Temporal Expression MicroRNA-21 in Serum of Patients with Spinal Cord Injury

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Keywords: Spinal cord injury, Serum, MicroRNA, MicroRNA-21

Abstract. Objective: To investigate the levels of microRNA-21 (miR-21) in the serum of patients with spinal cord injury (SCI) and to determine whether there was a correlation with degree of injury. Methods: Quantitative real-time reverse transcription–polymerase chain reaction(q-RT-PCR)was used to measure the serum levels ofmiR-21 at 1, 7 and 28 days and 3 months after SCI 42 in male and female SCI patients (n = 48) and compare with age and sex-matched patients with non spinal cord injuries (NSCI, n = 60) and healthy volunteers (n = 70). Correlations between serum miR-21 levels and age, sex and degree of injury were investigated. Results: The data from the present study show that the serum level of miR-21 immediately increased and peaked on day 7 post-SCI and then declined to the control level. There were no differences between NSCI group and normal control group at each time point. The expression level o fmiR-21 was related to the lesion degree of SCI. The age and sex of the patients did not influence miR-21 expression after SCI. Conclusions: These finding might provide reference for diagnosis and treatment and contribute to the identification of selective and temporal drug targeted therapy after SCI.

Introduction

Traumatic spinal cord injury (SCI), one of the most leading causes of disability and mortality is one the most common and devastating injuries observed in spine and neurosurgery departments. It is usually caused by motor vehicle accidents, sports injuries, diving accidents, and violence, which can cause permanent disabilities such as paralysis and loss of movement or sensation. In addition, SCI initiates a variety of inflammatory and/or immune responses including the infiltration of leukocytes such as monocytes, macrophages, T-cells and NK cells, into the injured area,1 which are the key factors of the secondary damage occurring subsequent to SCI.1 The treatment of SCI remains one of the greatest challenges for the basic science and clinical investigators so far. Despite many therapies have been explored, all method demonstrated some limited efficacy thus far.2 The celluar mechanisms associated with SCI are complex and involve a multitude of signaling pathways and molecular dysfunctions.3,4 MicroRNAs (miRNAs) are a novel class of small noncoding RNAs that negatively regulate gene expression at the posttranscriptional level by binding to the 3'-untranslated region of target mRNAs leading to their translational inhibition or sometimes degradation.5 It was recently reported that miRNAs are estimated to regulate 60% of all genes in the human genome.6 thus, they may widely influence the signaling networks leading to pathological responses after SCI. Increasing reports shows that a large number of miRNAs are expressed in the central nervous system (CNS).7,8 Some miRNAs are involved in several neurological disorders, such as including traumatic CNS injuries and neurodegenerative diseases.9–15 Therefore, miRs could become attractive novel therapeutic targets for the treatment of SCI.

MicroRNA-21 (miR-21) is an important member of the miRNA family. Recently, some studies have showed that miR-21 was increased following several types of CNS injuries, such as traumatic brain injury and brain ischemia.1 3–16 Additionally, some studies have shown the involvement of miRs in the pathogenesis of SCI in a rat contusion SCI model,10–12 with miR-21 emerging as one of the most dysregulated miRs.17 However, the expression level of miR-21 on patients with SCI are still unknown. Hence, the present study is aimed to determine the serum miR-21 in SCI patients at different time points after SCI and analyze the differential expression pattern of the miR-21 based on the time after injury, age and sex between SCI patients and age- and sex-matched non-SCI (NSCI) patients and controls. The outcome should help designing time dependent specific drug targeted therapy to SCI.

