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Relationship between genetic mutations and diabetes in non-insulin dependent diabetic mellitus (NIDDM)

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【Abstract】 A simple and rapid FoLT(formamide low temperature)-PCR, whereby human genomic DNA from blood can be amplified without DNA preparative stps, is described using human insulin genes. By applicatin of FoLT-PCR in human insulin genes, intragenic polymorphism in non-coding regions of the human insulin gene was shown after amplification and analysis by restriction enzyme digestion. All nucleotide sequences were the same as the reported, and four necleotides, at 4 different positions were polymorphic, and polymorphic alleles α 4, α5, α6, and β2 were identified. The new alleles were originated from homologous recombination between the α1 and β1 alleles, and the alleles were founded in heterozygotes only. Althouugh allele α1 was dominant, the new alleles and β1 were recessive. From this results, it was suggested that the new method of FoLT-PCR was highly applicable in genetic variation analysis.

key Words : Genetic divergence; Insulin gene; Homologous recombination; Non-insulin dependent diabetic mellitus

I. INTRODUCTION

Polyemrase chain reaction (PCR) is an in vitro method for gene amplification. In general, the DNA or target purification step(s) is necessary before the PCR can be applied. FoLT (formamide low temperature) -PCR is a protocol for amplifying DNA directly withou any preparative steps. It involves the use of formamide as well as reduced incubation temperature. We have expanded the human insulin gene analysis using the FoLT-PCR to elucidate the relationships between the genetic variations and diabetic disorders.

An intragenic recombination event was first

demonstrated in rosy cistron 1 of *Drosophila melanogaster*.[1] This recombination event has been extensively studied in an ettempt to explain allelic divergence of genes in mice and humans.[2,3,4,5,6] Intragenic polymorphism has the advantage of offering an assemblage of a large number of genetic markers in humans. These markers can be used to trace human gene divergence and they also allow analysis of the segregation of some disease genes with polymorphic markers near, or within, potentially defective genes.

As a model of intragenic recombination, the human insulin gene has long been studied to elucidate the etiology of diabetes.[7,8,9] The gene, which is located on chromosome 11p, consists of three exons and two introns, and its primary structure and regulation have been studied.[10,11,12]. Specific nucleotide substitutions in the coding region of human insulin gene were found,[13,14,15] and nucleotide variations were identified at two positions in intervening sequences and at two positions in the 3' untranslated region of human insulin gene. These variations revealed two nucleotide arrangements which were designated as alleles α1 (A_{-216}-C_{-1045}-C_{-1357}-C_{-1380}; A-C-C-C) and β1 (T_{-216}-G_{-1045}-T_{-1367}-A_{-1380}; T-G-T-A). However, there is no an adequate understanding on the polymorphic alleles of human insulin gene.

In this study, human insulin gens were analysed for genetic variation using the FoLT-PCR. New divergence of alleles, which provides evidence for an intragenic recombination events between alleles α1 and β1 of human insulin gene, was identified. This result is evidence for allelic divergence in human insulin gene, which may be the result of intragenic recombination.

Ⅱ. MATERIALS AND METHODS

1. DNA sources.

1) Blood

Two hundred μl whole blood was washed with 1 ml of 1% Triton X-100 solution by centrifugation and then pellet was resuspended in 200 μl of the same solution. Genomic DNA from unrelated Korean subjects, including IDDM and NIDDM patients, was purified from peripheral white blood cells. Genomic DNA from NIDDM-affected family was purified as described.[16]

2) Chemicals and solutions

Triton X-100 (Bio-Rad, USA), formamide (Sigma, USA), strile water, Taq DNA polymerase (Promega, USA; Pharmacia Inc., USA; Takara, Japan), allele ladder solution (Promega), mineral oil were supplied by manufacturers.

