Study on The Responsiveness to Oriental Medicine Therapy and Single-Nucleotide Polymorphism in Korean Cerebral Infarction Patients

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Ischemic brain injury such as cerebral infarction is characterized by acute local inflammatory response mediated by cytokines. The mechanism of cytokines involved in cerebral infarction progression are incompletely revealed yet. We investigated to find out the relationship between single nucleotide polymorphism (SNP) of interleukin 4 receptor (IL4R) and Oriental Medicine therapy efficacy in patients with cerebral infarction for 2 weeks. Oriental Korean Medicine therapies (herbal medicine and acupuncture) were applied daily and motor functions of patients were assessed using the modified cerebral vascular accident (MCVA) scores. Genotyping for IL4R polymorphism was done by pyrosequencing analysis. In IL4R genotypes and the frequency of alleles, there was no significant difference between cerebral infarction patients (n=124) and controls group (n=175). And there was also no significant difference among good and bad responders in cerebral infarction patients. In this study the IL4R genotype might not be the risk factor or a good predictive genetic marker for good and bad responders in cerebral infarction patients in Korean. Further studies including different cytokine genes will be necessary for the exact genetic markers.

Key words : Interleukin 4 Receptor (IL4R), gene, polymorphism, cerebral infarction, modified cerebral vascular accident (MCVA) scores, Oriental Medicine therapy

Introduction

Stroke is a clinical concept of neurological disorder characterized by an acute faint, unconsciousness, excessive phlegm, hemiparesis, dysphasia, facial palsy and motor disorder, etc. and is called cerebro-vascular accident (CVA) in Western Medicine11. Cerebral infarction is a multifactorial disease and various factors such as atherosclerosis, hypertension, diabetes mellitus, hyperlipidemia and smoking interact to increase the risk of developing cerebral infarction2-4).

In Oriental Medicine, cerebral infarction has been called wind-stroke syndrome (Chungpung). It is syndrome due to external affection by the pathogenic wind5. Acupuncture, moxibustion and herbal medicines have been used to treat wind-stroke syndrome6-9).

Genetic factors appear to contribute to virtually every human disease, conferring susceptibility or resistance, affecting the severity or progression of disease, and interacting with environmental influences8-10). Trying to get the information about genetic variation is important for understanding how genes function or malfunction, and how genetic and functional variation are related.

Recently in cerebral infarction many polymorphism were investigated and some polymorphism such as α1-antichymotrypsin gene was associated12). But some polymorphism such as promoter of lipopolysaccharide receptor CD14 was not related13).

About the association of interleukin 4 receptor (IL4R) polymorphism with stroke it was reported that the combination of TNF -308 G/G homozygosity and the IL4R 503P variant carrier status was associated with a particularly strong predisposition to large vessel stroke14).

Including herbal medicine and acupuncture Oriental Medicine therapy has been utilized to treat cerebral infarction for thousands of years in Korea15). But, although Oriental Medicine has therapeutic effect, all patients doesn’t respond to the therapy16). It would be very useful to predict which patients will have good improvement or not with Oriental Medicine therapy.

Response to therapies can also be affected by genetic differences17). So far there are few reports of gene polymorphisms predicting response to Oriental Medicine therapy in Korean cerebral infarction patients. Advances in
genotyping technology have facilitated the investigation of genetic factors in both disease susceptibility and severity[18,19].

In this study, we investigated the relationship between single-nucleotide polymorphism (SNP) of IL4R and Oriental Medicine therapy efficacy in patients with cerebral infarction for 2 weeks. This is the report to have shown the association of IL4R gene polymorphisms with cerebral infarction by use of brain computed tomography (CT) or magnetic resonance imaging (MRI) findings.

Subjects and Methods

1. Subjects

The control group consisted of 175 apparently healthy Korean. Controls were selected from healthy subjects who visited for the health examinations at Daegu Haany University medical center from March 2004 to December 2006. The group of 124 are cerebral infarction patients.