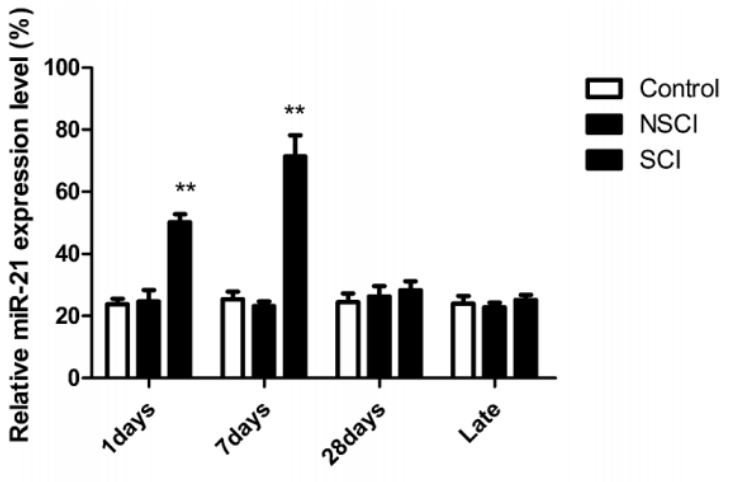

Figure1. Relative level of microRNA-21 (miR-21) in the serum after spinal cord injury (SCI) (n = 90) and non-spinal cord injuries (NSCI) (n = 70). Serum from SCI patients and control individual (NSCI and healthy volunteers) were collected 1, 7, 28 days and at three months (>30 days) after a sustained SCI (late). Data are represented as mean ± SEM (two-way ANOVA test and Tukey HSD post hoc analysis).

*P < 0.05, **P < 0.01 for SCI patients Vs control.

Material and Method

Subjects All the studies with SCI patients (n = 90) and NSCI patients (n = 60) and healthy volunteers (n = 70) were approved by Jilin university ethical committee. Written consent forms were filled out prior to the blood sample collection. This study retrospectively enrolled patients who had previously undergone SCI between July 2010 and August 2012 that were identified from a search of the archival surgical pathology files of the China-Japan Union Hospital, Jilin University, Changchun, Jilin Province, China.Expert neurologist and neurosurgeon were involved in the validation of SCI based on the clinical findings (MRI and CT scan) and paraclinical manifestations. Age- and sex matched healthy control volunteers were recruited from among individuals who sought a routine health check-up at the Physical Health Examination Centre of Jilin University, who had not previously been diagnosed with SCI. Patients with no injury to CNS were recruited (age- and sex-matched to SCI cohort) to study the effect of NSCI on miR-21 levels. NSCI group (n = 60) included 34 arm injuries and 36 leg injuries. Serum preparation A 4 ml sample of peripheral venous blood as drawn from all study participants after an overnight fast and placed at room temperature for 60 min. Then the blood samples were centrifuged at 700g for 10 min at 4 ℃ in a centrifuge, and then the serum was immediately separated from the

blood, frozen and stored at-80℃ until used. During the sample storage, repeated freeze–thawing was avoided to ensure the quality of the samples. RNA extraction and quantitative RT–PCR analysis of miR-21 Total RNA was isolated using TRIzol (Invitrogen, CA) and miRNeasy mini kit (Qiagen, West Sussex, UK) according to manufacturer's instructions. This efficiently recovered all RNA species, including mi-RNAs. RNA quality and quantity was measured using a nanodrop spectrophotometer (ND-1000, Nanodrop Technologies) and RNA integrity was determined by gel electrophoresis.Total RNA from each sample was reverse-transcribed to cDNA using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan) and qRT-PCR was performed using the SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan) and miRNA-specific primers for miR-21 (Ribobio, Guangzhou, China). The relative microRNA levels were normalized to U6 expression for each sample. The cycle threshold (Ct), which was defined as the number of PCR cycles required for the fluorescent signal to be higher than a threshold indicating baseline variability, was recorded. Relative changes of gene expression were represented by $2-\Delta\Delta Ct$, the difference between the original copy number of miR-21 in the NSCLC group and that in the control group. $\Delta\Delta Ct = (Ct\ miR\text{-}21 - Ct\ U6)$ of the SCI group − (Ct miR-21−Ct U6) of the control group. Three repeated wells were set. Statistical analyses Statistical analyses were undertaken using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA) and the SPSS® statistical package, version 16.0 (SPSS Inc., Chicago, IL, USA) for Windows® . Statistical comparisons between data sets were made based on the representation of the mean ± SD of data. Statistical analyses were performed using two-way repeated measurments analysis of variance (ANOVA) with Tukey HSC post hoc test for multiple comparisons. Significance level of p-value <0.05 was adopted.