2. Standard PCR amplification and DNA sequencing.

Three different sets of forward and reverse primers were used to give three amplified DNA fragments, as follows:

P1: sense
:5'-agcgcaaagagccccgcctgcagcctcca-3';
antisense
:5'-aggtagagagcttccaccaggtgtgagcca-3'

P2: sense
:5'-cgtgaagcttgtgggggtgagcccaggggccccaagg-3'
antisense :
5'-gaggctggcgacaggggtctggcccactca3'

P3: sense
:5'-gttttgttaagtaaagtcctgggtgacctgggg3'
antisense
:5'-ccagagagcgtggagagctgggaggggc-3'

The primers were synthesized using an Applied Biosystems DNA Synthesizer, model 381A (Takara, CO., LTD, Tokyo, Japan). PCR was performed on a Perkin Elmer Cetus instrument by standard conditions, which consisted of 30 cycles of denaturation at 94℃ for 1 minute, annealing at 55℃ for 1 minute, and extension at 72℃ for 1.5 minutes. Initial denaturation before PCR cycles proceded at 94℃ for 5 minutes, then *Taq* DNA polymerase (Pharmacia Inc.) was added and the products were analyzed by agarose gel electrophoresis.

All amplified DNA fragments were recovered using 1 % SeaPlaque agarose gel (FMC BioProducts) according to the general procedures.[17] Recovered DNA fragments were used directly as sequencing templates using Promega's fmol DNA Sequencing System. Sequencing primers, as nested primers for each fragment, were end-labelled with [γ-^{32}P]dATP. The thermocycling conditions for the sequencing reaction were modified as follows: After an initial denaturation step at 94℃ for 3 minutes, 30 cycles of denaturation, annealing, and extension were performed at 94℃ for 30 seconds, 65℃ for 20 seconds, and 72℃ for 1 minute. The reaction solutions were heated at 90℃ for 3 minutes, then loaded on 6 % polyacrylamide sequencing gel. Once polymorphism was found from a fragment of a subject, the PCR fragment was sequentially cloned into the *Sma*I site of plasmid pUC19. Each recombinant plasmid DNA was sequenced using Pharmacia's T7 Sequencing Kit with [α-^{35}S]dATP.

3. Restriction fragment length polyporphism (RFLP) analysis.

All amplified DNAs of PCR 1 and 3 fragments were analyzed using appropriate restriction endonucleases in order to identify the presence of their sites in polymorphic positions.[8] Approximately 100 ng of each fragment was digested. *Hph*I was used for PCR 1 fragments, and *Dra*III or *Pst*I was used for PCR 3 fragments. Reaction mixtures were analysed on 2.5 % NuSieve agarose gel.[19-22]

Ⅲ. RESULTS AND DISCUSSION

It is presently unknown whether a specific insulin gene allele may be correlated with diabetic pathology. It has been postulated that the 3′ untranslated region may be important for mRNA stability.[18] Nucleotide sequences of the 1.9 kb DNA fragments, including the 5′ to 3′ flanking regions of insulin genes, were determined by direct sequencing of the amplified DNAs. A common sequence of insulin genes was determined, and all sequences of the coding regions agreed with published data.[11]. However, nucleotides at the non-coding positions -85, -883, +1445, +1455, and +1469 were, in part, different from reported insulin genes sequences.[11,23] This may reflect genetic characteristics between ethnic groups (Table 1).

Two nucleotides, at +216 and +1045 in the intervening sequence, and two nucleotides at +1367 and +1380 in the 3′ untranslated region, were polymorphic. The arrangement of the dominant nucleotide residue at these four positions was A-C-C-C (allele α1). The recessive nucleotide arrangement in polymorphs was T-G-T-A (allele β1). Different nucleotide frequencies at the four positions suggest the presence of allele types other than alleles α1 and β1. Sequencing analysis produced primary structural information and identified variant

Table 1. Comparision of non-coding nucleotide in human insulin genes.

Source	N**	Nucleotide position*				
		-85	+883	+1445	+1455	+1469
Present study	12	AGG	GG	CTG	T	C
Kim et al.	48	AGG	GG	CTG	T	C
Ullrich et al.	2	AGG	GG	CTG	Δ	nd
Bell et al.	2	G	G	TC	Δ	Δ
Raben et al.	10	nd	nd	CTG	T	C

* Number on nucleotide starts from the first nucleotide of transcript of INS.

** "N" indicates the number of haplotype examined.

nd, notdetermined; Δ, deleted as compared to the present result.

nucleotides in polymorphic positions as being either homozygous or heterozygous. The sequencing of the heterozygous ·sequence determined the single allelic sequence. New arrangements were identified as A-C-C-A, A-G-C-C, and A-C-T-C at the gametic level. In addition, four heterozygous subjects exhibited A/T and C/A at +216 and +1380, respectively, while the other two positions were C/C. This indicates that the 4 subjects were heterozygous for α1 and T-C-C-A, or for A-C-C-A and T-C-C-C.

