원저

Interleukin 4 Receptor (IL-4R) Gene Polymorphism in Korean Stroke Patients by Using Pyrosequencing

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국문초록

Pyrosequencing을 이용한 한국인 중풍 환자의 Interleukin 4 Receptor (IL-4R) 유전자 다형성

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목적 : 본 연구는 Interleukin 4 Receptor (IL-4R) 유전자 다형성이 중풍의 발병과 관련이 있는지 알아보기 위해 수행되었다.

대상 : 대구한의대학교부속 한방병원에 입원한 중풍환자 56명과 종합건진센터에 내원한 중풍 기왕력이 없는 건강인 83명을 대상으로 하였다.

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방법 : 각 그룹에서 개개인마다 DNA를 분리 정제한 후 Taq polymerase로 증폭하여 한천 겔에서 전기영동을 하여 PCR 산물을 확인하였다. PCR 산물은 Pyrosequencing 과정을 통하여 IL4R의 유전형이 자동으로 판정되었다.

결과 : A/A, A/G, G/G의 세가지 유전자형이 검출되었으며 중풍군과 대조군 사이에 유의성 있는 차이가 발견되었다(p=0.005). 그러나 개별 allele 빈도에 있어서는 중풍군과 건강인 사이에 통계적인 유의성이 나타나지 않았다(p=0.995).

결론 : 이상의 결과를 통하여 IL4R 유전자 다형성은 중풍의 발병과는 관련성이 있는 것으로 사려되지만 더 많은 환자를 대상으로 다른 환경요인 또는 유전자와의 연관성에 대한 심도있는 연구가 필요하다고 하겠다.

Key words : Stroke, Interleukin 4 Receptor (IL-4R), Gene, Polymorphism

I. Introduction

Stroke is a leading cause of morbidity and mortality in Korea. Physical and psychological impairment from stroke may negatively affect quality of life. Stroke is a multifactorial disease and various factors such as atherosclerosis, hypertension, diabetes, hyperlipidemia and smoking interact to increase the risk of developing stroke. Lou et al revealed platelet hyperaggregability was seen in young patients with completed stroke[1].

IL-4 is a anti-inflammatory cytokine, which reduces the production of proinflammatory cytokines and destructive enzymes[2-3]. For many cytokines and their receptors, genetic variants have been described[4-8]. The IL-4R chain gene polymorphism was associated with IgE secretion and atopy in familial studies[9].

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, genetic polymorphism in the 5′-flanking region of the IL4R gene has not been described in stroke. We hypothesized that the IL4R gene is important candidate in the development of stroke.

In this study, we investigated the SNP (single-nucleotide polymorphism) of IL4R in patients with stroke. The present study was undertaken to see if specific genotypic and allelic variations are associated with stroke in the Korean population.

II. Subjects and Methods

1. Study Population

The control group consisted of 83 apparently healthy Korean. Controls were selected from healthy subjects who visited for the health examinations at Jehan medical center in Daegu from March 2002 to May 2003. The patient group consisted of 56 Korean stroke patients. At first 73 stroke subjects were selected from November 2001 until May 2003, who were admitted to the stroke service of the department of acupuncture & moxibustion, college of Oriental Medicine, Daegu Haany University. Of these patients, 17 subjects were excluded from this study (11 were transported to other hospitals, and 6 declined to give consent). Ultimately, 139 Koreans were enrolled in the current analysis.

2. Definition of Stroke

We included cerebral infarction patients with neurological symptoms lasting >24 hours accompanied by corresponding focal density changes detected by brain CT or MRI, and excluded patients suffering from epidural (or subdural) hematoma, brain tumors, and accidental or iatrogenic stroke. Intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH) was also excluded.

Final diagnosis of stroke was confirmed by serial CT or MRI findings. In case of CT, cerebral infarction was identified by gradual or sometimes rapid development of focal neurological symptoms and signs, such as hemiparesis, sensory impairment, and a low-density area in the CT image.

3. Blood Sample Collection

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 the medical research institute, Jehan medical center. Informed consent was obtained from all subjects. If patients were incommunicative, it was obtained from close relatives.

4. DNA Preparation and Genotyping

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 (Boehringer Mannheim, IN, USA). The extracted DNA was amplified by polymerase chain reaction (PCR), according to the method of Lesch et al, with minor modifications[10]. The IL4R gene (110-bp) was amplified using 25 ng of DNA, 5 pmol of each primer. IL4R forward was 5'-GAAACCTGGGAGCAGATCCT-3' and IL4R reverse was 5'-TCCACCGCATGTACAAACTC-3'. The polymerase chain reaction (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 minute at 95℃, 30 cycles of 30 seconds at 95℃, 30 seconds at 60℃, and 30 seconds at 72℃ with a Gene-Amp PCR System 9600 (Perkin-Elmer, Foster City, CA, USA). 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.

DNA Preparation for pyrosequencing was performed according to manufacturer's standard protocol (Pyrosequencing AB, Uppsala, Sweden)[11]. The streptavidin sepharose beads (Streptavidin Sepharose HP, Amersham Pharmacia Biotech, Uppsala, Sweden) were immobilized to PCR products. The sequencing primer of IL4R was 5'-CCCACCAGTGGCTAT-3' and it was designed so that the terminal residue hybridized to the base immediately adjacent to the A/G mutation from Pyrosequencing AB(http://www.pyrosequencing.com)[11]. 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[12]. 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)[13]. By using PSQ 96 Sample Prep Thermoplate (Pyrosequencing AB, Uppsala, Sweden) the PSQ 96 plate containing the samples was heated at 90℃ 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)[14]. 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 that was generated when a nucleotide is incorporated into a growing DNA strand[15]. From this process the polymorphism of the IL4R was genotyped automatically.