Results

Data on the age of SCI patients and the cause and nature of the trauma are given in Table 1. Statistical analysis of demographic parameters included the mean age and gender. The mean age of the surviving patients was 35.12 ±3.46 years. Of these, 36 patients age were above 35(older), the other were below 35(younger). Most of the patients (71.11%) encountered SCI from automobile accidents and the rest from falling. 35.56% of the patients had cervical SCI and the rest had SCI at all other levels of the spine. Of these, 48 patients were complete paralysis of spinal and 42 48 patients were incomplete paralysis of spinal. We have recorded 8.89% mortality of SCI patients during the study period. NSCI patients were recruited to age- and sex-matched with the SCI cohort. NSCI group had mean age 39.44 ± 4.75 years (26 female and 34 male) and included 32 arm injuries and 28 leg injuries. The control group (n = 70) was composed of 40 males and 30 females (mean ± SD age 37.3 ± 3.4 years). There was no significant difference in age and sex distribution between the patients with SCI and the control subjects. In the present study, we have quantitated the serum level of miR-21 of SCI patients at 1days, 7 and 28 days, and 3 months (Late) after the trauma. The data are given in Figure1. In the SCI patients, the serum level of miR-21 significantly increased 1day following the trauma. The increase in the serum level of miR-21 reached a peak on day 7 after SCI. After that period, the level of miR-21 returned back to the control level. However, there was no change in the serum miR-21 in NSCI patients and health control. There was no correlation between the level of miR-21 and patients gender (Figure2A). In addition, the expression the level of miR-21 has no correlation with patients age (Figure2B). There was a significant

difference between the serum levels of miR-21 and different degree of injury ($p < 0.001$). The serum level of miR-21 in complete paralysis of spinal group was significantly higher than those of incomplete paralysis of spinal group at 1day and 7day after SCI.

Table1. Demographics of SCI patients (n = 90) involved in the present study.

Factor		Number of patients			Age year (mean±SD)
		Male	Female	All	
Cause of injury	Vehicle	46	18	64	36.12±6.78
	Falling	16	10	26	34.34±5.12
Site of injury	Neck	20	12	32	37.41±4.33
	Others	42	16	58	35.20±5.56
Outcome	Death	5	3	8	46.55±4.89
	Survived	57	25	82	35.12±3.46
Degree of injury	Complete paralysis group	30	18	48	37.14±6.73
	Incomplete paralysis group	32	10	42	35.22±4.98

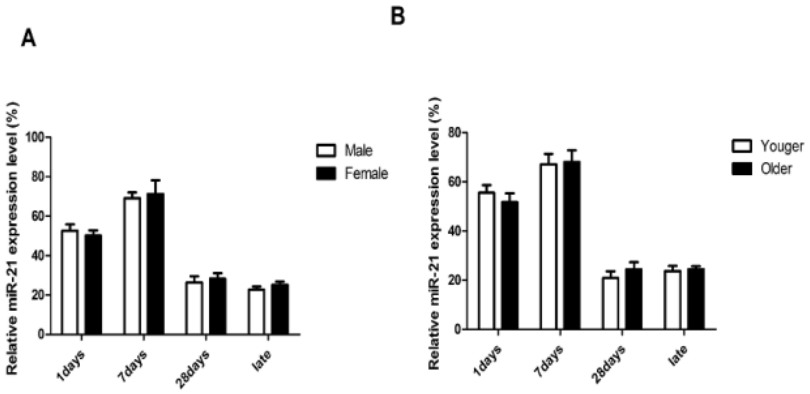

Figure2. Relative level of microRNA-21 (miR-21) in the serum after spinal cord injury (SCI) at different sex(A) and different age(B).Serum from SCI patients were collected 1, 7, 28 days and at three months (>30 days) after a sustained SCI (late).