To identify new alleles by a simple RFLP analysis, amplified DNA was used. PCR 1 fragments were digested with *Hph*I restriction endonuclease to determine the presence of the "T" nucleotide (+216) in the *Hph*I site. Likewise, either *Dra*III or *Pst*I restriction endonuclease was used to confirm the presence of nucleotides "G" (+1045) or T (+1367) in PCR 3 fragments (Fig. 1). RFLP analysis, showing the presence of the restriction sites at nucleotide positions +216, +1045, and +1367, identified the new alleles (Fig. 2).

An NIDDM affected family having parental genotypes α1/α1 and α1/β1 was also examined. No linkage was observed between NIDDM family members and an allele in the family. Three offspring were maternal type and one offspring was paternal type. However, one offspring was neither paternal nor maternal type. He was heterozygous possessing alleles A-C-C-C (α1) and T-C-C-C (Fig. 3). The occurence of allele T-C-C-C may originate from an intragenic recombination event between maternal alleles α1 and β1, and not from a substitution of nucleotide T for A in allele α1.

New variant alleles in insuline gene can be classified into two categories, based on the extent of allelic recombination. When intragenic recombination occurs between the X (+216 to +1045), Y (+1045 to +1367), and Z (+1367 to +1380) regions in alleles α1 and β1 (Fig. 4), it initially produces new types of allelic variants which are primary recombinants. Variant alleles at positions +1367 and/or +1380 can lead to less mRNA being translated and, hence, to hypoinsulinaemia causing NIDDM. With regard to the relationship between diabetes and allelic variation, the susceptibility of a person possessing a specific allele of insulin genes remains to be studied.

Molecular variation in non-coding regions

of eukaryotic genes has so far not been emphasized, especially for elucidation of the relationship between genetic disorders and mutations. Therefore, these results provide some insight into molecular variation causing genetic disorders.

REFERENCES

1. Chovnick, A., Schalet, A., Kernaghan, R. P. and Krauss, M. (1964) The rosy cistron in Drosophila melanogaster: genetic fine structure analysis. Genetics 50:1245-1259
2. Rose, S. M. and Cullen, S. E. (1981) A variant α-chain in an Ia molecule from the I-A subregion of the mouse major histocompatibility complex: a possible intragenic recombination. J. Immunol. 127:1472-1477
3. Higashi, Y., Tanae, A., Inoue, H. and Fujii-Kuriyama, Y. (1988) Evidence for frequent gene conversion in the steroid 21-hydroxylase P-450(C21) gene: implications for steroid 21-hydroxylase deficiency. Am. J. Hum. Genet. 42: 17-25
4. Oudet, C., Heilig, R., Hanauer, A. and Mandel, J. L. (1991) Nonradioactive assay for new microsatellite polymorphisms at the 5′ end of the dystrophin gene, and estimation of intragenic recombination. Am. J. Hum. Genet. 49:311-319
5. Siemens, I., Brenden, M., Mauff, G., Abbal, M., Toit, E. D., Bertrams, J. and Geserick, G. (1992) Apparently non- expressed alleles of factor B (BF) code for hypomorphic proteins. Immunogenetics 37:24-28
6. Tanzi, R. E., Vaula, G., Romano, D. M., Mortilla, M., Huang, T. L., Tupler, R. G. and Wasco, W. (1992) Assessment of amyloid β-protein precursor gene mutations in a large set of familial and sporadic Alzheimer disease cases. Am. J. Hum. Genet. 51:273-282
7. Awata, T., Iwamoto, Y., Matsuda, A. and Kuzuya, T. (1988) Identification of nucleotide substitution in gene encoding [LeuA3]insulin in third Japanese family. Diabetes 37:1068-1070
8. Hitman, G. A., Kambo, P. K., Viswanathan, M. and Mohan, V. (1991) An analysis of amplified insulin gene products in diabetics of Indian origin. J. Med. Genet. 28:97-100.
9. Hoban, P. R. and Kelsey, A. M. (1991a) A MaeIII polymorphism within intron A of the insulin (INS) gene detectable by PCR. Nucl. Acids. Res. 19:5091.
10. Bell, G. I., Pictet, R. L., Rutter, W. J., Cordell, B., Tischer, E. and Goodman, H. M. (1980) Sequence of the human insulin gene. Nature 284:26-32.
11. Ullrich, A., Dull, T. J., Gray, A., Brosius, J. and Sures, I. (1980) Genetic variation in the human insulin gene. Science 209:612-615.
12. Ohlsson, H. and Edlund, T. (1986) Sequence-specific interactions of nuclear factors with the insulin gene enhancer. Cell 45:35-44.
13. Kwok, S. C. M., Steiner, D. F., Rubenstein, A. H. and Tager, H. S. (1983) Identification of a point mutation in the human insulin gene giving rise to a structurally abnormal insulin (Insulin Chicago). Diabetes 32:872-875.
14. Haneda, M., Chan, S. J., Kwok, S. C. M., Rubenstein, A. H. and Steiner, D. F. (1983) Studies on mutant human insulin genes:identification and sequence analysis of a gene encoding [SerB24] insulin. Proc. Natl. Acad. Sci. USA 80: 6366-6370.
15. Chan, S. J., Seino, S., Gruppuso, P. A.,

