1, 2008 to September 1, 2011. Patients meeting the following inclusion criteria were considered eligible for this study: (1) adults ≥18 years; (2) severe brain injury with Glasgow Coma Scale (GCS) score ≤8 at admission; (3) remaining in a coma state two weeks after the initial injury (GCS ≤ 8); and (4) normal intracranial pressure or without radiological evidence of elevated intracranial pressure. Subjects were excluded if they suffered severe extracranial injury or multi-organ dysfunction. Adults who received a lumbar puncture for diagnostic purposes and without confirmed central nervous system diseases were recruited as controls.

### Preparation of CSF

CSF samples were obtained from included patients by lumbar puncture as part of intensive care treatment. CSF samples from adults who received a lumbar puncture for diagnostic purposes were collected as control (described above). The volume of every sample varied from 6 to 8 ml. Samples were firstly subjected to cytological examination and only those with normal findings were included. Then each CSF sample was centrifuged at 3000 × g for 10 min, the sediment was discarded and the supernatant was stored at −80 °C for further processing.

### RNA extraction and chip hybridization and rinsing

Total RNA containing low molecular weight RNA was extracted with TRIZOL reagent (Invitrogen-Life Technologies, USA) from CSF samples according to the manufacturer's instructions, and the purity of total RNA was measured by the ratio of $A_{260}/A_{280}$. An absorbance ratio greater than 1.9 is usually considered as an acceptable indicator of RNA purity. The integrity of total RNA was qualified using an Agilent Bioanalyzer 2100 capillary electrophoresis (Agilent Technologies, USA), and samples fell within Asuragen's acceptable limits: RNA integrity number (RIN) greater than 7.[13,14]

Affymetrix platform for microRNA expression analysis (GeneChip® miRNA 3.0 Array), based on mirBase version 17 (http://www.mirbase.org/), was used to obtain microRNA expression profiles. This version contains 19 913 probe sets including 5818 human premature and mature microRNAs. A total of 500 ng of total RNA was labeled using FlashTag™ Biotin HSR RNA Labeling Kit (Genisphere, USA) according to the manufacturer's procedure. Labeled miRNAs were hybridized to GeneChip miRNA 3.0 Array as recommended by the manufacturer.[15] The array was washed and stained using Fluidics Station 450 (Affymetrix) with the fluidics protocol FS450-0004.

### Imaging and data processing

Images were scanned using a Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, USA). A 2-fold change in expression level was chosen as a cutoff for categorizing a significant change in expression level.[13]

### Bioinformatic prediction of SNPs

First, we verified the site information and sequence accuracy of the specific microRNAs detected in this study. MicroRNAs could be classified either as intergenic or as intragenic. Intragenic microRNAs are located within other transcriptional units (host genes) and are transcribed in parallel to their host genes, suggesting that they share promoters with host genes. Intergenic microRNAs are located between other transcriptional units and therefore intergenic microRNAs have their own transcriptional units and promoters.[16] The sequence containing 2000 bp upstream and 500 bp downstream of transcription start site (TSS) was defined as the promoter region, and it was downloaded from Eukaryotic Promoter Database.

### Prediction of transcription factors and corresponding motifs

The putative transcription factors of the promoter region and the corresponding motif areas were identified using TRANSFAC® database (BIOBASE), which was available at http://www.biobase.de. The algorithm was Match™ which used a library of position weight matrices collected in TRANSFAC® database and therefore offered the possibility of searching for a great variety of transcription factor binding sites.[17]

## Identification of SNPs in the motif areas

For computational identification, single Nucleotide Polymorphism Database (dbSNP) (http://www.ncbi.nlm.nih.gov/SNP/) was used to identify SNPs in the motif areas. The database also provided detailed information about SNPs.[18]

## Statistical analysis

To determine whether microRNAs detected by microarray analysis had altered expression levels in coma patients, the ratio of the two sets of detected signals (coma patients vs controls, $\log^2$ transformed, balanced) was calculated. Two-sided student's t tests were used for group comparison. $p < 0.05$ was considered as statistical significance. All statistical analyses were performed using the statistical package SPSS 19.0.

## Results

A total of 26 (14 males and 12 females) coma patients and 21 (12 males and 9 females) controls were included. Controls were consisted of 9 patients diagnosed with headache, 5 patients with vertigo, 5 patients with spinal pain and 2 patients with tinnitus. Age ($49.1 \pm 8.07$ vs $50.4 \pm 8.66$, $p > 0.05$) and gender showed no significant difference between the two groups. (Tables 1 and 2).