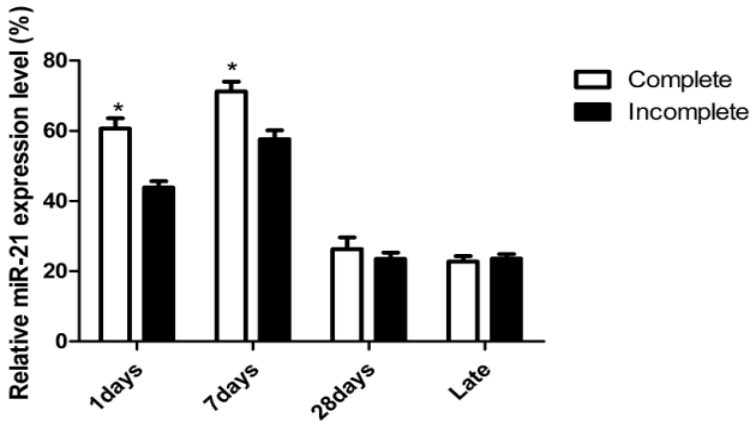

Figure3. Relative level of microRNA-21 (miR-21) in the serum after spinal cord injury at complete paralysis of spinal group and incomplete paralysis of spinal group. *P < 0.05.

Discussion

Traumatic spinal cord injury (SCI) could induces widespread molecular and biochemical changes, such as altered mRNA and protein expression,production of free radicals, axonal plasticity, inflammatory activation and neuronal cell death.18,19 Recently, it has been demonstrated that individual miRNAs can regulate hundreds of genes simultaneously by targeting RNA-induced silencing complexes (RISC) to mRNAs where they acted to inhibit translation or direct destructive cleavage.20 Therefore, altering the expression of miRNAs may greatly affect SCI pathophysiology and functional outcome. In present study, we found that the serum levels of miR-21 at different time points has different changs after SCI, which further showed that miRNAs may greatly affect SCI pathophysiology and functional outcome. Recently, several other studies have demonstrated aberrant expression of miR-21 in many injury models. For example, microarray analyses demonstrated that the levels of many miRNAs were altered at several time points (6–72 h) after traumatic brain injury, and that miR-21 was upregulated at all times in the rat cerebral cortex,1 3 Similarly, Redell et al.found that the expressions of many miRNAs were altered in the hippocampus after traumatic brain injury, and miR-21 expression was significantly upregulated in the hippocampus with expression levels peaking 3 days post-injury, and returned to near normal levels days post-injury.21 In addition, recently, several studies demonstrated that miR-21 was upregulated following contusion SCI.10–12 Recently Hu et al reported miRNA signatures after SCI have revealed that multiple miRs are aberrantly expressed via microarray analyses. Among them, miR-21 expression upregulate and contributes to SCI. Knockdown of miR-21 in vivo exacerbates the functional deficit, aggravates tissue damage, and increases apoptotic cell death in rats following SCI.22 However, these report mainly focused on miR-21 expression in rat model, which have some different from patients. In the present, we selected patients as study objective and found the serum levels of miR-21 at different time points has different changs after SCI, which might contribute to the identification of selective and temporal drug targeted therapy after SCI. In conclusion, in the present study, our data showed that the serum levels of miR-21 at different time points has different changes after SCI and serum miR-21 levels has significantly correlated with degree of injury in SCI patients. These findings might contribute to the identification of selective and temporal drug targeted therapy after SCI.

Declaration of conflicting interest. The authors declare that there are no conflicts of interest.

Funding. The authors gratefully acknowledge the financial support provided by The Development of Science and Technology Plan Projects of Jilin (no. 2011Z099).

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