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원 저

A Study on Single Nucleotide Polymorphisms of Interleukin 10 in Bell's Palsy Patients by Pyrosequencing

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국문초록

구안와사 환자에서의 Interleukin 10 단일염기다형성 연구-Pyrosequencing

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목적 : 본 연구는 Interleukin 10 (IL10) 유전자 다형성이 구안와사의 발병과 관련이 있는지 알아보기 위해 수행되었다.

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대상 : 대구한의대학교부속 한방병원에 내원한 구안와사 환자 62 명과 종합건진센터에 내원한 구안와사 기왕력이 없는 건강인 104 명을 대상으로 하였다.

방법 : 각 그룹에서 개개인마다 DNA를 분리 정제한 후 Taq polymerase로 증폭하여 한천 겔에서 전기영동을 하여 PCR 산물을 확인하였다. PCR 산물은 Pyrosequencing 과정을 통하여 IL10의 유전형이 자동으로 판정되었다.

결과 : A/A, A/C의 두가지 유전자형이 검출되었으며 구안와사군과 대조군 사이에 유의성 있는 차이가 발견되지는 않았다(p=0.052). 또한 개별 allele 빈도에 있어서도 구안와사군과 건강인 사이에 통계적인 유의성이 나타나지 않았다(p=0.064).

결론 : 이상의 결과를 통하여 IL10 유전자 다형성은 구안와사의 발병과는 관련성이 없는 것으로 사려된다. 그러나 더 많은 구안와사 환자를 대상으로 IL10 유전자와의 연관성에 대한 후속 연구가 필요하다고 하겠다.

Key words : Bell's Palsy, Interleukin 10, Single Nucleotide Polymorphisms (SNP)

I. Introduction

Most of peripheral 7th cranial nerve palsies remain without an identified aetiology, and will be diagnosed as idiopathic or Bell's palsy. Some characteristics of this condition may be feature of a viral infection. Recently an increased interest and focus on the possible herpes simplex virus (HSV) aetiology in idiopathic facial paralysis has been seen. Infection of the facial nerve with HSV induces edema and paralysis of the nerve[1-2].

In recent some study the interleukin 6 (IL6), interleukin 8 (IL8) and Tumor necrosis factor-alpha (TNF-α) levels were significantly higher in the Bell's palsy than in the control. As in every infectious and immune reactions, cytokines should also be involved in Bell's palsy[2].

For many cytokines and their receptors, genetic variants have been described[3-7]. 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.

IL10 is one of the immunomodulatory cytokines and it has anti-inflammatory capabilities[8]. To date, genetic polymorphism in the 5'-flanking region of the IL10 gene

has not been described in Bell's palsy. We hypothesized that the IL10 gene is important candidate in the development of Bell's palsy and specific genotypic and allelic variations should be associated with Bell's palsy in the Korean population. In this study, we assessed the SNP (single-nucleotide polymorphism) of IL10 in patients with Bell's palsy.

II. Subjects and Methods

1. Study Population

62 patients with Bell's palsy were selected from the subjects who visited for the Bell's palsy service of the department of acupuncture & moxibustion, college of Oriental Medicine, Daegu Haany University from May 2002 to May 2003. Diagnosis of Bell's palsy was made by neurologic examination, Additional otolaryngologic and radiological examinations such as cranial or temporal magnetic resonance imaging or temporal bone computed tomography were performed in case of requirement.

The control group consisted of 104 healthy volunteers who visited for the health examinations at Jehan medical center in Daegu from May 2002 to May 2003. They had no history of chronic systemic disease, drug use, or facial paralysis. The physical examination was normal in the controls. They did not have an acute infection in the past 1 month. Ultimately, 168 Koreans were enrolled in the current analysis.

2. 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.

3. 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). The IL10 gene (113-bp) was amplified using 25 ng of DNA, 5 pmol of each primer. IL10 forward was 5′-GGGTAAAGGAGCCTGGAACAC-3′ and IL10 reverse was 5′-GGGTGGGCTAAATATCCTCAAAGT-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)[9]. The streptavidin sepharose beads (Streptavidin Sepharose HP, Amersham Pharmacia Biotech, Uppsala, Sweden) were immobilized to PCR products. The sequencing primer of IL10 was 5′-CTGGCTTCCTACAG-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)[9]. 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[10]. In 55 ul of 4 M acetic acid containing 0.35 uM of IL10 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)[11]. 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)[12]. 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 stran[13]. From this process the polymorphism of the IL10 was genotyped automatically.