## Microarray analysis

Samples from controls and coma patients were hybridized to microarrays containing the known human microRNAs. Expression of 14 microRNAs in coma group differed from that of controls ($p < 0.05$); 10 out of the 14 had higher expression (Table 3) and 4 showed lower expression compared with controls (Table 3). Results were given as fold-change and p values. Of the up-regulated microRNAs, miR-141 and miR-257 displayed the greatest fold-changes at 4.62 and 3.05 times of that of controls, while the remaining microRNAs increased from 2.0 to 2.5-fold. Of the down-regulated microRNAs, miR-1297 had the greatest fold-change at −3.44 times in coma group compared with controls.

**Table 1**
Baseline characteristics of traumatic coma patients.

| | |
|---|---|
| Number of patients (male:female) | 26 (14:12) |
| Age (years, mean ± SD) | 49.1 ± 8.07 |
| GCS in admission (n) | |
| 3 | 5 |
| 5 | 4 |
| 6 | 7 |
| 7 | 3 |
| 8 | 7 |
| GCS two weeks after brain injury (n) | |
| 3 | 3 |
| 4 | 1 |
| 5 | 3 |
| 6 | 6 |
| 7 | 5 |
| 8 | 8 |
| Injury characteristics (n, %) | |
| Mesencephalic cisterns compressed or absent on initial CT | 15 (58) |
| Midline shift (≥5 mm) detected on initial CT | 12 (46) |
| Signs of intracranial hypertension detected on initial CT | 26 (100) |

**Table 2**
Baseline characteristics of controls.

| | |
|---|---|
| Number of patients (male:female) | 21 (12:9) |
| Age (years, means ± SD) | 50.4 ± 8.66 |
| Definitive diagnosis (n) | |
| Headache | 9 |
| Vertigo | 5 |
| Spinal pain | 5 |
| Tinnitus | 2 |

**Table 3**
Up-regulated and down-regulated microRNAs.

| Up-regulated microRNAs | | | Down-regulated microRNAs | | |
|---|---|---|---|---|---|
| microRNA | Fold-change | p value | microRNA | Fold-change | p value |
| miR-141 | 4.62 | 0.005 | miR-1297 | −3.44 | 0.016 |
| miR-572 | 3.05 | 0.002 | miR-33b | −2.61 | 0.009 |
| miR-181a-star | 2.47 | 0.001 | miR-933 | −2.59 | 0.042 |
| miR-27b-star | 2.37 | 0.012 | miR-449b | −2.14 | 0.022 |
| miR-483-5p | 2.36 | 0.003 | | | |
| miR-30b | 2.18 | 0.030 | | | |
| miR-1289 | 2.13 | 0.006 | | | |
| miR-431-star | 2.12 | 0.021 | | | |
| miR-193b-star | 2.09 | 0.002 | | | |
| miR-499-3p | 2.01 | 0.013 | | | |

## SNPs of motif areas

We searched the motif areas of the microRNA promoter regions by querying the dbSNP and identified one SNP (rs11851174 allele: C/T) within the motif area of microRNA has-miR-431-3P gene promoter region. The SNP site was within chromosome 14 strand +, with a mutation from standard base C to T.

## Discussion

In the present study, we used microarray platforms to examine the differential microRNA expression levels in CSF of coma patients two weeks after brain injury, a time point when the traumatic pathological features were transited from acute phase to subacute stage with impaired consciousness. The major findings were that expression levels of specific microRNAs in CSF of traumatic coma patients were significantly up- or down-regulated compared with controls. In addition, one SNP with a potential function of modulating the outcome after TBI was identified in the motif area of microRNA has-miR-431-3P promoter region.

More specifically, the expression levels of 10 microRNAs were significantly elevated while 4 microRNAs were decreased, which was quite different from previous studies reporting more down-regulated than up-regulated microRNAs after TBI.[11,19] Several factors may contribute to the divergence in experimental results. First, this study relied on clinical samples rather than on animal models.[10] Second, the time point of sample collection in this study was two weeks after the initial injury, a subacute phase of TBI, while most of the other studies used samples from the acute stage.[10,11] Finally, the samples used for microarray analysis in the present study were CSF rather than the cerebral cortex or hippocampus.

The most highly enriched microRNA in CSF of coma patients was miR-141, which also played a role in suppressing human osteosarcoma.[20] In addition, most of the up-regulated microRNAs were associated with oncogenesis. For example, miR-431 was related to the viability of medulloblastoma and glioblastoma cells[21]; miR-30b regulated migration and invasion of human colorectal cancer via

SIX1[22]; miR-483-5p was a candidate serum biomarker for adrenocortical tumors[23]; miR-181 was suggested as having a role in neuroinflammatory responses of astrocytes.[24] Because a single microRNA can regulate the expression of hundreds of target genes,[11] alteration in a panel of microRNAs could greatly affect the pathophysiology and outcome of TBI, including post-traumatic unconsciousness. Some of the predicted target genes of hsa-miR-431-3p, such as MTRNR2L1, were involved in the process of ischemia and reperfusion injury of cortical neurons that was initiated after TBI, also indicating that this specific microRNA might contribute to the pathophysiology of TBI.

