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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

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ABSTRACT

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.

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,

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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 $\geq$18 years; (2) severe brain injury with Glasgow Coma Scale (GCS) score $\leq$8 at admission; (3) remaining in a coma state two weeks after the initial injury (GCS $\leq$ 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 $\times$ 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]