4. Statistical Analysis

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

Ⅲ. Results

1. Characteristics of the subjects

The characteristics of the Bell's palsy patients and controls are shown in Table 1. There was no significant difference between

Table 1. Clinical Characteristics of Bell's Palsy Patients and Controls

	Controls	Patients	P value
Age	44.5 ± 12.8	46.1 ± 13.9	0.603
No. of Male	23	33	
No. of Female	81	29	p<0.001

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

the patients and controls as for age (p=0.603) but there was significant difference as for sex (p<0.001). Mean age of the controls and patients was 44.5 ± 12.8 and 46.1 ± 13.9 years. The number of male / female of the controls and patients was 23 / 81 and 33 / 29.

2. IL10 genotype Distribution

There was no statistically significant genotypic distribution difference between control and Bell's palsy group (p=0.052, OR (95% CI) ; 2.23 (0.98-5.98)). The frequencies of A/A homozygotes and A/C heterozygotes among control subjects were 91 (87.5%) and 13 (12.5%). The frequencies of A/A and A/C among the Bell's palsy patients were 47 (75.8%) and 15 (24.2%). These results are shown in Table 2.

Table 2. Comparison of IL10 Genotype Distribution between Bell's Palsy and Control Participants

Genotype	No. of Controls	No. of patients	OR (95% CI)	P value
A/A	91 (87.5)	47 (75.8)	2.23	0.052
A/C	13 (12.5)	15 (24.2)	(0.98-5.98)	

x^2 test was used to compare values of Bell's palsy patients and controls

3. Allele Frequencies Distribution

There was not statistically significant allelic frequency difference between control and Bell's palsy group (p=0.064, OR (95% CI) ; 2.06 (0.94-4.49)). The allelic frequency of A and C was 195 (93.8%) and 13 (6.2%) among the control subjects and 109 (87.9%) and 15 (12.1%) in Bell's palsy patients, respectively (Table 3).

Table 3. Comparison of Allele Frequencies of IL10 between Bell's Palsy and Control Participants

Allele	No. of Controls	No. of patients	OR (95% CI)	P value
A	195 (93.8)	109 (87.9)	2.06 (0.94-4.49)	0.064
C	13 (6.2)	15 (12.1)		

x^2 test was used to compare values of Bell's palsy patients and controls

IV. Discussion

Normal facial movement is required for speaking, chewing, swallowing and protecting the eye. Bell's palsy is an acute peripheral monosymptomatic facial palsy with undetectable causes. The aetiology of Bell's palsy remains unclear although genetic, vascular, infective and immunological causes have all been provided[14]. Recently, it has been suggested that Bell's palsy is caused by reactivation of HSV in the geniculate ganglia[15-16]. Bell's palsy causes most cases of unilateral and acute facial palsy. Symptoms improve in nearly all patients with Bell's palsy, but some patients are left with cosmetic deficits or malfunction[17].

In Bell's palsy, there is an inflammatory reaction which is compressing the facial nerve in the fallopian canal, particularly in the labyrinthine segment. In decompression surgeries, it is common to encounter an inflammatory reaction in this labyrinthine segment. It is likely that there is demyelination in Bell's palsy. This inflammation or demyelination is possibly caused by HSV infection[2-18].

Cytokines take important part in the infectious and inflammatory processes. They serve to transduce antigen specific signals, and focus inflammatory response where it is needed. That is, as in other disease states, some of the cytokines play a role in Bell's palsy, but importance of this role has not been established delicately to date. Interleukines and TNF-a are the cytokines which are released by certain cells (mononuclear phagocytes, endothelial cells, epithelial cells, T cells, natural killer cells, etc.), and act in the generation of immune response, inflammation, acute phase reaction, fever, etc[18].