Mature microRNAs are sequentially processed from pre-microRNAs, which in turn are processed from pri-microRNAs, the primary transcripts of microRNA genes.[25] The production of microRNAs can be altered by variation in DNA sequence within their genes, including copy number variation, insertion of the DNA sequence and SNPs.[26] Previous studies have shown that SNPs in microRNA genes may be related to the clinical prognosis of various diseases.[27] Researchers often adopt a cohort study or case—control study to reveal the associations between SNPs and various diseases, and it is advisable to sort out the possible functional SNPs before planning a larger population study.[28] However, the possibility of numerous SNPs in these genes opens the question of which part should be targeted for scanning as potentially functional SNPs affecting microRNA expression. Although the regulation of microRNA production involves a complex mechanism and has not been thoroughly elucidated yet, it has been postulated that the promoter areas of microRNA genes, especially the transcription factor binding motifs, play a pivotal role in the biogenesis of microRNA. In addition, functional SNPs probably sit within the promoters of microRNA genes. Because targeting SNPs within the motif area of promoter regions might most effectively identify the potentially functional SNPs, we scanned the motif areas of these microRNA genes. This scan yielded one relevant SNP (rs11851174 allele: C/T) within the motif area of the microRNA hsa-miR-431-3P gene promoter, chromosome 14, and strand +. A query of the data from HapMap project (version 2010-08_phaseII+III) resulted in a brief overview of the distribution of these alleles among different regions and ethnicities. These data revealed that the mutation rate of allele C to allele T is the lowest (4.5%) in Gujarati Indian population in Houston, and the highest in those of African ancestry (39.5%) in Southwest USA. However, no studies have suggested an association between this SNP (rs11851174 allele: C/T) and any pathophysiological processes.

Using a bioinformatic approach, we identified one SNP in the motif areas of the 14 differentially expressed microRNA gene promoter sequences. The SNP rate in these motif areas seemed low; however, several important points should be made. The regulation of microRNAs in CSF of coma patients after TBI is a multifactorial process influenced by both environmental and genetic factors, so one SNP would not be expected to explain all the differentially expressed microRNAs. In addition, the identified SNP (rs11851174) may play a role in regulating the expression level of microRNA hsa-miR-431-3P by interfering with the binding of transcription factors to motif areas. One of the predicted target genes of hsa-miR-431-3P is MTRNR2L1, which encodes Humanin, a peptide that can protect cortical neurons from ischemia and reperfusion injury, a pathological process involved in brain injury. Thus, the identified SNP (rs11851174) could plausibly be related to the consequences of TBI.

This study was not the first to suggest the associations between genetic polymorphisms and outcome after TBI.[29,30] In recent years, emerging evidence has shown that genetic factors play a role in these outcomes, and apolipoprotein E (APOE) has received considerable attention. Using a variety of measures, several investigators have reported that E4 allele of APOE is associated with a

poor outcome after TBI.[31] In addition, genes involved in other pathophysiological processes, such as cytokine genes, are now being implicated. For example, researchers have found that interleukin-6 promoter polymorphism is a risk factor for poor outcome after ischemic stroke and may affect TBI.

To our best knowledge, this study was the first to demonstrate an alteration in microRNAs in CSF of coma patients at subacute stage after TBI. Future studies that use genetic approaches or specific microRNA mimics/inhibitors may help address whether or not alterations in these microRNAs can be causally linked to cognitive dysfunction or other pathologies observed after TBI. With the development of specificity and delivery of mimetic and inhibitor compounds, microRNAs altered in CSF of comatose patients after TBI could be targeted for therapeutic intervention, which may be useful for post-traumatic coma.

This study had a few limitations, especially the small sample size and potential consequences for the quality of the statistical results. In addition, microarray testing is a relatively new technology that has been applied for cancer diagnosis, prognosis and treatment planning but also attracts criticism for a lack of reliability and consistency.[32] Thus, an altered microRNA expression profile detected by microarray platforms required further validation using other experimental methods like RT-PCR. Furthermore, our detection of microRNA expression levels in CSF was performed on TBI patients, and some therapeutic interventions could have interfered with microRNA expression and thus affected our results. Finally, the motif areas of promoter regions were based on computational prediction and required experimental confirmation.

The results of this study showed that the expression levels of specific microRNAs were changed in CSF of patients who had remained in a coma state for two weeks following TBI. In addition, one SNP (rs11851174 allele: C/T) was identified by bioinformatic methods in the motif area of the microRNA has-miR-431-3P gene promoter region. However, the exact associations of the specific microRNAs and SNP (rs11851174) with decreased consciousness after TBI remain unclear. Nevertheless, this discovery was encouraging and could serve as a basis for designing either a case—control or a cohort study to examine associations between the identified SNP (rs11851174) and traumatic coma.

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