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Initial research on the relationship between let-7 family members in the serum and massive cerebral infarction

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ABSTRACT

Eighty-eight ischemic stroke patients with massive cerebral infarction (MCI) who met our selection criteria were included in this study. MCI was assessed using the Glasgow Coma Scale (GCS) at hospital admission and at 2 weeks. The sera of all patients and controls were sampled at 48 h after the patients' attacks, and the sera of patients with MCI who had no severe cardiopulmonary complications, including those with hemorrhagic transformation (HT), were sampled again at 2 weeks. The relative expression of let-7 miRNA in the serum was determined by real-time qRT-PCR, and the blood levels of lipids, glucose, high-sensitivity C-reactive protein (hs-CRP), homocysteine and blood pressure were measured at admission. Interleukin-6 (IL-6) levels were detected by ELISA, and a luciferase assay was performed to confirm that IL-6 was a gene target of let-7. The relative expression of let-7f was significantly down-regulated in MCI without HT patients compared with controls ($P < 0.001$), and it was positively correlated with GCS ($P < 0.01$) and negatively correlated with hs-CRP ($P < 0.01$). The relative expression of let-7f was significantly up-regulated in MCI patients with HT ($P < 0.01$). IL-6 is a direct target gene for let-7f, and IL-6 expression was increased in MCI without HT patients compared to controls ($P < 0.01$). The expression of let-7f in serum is associated with MCI without HT, which specifically inhibits IL-6. This suggests that let-7f may control inflammation in patients with MCI without HT.

1. Introduction

Acute ischemic stroke involving the entire vascular distribution of a carotid or middle cerebral artery can cause massive cerebral infarction (MCI) [1], which has a poor prognosis both in the short and long term, resulting in death in most patients and severe neurological defects in patients who survive [2–4]. MCI cases with massive middle cerebral artery (MCA) infarction account for 10% to 15% of all MCA infarctions [5].

MicroRNAs (miRNAs), which are single-stranded, small, non-coding RNAs, regulate various pathophysiological processes, including the occurrence of tumors, cell proliferation, hematopoiesis, metabolism, inflammation, embryo formation, etc. [6–8]. The let-7 family was the second microRNA group to be discovered in *Caenorhabditis elegans*, and later studies found that it has important regulatory roles, such as cell growth, differentiation, and apoptosis [9–11]. Abnormal expression of let-7 is closely related to many human diseases, such as cancer and cardiovascular diseases [12–13]. The let-7 family may be regulated during acute ischemic stroke, according to the results of microarray assays in previous studies [14–15]. Low-grade chronic inflammation has been widely recognized to play an important role in the process of atherogenesis [16], and the levels of inflammatory markers (e.g., IL-6) are associated with the risk of experiencing cerebrovascular events [17–18]. The aim of this study was to determine whether let-7 family members in the serum are related to MCI and the potential regulatory mechanism of let-7 for IL-6 expression.

2. Materials and methods

2.1. Clinical data

Between 2012 and 2014, 88 patients with MCI were selected for this study. The selection criteria were as follows: 1) age > 18 years; 2) within 48 h after stroke attack; 3) based on CT or MRI, the patient had an infarct of at least 67% of the middle cerebral artery (MCA) territory, with or without the additional infarction of the anterior or posterior cerebral artery on the same side [19]. The exclusion criteria were as follows: 1) unconsciousness due to metabolic disturbances or medication; 2) any sedation or surgery; 3) a pre-stroke score on the modified Rankin Scale (mRS) of more than 2; and 4) the presence of a concurrent serious illness which may affect the patient's outcome, such as severe cardiopulmonary complications. Healthy persons (N = 45) and patients

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who suffered from ischemic stroke without MCI (IS without MCI, N = 40) and intracerebral hemorrhage (ICH, N = 45) were used as controls.

For patients with MCI who had no severe cardiopulmonary complications at 2 weeks after the attack, CT was repeated to determine the occurrence of hemorrhagic transformation (HT) (N = 22) or the absence of HT (N = 34). The patients with MCI were assessed by Glasgow Coma Scale (GCS) at hospital admission and at 2 weeks, and lipid, glucose, high-sensitivity C-reactive protein (hs-CRP), homocysteine blood levels and blood pressure were measured at those times.

Ethics statement: This study was approved by Medical Ethics committee of the First Affiliated Hospital of Zhengzhou University, and was performed according to the principles of the Declaration of Helsinki and the amendment of Somerset West (1996). All participants provided written informed consent.

2.2. Serum collection

Fresh blood samples were obtained from groups as previously described and were collected into non-anticoagulant tubes. Fresh serum samples were obtained by the centrifugation of blood samples at 200 × *g* for 15 min at 4 °C. The supernatants were removed and collected into 1.5 mL polypropylene tubes at −80 °C.