In one study, The IL-6, IL-8 and TNF-α levels were significantly higher in the Bell's palsy than in the control ($p<0.01$ and $p=0.017$, respectively) while the IL-1b and IL-2r levels were similar in both groups. The mean IL-6 levels were 8.6±3.1 and 5.3±0.5 pg/ml in the patients and controls,

respectively. The mean IL-8 levels were 9.3±6.3 and 6±0.8 pg/ml in the patients and controls, respectively. The TNF-α levels were 10.1±6.2 and 4.3±0.8 U/ml in the patients and controls, respectively, which were statistically significant ($p<0.01$)[18].

IL10 is one of the immunomodulatory cytokines. IL10 is produced by a variety of cell types, including monocytes and B cells[19]. It is an up-regulator of B lymphocyte production and differentiation[20], but has anti-inflammatory abilities that can directly down-regulate TNF α, IL-1, IL-8 and interferon-γ production[21]. Three of IL10 SNPs have been studied in some detail: -1082(G to A), -819(C to T) and -592(C to A)[22-24].

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.

In this study, we investigated polymorphism in the IL10 gene region in Korean Bell's palsy patients. Our data did not provide any evidence for in vivo functional regulation of IL10 in between Bell's palsy subjects and control participants.

The overall analysis revealed no significant interactions between genotype ($p=0.052$). The frequencies of A/A and A/C among control subjects were 91 (87.5%) and 13 (12.5%). The frequencies of A/A and A/C among the Bell's palsy patients were 47 (75.8%) and 15 (24.2%).

And our data failed to show any allelic frequency difference between Bell's palsy and control Korean ($p=0.064$). The allelic frequency of A and C was 195 (93.8%) and 13 (6.2%) among the control subjects and 109 (87.9%) and 15 (12.1%) in Bell's palsy patients, respectively.

Genetic factors and environmental factors are both critical in the development of Bell's palsy. So far it is very difficult to apply the results from genetic studies to clinic patients.

There are some limitations of this study. Firstly, the IL10 serum of the patients was not taken, which makes the information somewhat heterogenous. And the sample size was too small. In the further studies, these limitations should be improved.

The cytokine IL10 may not be pathogenetic factors in Bell's palsy. But further studies including different cytokine gene can be a useful for predicting Bell's palsy. Establishment of more systemic approach and high quality of prospective cohorts will be necessary for the good prediction of genetic markers.

V. Conclusion

The IL-10 gene polymorphism was not

associated in Korean Bell's palsy patients. The present results indicate that IL-10 gene polymorphism would not be the possible contribution to Bell's palsy.

1. In IL10 genotypes, there was no significant difference between Bell's palsy patients and controls.
2. In the frequency of IL10 alleles there was no significant difference between Bell's palsy patients and controls.

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 IL10 or other genes and lifestyles with regard to Bell's palsy risk is required.

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Ⅵ. References

1. Steiner I,. Mattan Y, Bell's palsy and herpes viruses: to acyclovir or not to acyclovir?. J. Neurol. Sci. 1999 ; 170(1) ; 19-23.
2. Yilmaz M, Tarakcioglu M, Bayazit N, Bayazit YA, Namiduru M, Kanlikama M. Serum cytokine levels in Bell's palsy. J Neurol Sci. 2002 ; 197(1-2) : 69-72.
3. Bugawan TL, Mirel DB, Valdes AM, Panelo A, Pozzilli P, Erlich HA. Association and interaction of the IL4R, IL4, and IL13 loci with type 1 diabetes among Filipinos. Am J Hum Genet. 2003 ; 72(6) : 1505-1514.
4. Mirel DB, Valdes AM, Lazzeroni LC, Reynolds RL, Erlich HA, Noble JA. Association of IL4R haplotypes with type 1 diabetes. Diabetes. 2002 ; 51(11) : 3336-3341.
5. Bidwell J, Keen L, Gallagher G, Kimberly R, Huizinga T, McDermott M et al. Cytokine gene polymorphism in human disease: on-line databases. Genes Immun, 1999 ; 1 : 3-19.
6. Olavesen MG, Hampe J, Mirza MM, Saiz R, Lewis CM, Bridger S, Teare D, Easton DF, Herrmann T, Scott G, Hirst J, Sanderson J, Hodgson SV, Lee J, MacPherson A, Analysis of single-nucleotide polymorphisms in the interleukin-4 receptor gene for association with inflammatory bowel disease. Immunogenetics. 2000 ; 51(1) : 1-7.
7. Buchs N, Silvestri T, di Giovine FS, Chabaud M, Vannier E, Duff GW, Miossec P. IL-4 VNTR gene polymorphism in chronic polyarthritis. The rare allele is associated with protection against destruction. Rheumatology (Oxford). 2000 ; 39(10) : 1126-1131.
8. de Waal Malefyt R, Abrams J, Bennett