2. Definition of cerebral infarction

We included cerebral infarction patients with neurological symptoms lasting >24 hours accompanied by corresponding focal density changes detected by CT or MRI, and excluded patients suffering from internal cerebral hemorrhage, epidural (or subdural) hematoma, brain tumors, and accidental or iatrogenic cerebral infarction. Final diagnosis of cerebral infarction was confirmed by serial CT or MRI findings.

3. Methods of Oriental Medicine therapy

The acupuncture methods were applied so as to elicit the sensation of Qi, and the needles were removed after 15 minutes. Daily acupuncture therapy was administered for 2 weeks at the same time each day (around 10:00 am) at points GV20, GV26, CV24, LI11, LI4, TE5, ST36, LR3, GB34, GB31, and GB3920. The needles used were stainless steel filiform needles (0.25 mm diameter, 40 mm length, DongBang, Korea). All patients were treated with herbal medicines (Sopung-tang or Sosokmyeong-tang, 120cc, t.i.d. p.o.) and Oriental physical therapy (Tens or IST, 20 minutes).

4. Assessments

Patients were assessed using the modified cerebral vascular accident (MCVA) scores. The MCVA scores consist of Glasgow Coma Scale(GCS), Fugl Meyer scale and NIH stroke scale[21-25]. Scores were determined by same protocol, and no more than one time at each test. Patients were tested two times: before treatment, after two weeks of treatment. The changes in MCVA scores of 28 patients were analyzed, so good responders were as patients who showed increase of MCVA scores, and bad responders were who showed decrease of MCVA scores.

5. Blood samples

Blood samples were obtained from the antecubital vein without regarding to the time of the last meal. This study was approved by the ethics review committee of Daegu Haany University medical center. Informed consent was obtained from all subjects. If patients were noncommunicative, it was obtained from close relatives.

6. Polymerase Chain Reaction Preparation

Blood samples from all subjects were obtained for DNA extraction and collected in EDTA tube. Genomic DNA was extracted using DNA isolation kit for Mammalian Blood (Roehringer Mannheim, IN, USA). The extracded DNA was amplified by polymerase chain reaction (PCR), according to the method of Lesch et al., with minor modifications[26]. The IL4R gene (110-bp) was amplified using 25 ng of DNA, 5 pmol of each primer. IL4R forward was 5'-GAAACCCTGGGAGCACAGTCC-3' and IL4R reverse was 5'-TCCACCGCATGTACAAACTC-3'. The PCR amplification was performed by using 0.5 unit Taq polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom). The 30 ul of PCR reaction mixtures were 10 mM Tris-HCL, pH 9.0, 1.5 mM magnesium chloride, 50 mM potassium chloride, 0.1% Triton-X 100, 0.01 % [v/v] stabilizer, 0.25 mM of each deoxynucleotide triphosphate (dNTP), 0.1 M of each oligonucleotide primer. The PCR steps were denaturation of 5 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C with a Thermocycler (Astech, Fukuoka, Japan). The reverse primer was biotinylated to allow the preparation of single stranded DNA. The quality of PCR products was controlled by 1.5% of agarose gel electrophoresis.

7. Genotyping and pyrosequencing

DNA Preparation for pyrosequencing was performed according to manufacturer’s standard protocol (Pyrosequencing AB, Uppsala, Sweden)[29]. The streptavidin sepharose beads (Streptavidin Sepharose HP, Amersham Pharmacia Biotech, Upppsala, Sweden) were immobilized to PCR products. The sequencing primer of IL4R gene (refSNP ID: rs1801279) from Pyrosequencing AB. By incubation at room temperature for 10 minutes, 20 ul of biotinylated PCR products were immobilized onto streptavidin coated sepharose beads, the immobilized
PCR products were transferred to a Millipore 96-well filter plate (Millipore, Bedford, MA, USA). Vacuum was used to eliminate the different solutions and reagents to obtain pure, single stranded DNA while the beads remained in the wells\(^{26}\). In 55 ul of 4 M acetic acid containing 0.35 uM of IL4R sequencing primer the beads with the immobilized template were resuspended. Then the 45 ul of suspension was transferred to a PSQ 96 plate (Pyrosequencing AB, Uppsala, Sweden)\(^{27}\). By using PSQ 96 Sample Prep Thermoplate (Pyrosequencing AB, Uppsala, Sweden) the PSQ 96 plate containing the samples was heated at 90°C for 5 minutes for sequencing primer annealing, and moved to room temperature for 10 minutes. Then the PSQ 96 Plate was placed into the process chamber of the PSQ 96 instrument (Pyrosequencing AB, Uppsala, Sweden)\(^{28}\). The enzymes, substrates, and nucleotides were dispensed from a reagent cassette into the wells by using the PSQ 96 SNP Reagent Kit (Pyrosequencing AB, Uppsala, Sweden). The light was generated when a nucleotide is incorporated into a growing DNA strand\(^{29}\). From this process the polymorphism of the IL4R was genotyped automatically.