Schwartz, R. and Steiner, D. F. (1987) A mutation in the B chain coding region is associated with impaired proinsulin conversion in a family with hyperproinsulinemia. Proc. Natl. Acad. Sci. USA 84:2194-2197.

16. Kunkel, L. M., Smith, K. D., Boyer, S. H., Borgaonkar, D. S., Wachtel, S. S., Miller, O. J. and Breg, W. R. (1977) Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. Proc. Natl. Acad. Sci. USA 74: 1245-1249.
17. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 6, 30-31.
18. Ko, J. H., Kwon, S. T. and Kim, C. H. (1994) Metal affinity engineering of proinsulin carrying genetically attached (His)10 tail. Biosci. Biotech. Biochem. 58: 1694-1699.
19. Kim, Y. S., Kim, J. K. and Kim, C. H. (1997) Genetic polymorphism in non-coding regions of the human insulin gene. Dongguk Journal: Natural Science. 16, 371-385.
20. Kim, Y. J., Tae-Wha Chung, In-Seong, Choe, Lee, Y. C. and Kim, C. H. (1996) Sequence analysis of the 5'-klanking region of the gene encoding human GNT. Gene 170, 281-283.
21. Lee, Y. C. and Kim, C. H.(1998) Cloning and expression of cDNA for human sia α-2, 3-Galβ1.4 GlcNAc. Arch, Biochem, Biophys, 360, 41-46
22. Raven, N., Barbetti, F., Cama, A., Lesniak, M. A., Lillioja, S., Zimmet, P. and Serjeantson, S. W. (1991) Normal coding sequence of insulin gene in Pima Indians and Nauruans, two groups with highest prevalence of type II diabetes. Diabetes 40:118-122.

Legends to Figures

Fig. 1. RFLP analysis of human insulin gene with PCR products by restriction endonucleases *Hph*I (+216), *Dra*III (+1045), and *Pst*I (+1367).

2.5 % NuSieve agarose gels (FMC BioProducts) were stained in ethidium bromide and were transilluminated with ultraviolet light. In photographs, lane M is 1 kb ladders (BRL Inc., USA). First Lanes in gel pictures are the amplified DNA fragments of PCR 1 (for +216) or PCR 3 (for +1045 and +1367). Each second lane is homozygous DNA fragments of allele α1, and each third lane is one of the heterozygous alleles α1/β1. Lane 4 is a control of amplified DNA fragments of INS allele β1 obtained from a recombinant plasmid. The bands corresponding to 51, 35, and 27 bp fragments appear faint in photographs.

Fig. 2. Schematic cleavage positions of restriction endonucleases *Hph*I (+216), *Dra*III (+1045), and *Pst*I (+1367).

Arrows indicate the presence of *Hph*I, *Dra*III, and *Pst*I restiction endonuclease sites.

Fig. 3. Pedigree of a NIDDM affected family.

Solid squares and circles designate subjects affected. Parental genotypes are α1/α1 and α1/β1. Among offspring examined, three offsprings are α1/β1 and one is α1/α1. Another offspring possesses INS alleles α1/β2 (indicated by arrow).