B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med, 1991 ; 174 : 1209-1220.
9. http://www.pyrosequencing.com
10. Wieser F, Fabjani G, Tempfer C, Schneeberger C, Sator M, Huber J, Wenzl R. Analysis of an interleukin-6 gene promoter polymorphism in women with endometriosis by pyrosequencing. J Soc Gynecol Investig. 2003 ; 10(1) : 32-36.
11. Gruber JD, Colligan PB, Wolford JK. Estimation of single nucleotide polymorphism allele frequency in DNA pools by using Pyrosequencing. Hum Genet. 2002 ; 110(5) : 395-401.
12. Pettersson M, Bylund M, Alderborn A. Molecular haplotype determination using allele-specific PCR and pyrosequencing technology. Genomics. 2003 ; 82(3) : 390-396.
13. Pacey-Miller T, Henry R. Single-nucleotide polymorphism detection in plants using a single-stranded pyrosequencing protocol with a universal biotinylated primer. Anal Biochem. 2003 ; 317(2) : 166-170.
14. Rowlands S, Hooper R, Hughes R, Burney P. The epidemiology and treatment of Bell's palsy in the UK. Eur J Neurol. 2002 ; 9(1) : 63-7.
15. Murakami S, Mizobuchi M, Nakashiro Y, Doi T, Hato N, Yanagihara N. Bell palsy and herpes simplex virus: identification of viral DNA in endoneurial fluid and muscle. Ann Intern Med. 1996 ; 124(1Pt1) : 27-30.
16. Ohtani F, Furuta Y, Horal P, Bergstrom T. Rapid strip assay for detection of anti-herpes simplex virus antibodies: application to prediction of varicella- zoster virus reactivation in patients with acute peripheral facial palsy. J Med Virol. 2000 ; 62(1) : 37-41.
17. Morrow MJ. Bell's Palsy and Herpes Zoster Oticus. Curr Treat Options Neurol. 2000 ; 2(5) : 407-416.
18. Edstrom S, Hanner P, Andersen O, Rosenhall U, Vahlne A, Karlsson B. Elevated levels of myelin basic protein in CSF in relation to auditory brainstem responses in Bell's palsy. Acta Otolaryngol. 1987 ; 103(3-4) : 198-203.
19. Burdin N, Peronne C, Banchereau J, Rousset F. Epstein?Barr virus transformation induces B lymphocytes to produce human interleukin 10. J Exp Med, 1993 ; 177 : 295-304.
20. Rousset F, Garcia E, Defrance T et al. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc Natl Acad Sci, 1992 ; 89 : 1890-1893.
21. Katsikis PD, Chu CQ, Brennan FM, Maini RN, Feldmann M. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J Exp Med 1994 ; 179 : 1517-1527.
22. Crawley E, Kay R, Sillibourne J, Patel P, Hutchinson I, Woo P. Polymorphic haplotypes of the interleukin-10 5′ flanking region determine variable interleukin-10 transcription and are associated with particular

phenotypes of juvenile rheumatoid arthritis. Arthritis Rheum, 1999 ; 42 : 1101-1108.

23. Eskdale J, Keijsers V, Huizinga T, Gallagher G. Microsatellite alleles and single nucleotide polymorphisms (SNP) combine to form four major haplotype families at the human interleukin-10 (IL-10) locus. Genes Immun, 1999 ; 1 : 151-155.

24. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet 1997 ; 24 : 1-8.