8. Statistical analysis

X2 tests or Fisher’s exact test was used to compare sex, the distribution of the genotypes and the frequencies of alleles between cerebral infarction patients and controls, good responders and bad responders. To compare the age of cerebral infarction patients and controls Student’s t-test was used.

The genotype data were tested for Hardy-Weinberg equilibrium using the X2 goodness-of-fit test. The changes in the MCVA scores of the two groups after 2 weeks of treatment were analyzed by paired t-test. P-values of less than 0.05 were considered significant. The statistical package used in this study was SAS (release 8.1, SAS Institute Inc, Cary, NC, USA).

Results

1. Characteristics of subjects

The patient group consisted of Korean cerebral infarction patients. At first 132 cerebral infarction subjects were, who were admitted to the department of acupuncture & moxibustion, hospital of Oriental Medicine, Daegu Haamy University. Of these patients, 8 subjects were excluded from this study (6 were transported to other hospitals, and 2 declined to give consent). Ultimately, 124 Koreans were enrolled in the current analysis. The characteristics of the patients and controls are shown in Table 1. There was no significant difference between the patients and controls as for age and sex.

| Table 1. Clinical Characteristics of Cerebral Infarction Patients and Controls |
|-------------------------------------------------|------------------|----------|--------|
| Controls (n=175)                               | Cerebral infarction patients (n=124) | P value |
| Male / Female                                  | 95 / 80          | 65 / 59  | 0.749  |
| Age, mean±SD                                    | 64.1 ± 5.2       | 63.7 ± 8.8 | 0.733  |

X2 test was used to compare values of cerebral infarction patients and controls for sex. Age was compared by Student’s t-test.

2. IL4R genotypes

The distribution of genotypes and allelic frequencies are shown in Table 2. The observed genotype frequencies of cerebral infarction patients and the control group did not show significant difference predicted by the Hardy Weinberg equation. As for IL4R genotypes distribution of A/A homozygotes, A/G heterozygotes, and G/G homozygotes and there was no significant difference between control and cerebral infarction group (p=0.557, Fig. 1-3). About allele frequencies distribution there was no significant difference between control and cerebral infarction group (p=0.235, Table 2).

<table>
<thead>
<tr>
<th>Table 2. Genotype and Allele Frequencies of Polymorphism in IL4R Gene in Cerebral Infarction Patients and Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype (%)</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>AA</td>
</tr>
<tr>
<td>127 (72.6)</td>
</tr>
</tbody>
</table>

X2 tests or Fisher’s exact test was used to compare genotypes and allele frequencies between controls and cerebral infarction patients.

![Fig. 1. Result of A/A homozygote genotype of the IL4R gene by using Pyrosequencing](image1)

![Fig. 2. Result of A/G heterozygote genotype of the IL4R gene by using Pyrosequencing](image2)
3. MCVA scores and Oriental Medicine therapy response

To compare the MCVA scores between good responders and bad responders to Oriental Medicine therapy Student's t-test was used. There was no significant difference between good responders and bad responders in both before treatment and after treatment (p=0.297, p=0.913 respectively).

Paired t-test was used to compare the changes in the MCVA scores of good responders and bad responders after 2 weeks of treatment. There was significant difference only in good responders between before treatment and after treatment (p=0.029, Table 3). The MCVA scores are shown in Table 4.