5. Statistical Analysis

To compare age of stroke patients and controls Student's t-test was used. To compare sex, the distribution of the genotypes and the frequency of alleles between Korean stroke patients and controls x^2 tests was used. The odds ratios (OR) and 95% confidence intervals (CI) were used to quantify the association with stroke. AS statistical package SAS program (release 8.2) was used.

Ⅲ. Results

1. Characteristics of the subjects

The characteristics of the patients and controls are shown in Table 1. There was significant difference between the patients and controls as for age ($p<0.001$) and sex ($p<0.001$). Mean age of the controls and patients was 43.3 ± 13.2 and 63.2 ± 11.8 years. The number of male / female of the controls and patients was 15 / 32 and 68 / 23.

2. IL4R genotype Distribution

There was statistically significant genotypic distribution difference between control and stroke group ($p=0.005$). The frequencies of A/A homozygotes, A/G heterozygotes, and G/G homozygotes among control subjects were 46 (55.4%), 37 (44.6%) and 0 (0.0%). The frequencies of A/A, A/G and G/G among the stroke patients were 34 (60.7%), 19 (34.0%) and 3 (5.3%). These results are shown in Table 2.

Table 1. Clinical Characteristics of Stroke Patients and Controls

	Controls	Patients	P value
Age	43.3 ± 13.2	63.2 ± 11.8	$p<0.001$
No. of Male	15	32	
No. of Female	68	24	$p<0.001$

Student's t-test was used to compare age of stroke patients and controls.
x^2 test was used to compare the sex of stroke patients and controls.

Table 2. Comparison of IL4R Genotype Distribution between Stroke and Control Participants

Genotype	No. of Controls	No. of patients	P value
A/A	46 (55.4)	34 (60.7)	
A/G	37 (44.6)	19 (34.0)	0.005
G/G	0 (0.0)	3 (5.3)	

x^2 test was used to compare values of stroke patients and controls.

3. Allele Frequencies Distribution

There was not statistically significant allelic frequency difference between control and stroke group (p=0.995). The allelic frequency of A and G was 129 (77.7%) and 37 (22.3%) among the control subjects and 87 (77.6%) and 25 (22.4%) in stroke patients, respectively (Table 3, OR (95% CI) ; 1.00 (0.56-1.78)).

Table 3. Comparison of Allele Frequencies of IL4R between Stroke and Control Participants

Allele	No. of Controls	No. of patients	OR (95% CI)	P value
A	129 (77.7)	87 (77.6)	1.00 (0.56-1.78)	0.995
G	37 (22.3)	25 (22.4)		

x^2 test was used to compare values of stroke patients and controls.

IV. Discussion

Stroke is the second most fatal disease following cancer in Korea. Stroke is a clinical concept of neurological disorder characterized by an acute faint, unconsciousness, excessive phlegm, hemiparalysis, dysphasia, facial palsy and motor disorder, etc. Stroke develops several complications, among which sequela of stroke like motor disorder affects the family as well as the patient with great psychological and financial stress.

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 influences. In 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 stroke many polymorphism were investigated and some polymorphism such as α1-antichymotrypsin gene was associated[16] but some polymorphism such as promoter of lipopolysaccharide receptor CD14 was not related[17]. This is the report to have shown the association of IL4R gene polymorphisms with stroke, especially cerebral infarction, by use of CT or MRI findings.

The IL-4R is composed of multiple chains, including a specific chain and a γc chain, In the IL-4R α-chain gene, an A→G transition at nucleotide 1902, causing a change from glutamine to arginine at codon

576, has been described and the presence of this rare allele has been associated with familial hyper-IgE syndrome and atopy[9].

In this study, we investigated polymorphism in the IL4R gene region in Korean stroke patients. Our data provide evidence for in vivo functional regulation of IL4 availability by IL4R in between stroke subjects and control participants. The overall analysis revealed significant interactions between genotype (p=0.005). The frequencies of A/A, A/G and G/G among control subjects were 46 (55.4%), 37 (44.6%) and 0 (0.0%). The frequencies of A/A, A/G and G/G among the stroke patients were 34 (60.7%), 19 (34.0%) and 3 (5.3%).

But our data failed to show any allelic frequency difference between stroke and control Korean (p=0.995). The allelic frequency of A and G was 129 (77.7%) and 37 (22.3%) among the control subjects and 87 (77.6%) and 25 (22.4%) in stroke patients, respectively.

The present results indicate the possible contribution of IL-4 gene polymorphism to stroke. But significant difference between genotypes maybe result from the different age and sex between stroke patients and controls.

Genetic factors and environmental factors are both critical in the development of stroke. So far it is very difficult to apply the results from genetic studies to clinic patients. Our results suggest that the investigated IL4R polymorphisms are somewhat susceptibility factors in the etiology of ischemia. The findings of this study need to be confirmed in larger patients samples and further studies. Additional epidemiologically based studies of the effects and relationship between IL4R or other genes and lifestyles with regard to stroke risk is required.

V. Conclusion

From above study the results can be summarized as followings.

1. In IL4R genotypes, there was significant difference between stroke patients and controls.
2. In the frequency of IL4R alleles there was no significant difference between stroke patients and controls.

Further studies including different cytokine gene can be a useful for predicting stroke. Establishment of more systemic approach and high quality of prospective cohorts will be necessary for the good prediction of genetic markers.

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