Fig. 4. Possible recombinant haplotypes.

Arrows indicate the alleles observed in the present analysis.

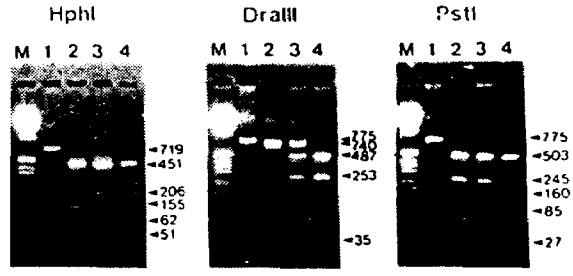


Fig. 1.

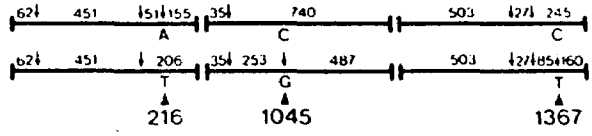


Fig. 2.

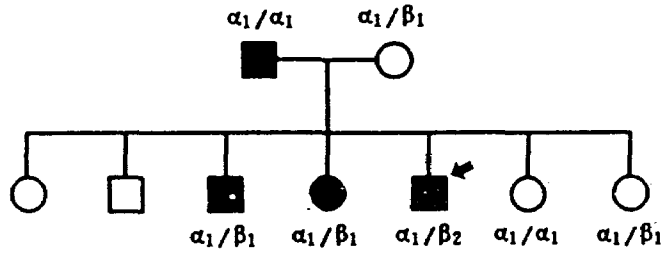
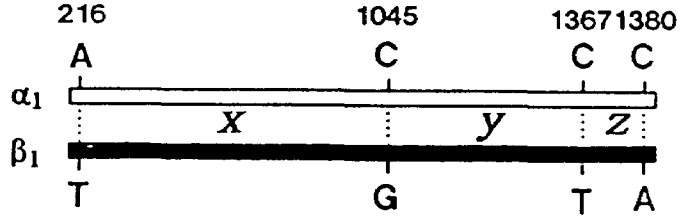


Fig. 3.



PRIMARY RECOMBINANT	A - G - T - A	T - C - C - C
	A - C - T - A	T - G - C - C
	A - C - C - A	T - G - T - C
SECONDARY RECOMBINANT	A - G - C - C	T - C - T - C
	A - C - T - C	T - G - C - A
	A - G - T - C	T - C - T - A
	A - G - C - A	T - C - C - A

Fig. 4.

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=초 록=

인슈린비의존성 당뇨병 (NIDDM)에서 유전적 변이와 체질의학적 관계

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FoLT-PCR 기술을 인체체질의학적 응용을 위하여 당뇨병연구에 사용하였다.
당뇨병은 제1형 및 제2형으로 나뉘는데 제1형은 인슈린비의존성(NIDDM)으로 당뇨병환자의 약60%이상을 차지하며, 제2형은 인슈린의존성(IDDM)으로 당뇨병환자의 30%미만을 차지한다. 이들은 대부분 후천적으로 환경중에서의 인슈린관련 유전자의 돌연변이에 의해 발병하는 것으로 알려져 있다. 이에, 본 연구에서는 당뇨병의 병인을 유전적변이와 체질의학적 관계에서 고찰하기 위하여 수행되었다. NIDDM환자와 IDDM환자를 대상으로 인슈린유전자를 증폭하여 제한효소절단 양상과 염기배열분석을 하였다. 비암호영역중 4개 위치 +216, +1045, +1367, 및 +1380에서 다형성을 보였으며 새로운 α4, α5, α6 및 β2이 α1과 β1가 이형(heterozygous)에서만 검출되며 α1은 우성이며 신규형들과 β1은 열성이었다. 이러한 당뇨병병인은 유전학적으로 체질의학과 깊은 관계를 가지는 것을 시사하였다.

중심낱말 : 유전적 다형성, 인슈린 유전자, 상동성 재조합, 인슈린비의론성 당뇨병

약 어 : IDDM, insulin dependent diabetic mellitus; NIDDM, non-insulin dependent diabetic mellitus; RFLP, restriction fragment length polyporphism; VNTR, variable number random repeat.