Table 3. Modified Cerebral Vascular Accident (MCVA) Scores and Response to Oriental Medicine Therapy

<table>
<thead>
<tr>
<th>Items</th>
<th>Good responders</th>
<th>Bad responders</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>63.7 ± 11.3</td>
<td>65.2 ± 11.9</td>
<td>0.779</td>
</tr>
<tr>
<td>Before treatment</td>
<td>18.6 ± 5.9</td>
<td>22.0 ± 8.3</td>
<td>0.597</td>
</tr>
<tr>
<td>After treatment</td>
<td>22.2 ± 5.5</td>
<td>21.9 ± 8.2</td>
<td>0.913</td>
</tr>
<tr>
<td>After treatment - Before treatment</td>
<td>3.6 ± 3.7</td>
<td>0.1 ± 0.2</td>
<td>0.001</td>
</tr>
</tbody>
</table>

To compare the MCVA scores between good responders and bad responders Student's t-test was used. Paired t-test was used to compare the changes in the MCVA scores of good responders and bad responders after 2 weeks of treatment (p=0.029, p=0.186 respectively).

Table 4. Modified Cerebral Vascular Accident scores

<table>
<thead>
<tr>
<th>Items</th>
<th>Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Level of consciousness (Glasgow coma scale)</td>
<td>15~3</td>
</tr>
<tr>
<td>2. Motor</td>
<td></td>
</tr>
<tr>
<td>(1) Arm</td>
<td>raise the arm 180° above the head so that the thumb is pointing towards the head of the bed</td>
</tr>
<tr>
<td>with straight arm</td>
<td>10</td>
</tr>
<tr>
<td>more than 90° with extended elbow</td>
<td>9</td>
</tr>
<tr>
<td>at 90° with extended elbow</td>
<td>8</td>
</tr>
<tr>
<td>at least 5° with extended elbow</td>
<td>7</td>
</tr>
<tr>
<td>start position (maximal abduction of shoulder, elbow flexed, radiocubital joint approximated)</td>
<td>move the hand towards the opposite knee, full extension on completion of the movement and full pronation in radiocubital joint</td>
</tr>
<tr>
<td>perform the complete movement</td>
<td>6</td>
</tr>
<tr>
<td>perform parts of the movement</td>
<td>5</td>
</tr>
<tr>
<td>if cannot perform the above movement, move the hand to the starting position above</td>
<td></td>
</tr>
<tr>
<td>perform the complete movement</td>
<td>4</td>
</tr>
<tr>
<td>perform parts of the movement</td>
<td>3</td>
</tr>
</tbody>
</table>

4. Genotyping and Oriental Medicine therapy response

The patients were divided into good responders and bad responders by the response to Oriental medicine therapy. The observed genotype frequencies of good responders and bad responders did not show significant difference predicted by the Hardy-Weinberg equation. As for IL4R genotypes distribution and allele frequencies distribution there was no significant difference between good responders and bad responders (Table 5, p=1.000, p=0.483 respectively).

Table 5. Genotype and allele frequencies of polymorphism in IL4R gene of good responders and bad responders in cerebral infarction patients and controls.

<table>
<thead>
<tr>
<th>Genotype (%)</th>
<th>Allele Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>AG</td>
</tr>
<tr>
<td>good responders</td>
<td>n=8</td>
</tr>
<tr>
<td>bad responders</td>
<td>n=10</td>
</tr>
</tbody>
</table>

X2 tests or Fisher's exact test was used to compare genotypes and allele frequencies between good responders and bad responders in cerebral infarction patients.

Discussions

Stroke is the second most fatal disease following cancer.
in Korea, and is the most frequent disease as the cause of admission most hospitals of Oriental Medicine\textsuperscript{30,31}. Cerebral infarction, as one of several stroke types, develops several complications, among which sequelia of cerebral infarction like motor disorder affects the family as well as the patient with great psychological and financial stress. Physical and psychological impairment from cerebral infarction may negatively affect quality of life\textsuperscript{32,33}.

Cerebral infarction is a multifactorial disease and various factors such as hypertension, diabetes mellitus, atherosclerosis, hyperlipidemia and smoking interact to increase the risk of developing cerebral infarction\textsuperscript{34}. Lou et al revealed platelet hyperaggregability was seen in young patients with completed stroke\textsuperscript{35}.