2.3. Evaluation for potential hemolysis of the blood samples

To determine whether hemolysis of the blood samples had occurred during the sample preparation, the expression level of two miRNAs was determined; miR-451, which was expressed in red blood cells, and miR-23a, which was relatively stable in serum and not affected by hemolysis. A delta Cp (miR-23a–miR-451) of more than five was an indicator of possible erythrocyte miRNA contamination and a delta Cp of 7–8 or more indicated a high risk of hemolysis [20]. These parameters were evaluated for some random samples from both the experimental and control groups. The results indicated that possibility of hemolysis of the blood samples were quite low.

2.4. Real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR)

The Reverse Transcription System and Gotaq qPCR Master Mix (Promega) were used. The 5 s ribosomal RNA (5 s rRNA) was used to normalize the samples. The quantitative PCR procedure was performed according to the instructions provided by the manufacturers. Amplification was performed using a LightCycler 1.5 Real-Time PCR System (Roche). The endpoint of qRT-PCR data is the comparative cycle threshold method (Ct). The relative changes in gene expression were quantified using the Livak method (also known as $2^{-\Delta\Delta Ct}$ method) after determining the Ct values for the reference and target genes in each sample set. All reactions were performed in triplicate.

2.5. Prediction of potential gene targets and ELISA

miRNAs can be predicted using a computational approach. First, the potential binding sites in the messenger RNA 3′-UTR were identified according to specific base-pairing rules. Then, cross-species conservation requirements were implemented. A predictive search for a potential gene target of let-7f was performed using the programs TargetScan (http://www.targetscan.org), Miranda (http://www.microrna.org) and DIANA-microT (http://diana.cslab.ece.ntua.gr). Using three different selection criteria, we identified interleukin-6 (IL-6) as a potential gene target of let-7f. Human interleukin 6 (IL-6) protein levels in the sera of patients from different groups were quantified using commercial ELISA kits (Lichen in Shanghai) according to the manufacturer's instructions.

2.6. Reporter vectors and luciferase assay

According to the Miranda system, segments of the 3′-UTR of IL-6 were amplified by genomic PCR and cloned between the XhoI–NotI sites of psicheck-2 (Promega). We cloned the segment of the IL-6 3′-UTR that contains a potential let-7f binding site and is located at nucleotides 316–322. The oligonucleotide sequences used for the PCR were IL6XhoIF: 5′-ccgctcgagCATGGGCACCTCAGATTGTTGTT-3′; and IL6NotIR: 5′-ataagaatgcggccgcTTGCTGAATTTTTTAAAATGCCATT-3′. The PCR conditions were 30 cycles of 98 °C for 15 s, 55 °C for 15 s, and 68 °C for 20 min. The site-directed mutagenesis was constructed at the binding site using the following sequences: mutIL6F: 5′-GATCATTTCTTGGAAAGTGTAGGCTATGGAGTAATAAATGGCTAACTTATACATATT-3′; and mutIL6R: 5′-AATATGTATAAGTTAGCCATTTATTACTCCATAGCCTACACTTTCCAAGAAATGATC-3′.

Next, 293T cells (2×10^4 cells/well) were seeded in 24 well-plates, and transfection was performed in triplicate at 50–60% confluency by adding 100 μl transfection solution, which contained 50 nM microRNA, 0.5 μg reporter plasmid in psiCHECK2 (Promega), and 1 μl Lipofectamine 2000 (Invitrogen). Then, the cells were incubated for 5 h in 5% CO^2 at 37 °C, and the transfection solution was replaced with complete medium. Two days after transfection, 100 μl Passive Lysis Buffer (Dual-Luciferase Reporter Assay System; Promega) was added. The relative amounts of Renilla and firefly luciferase were analyzed using a dual luciferase assay. The Renilla/firefly luciferase ratio was calculated and normalized to the control.

2.7. Statistical analysis

All data are presented as the means ± SD unless otherwise specified. To analyze significant differences, the normally distributed data were compared between the different groups using the two-tailed Student's t-test or one-way ANOVA for multiple comparisons. The α level was set at 0.006 (0.05/8) in accordance with the Bonferroni correction for multiple comparisons. The paired sample data were compared using the paired sample test. Discontinuous variable between groups were compared using the Chi-square test, and ordinal variables were compared using the Mann–Whitney U test. Correlation analysis was performed with the Pearson correlation coefficient. A *P*-value less than 0.05 was defined as statistically significant. All statistical analyses were performed with SPSS software for Windows, v. 19 (SPSS). The diagram was generated with GraphPad Prism 5.

3. Results

3.1. Clinical data

There were no significant differences in age or gender among the MCI (48 h), IS without MCI, ICH, and NC groups (Table 1). There were no significant differences in age and gender between the MCI without HT (2 weeks) and the MCI with HT (2 weeks) groups (Table 2).

Table 1
Demographic and clinical characteristics of the sample population at 48 h.

	MCI (48 h)	IS without MC	ICH	NC	*P*-value
Age, mean ± SD (years)	58.57 ± 3.2	58.15 ± 2.3	57.91 ± 2.6	57.82 ± 2.4	0.409
Male/female (cases)	55/33	26/14	29/16	26/19	0.895

Chi-square test for sex data, one-way ANOVA for age data in groups.