IL-4 is an anti-inflammatory cytokine, which reduces the production of proinflammatory cytokines and destructive enzymes\textsuperscript{36,37}. For many cytokines and their receptors, genetic variants have been described\textsuperscript{38,39}. Gene expression can be regulated by a number of genetic elements located in the 5'-upstream region of the gene. Variances in this upstream sequence can result in different level of gene expression. To date, the genetic polymorphism in the 5'-flanking region of the IL-4R gene has rarely been described in cerebral infarction.

The mechanism of cytokines involved in cerebral infarction progression are not revealed completely yet. But ischemic brain injury such as cerebral infarction is characterized by acute local inflammatory response mediated by cytokines\textsuperscript{40}. Recently it was reported that the mean IL-4 serum levels were significantly higher in the patients with cerebral infarction than in the normal groups\textsuperscript{41,42}.

Whether cytokines is a result or cause of the brain injury like cerebral infarction process is not certain. We hypothesized that the IL-4R gene would be an important candidate in the development of cerebral infarction. Cytokines in cerebral infarction may play an important role in the response to injury. Especially anti-inflammatory cytokines such as IL-4 may act in a feedback loop to inhibit continued proinflammatory cytokine production\textsuperscript{43}.

About the association of IL-4R polymorphism with stroke it was reported that the combination of TNF (-308) G/G homozygosity and the IL-4R 503P variant carrier status was associated with a particularly strong predisposition to large-vessel stroke\textsuperscript{44}. The IL-4R is composed of multiple chains, including a specific chain and a \(\gamma\)c chain. In the IL-4R \(\alpha\)-chain gene, an A\(\rightarrow\)G transition at nucleotide 1902, causing a change from glutamine to arginine at codon 576\textsuperscript{45}. Recently in cerebral infarction many polymorphism were investigated and some polymorphism such as 1-antichymotrypsin gene was associated\textsuperscript{46}, but some polymorphism such as promoter of lipopolysaccharide receptor CD14 was not related\textsuperscript{47}.

In the IL-4R genotypes distribution there was no significant difference between control and cerebral infarction group (\(p=0.557\)). Also about allele frequencies distribution there was no significant difference between control and cerebral infarction group (\(p=0.235\)).

We compared the MCVI scores between good responders and bad responders to Oriental Medicine therapy. There was significant difference only in good responders before treatment and after treatment (\(p=0.029\)). However, there was no significant difference between good responders and bad responders in both before treatment and after treatment (\(p=0.297\), \(p=0.913\) respectively).

Information about DNA sequence variation will thus have a wide range of application in the analysis of disease and in the development of diagnostic, therapeutic, and preventative strategies. With a better understanding of interactions between genotype and treatment response it would be possible to decide the treatments most likely to be efficacious for specific patients.

Predicting which cerebral infarction patients are likely to respond well to the treatment and undergo a good improvement course based on genotype data would be a major clinical point. It would ensure that cerebral infarction patients at highest risk of a severe outcome could be targeted with early aggressive therapies.

We followed up patients with cerebral infarction who were being treated with Oriental Medicine therapy, and tested whether the patients clinically respond to Oriental Medicine therapy or not. We divided the patients into good responders and bad responders by the response to Oriental Medicine therapy. However, there was also no significant difference between good responders and bad responders as for IL-4R genotypes and allele frequencies distribution (\(p=1.000\), \(p=0.483\) respectively).

In this study the IL-4R genotype might not be the susceptibility factor in cerebral infarction patients in Korean or a good predictive genetic marker for good and bad responders. Further studies including different cytokine genes will be necessary for the exact genetic markers in cerebral infarction. Additional studies about relationship among genetic markers and infarction region and preceding factors such as hypertension, diabetes mellitus, hyperlipidemia are required.

**Conclusion**

The results imply that the IL-4R polymorphism might not
be the risk factor in the cause of cerebral infarction. The findings of this study need to be confirmed in larger patients samples. Establishment of more systemic approach and high quality of studies are necessary for genetic markers of good prediction in cerebral infarction.

References