Variable Number of Tandem Repeat Polymorphism of the Endothelial Nitric Oxide Synthase Gene in Japanese Population

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ABSTRACT. We examined the variable number of tandem repeat (VNTR) polymorphism at intron 4 of the human endogenous nitric oxide synthase (eNOS) gene. The alleles were amplified from genomic DNA samples by the polymerase chain reaction (PCR), and analyzed by agarose gel electrophoresis. The PCR products showed one or three reproducible bands and two alleles and one extra band could be identified. The nucleotide sequence data revealed that the larger allele, allele 2, was in good agreement with that of the VNTR region, whereas the smaller one, allele 1, lacked one 27-bp core unit. The extra band, which showed the largest molecule, always appeared when heterozygote samples were used as templates, indicating a heteroduplex structure resulting from mismatch of the heterozygous alleles during annealing.

The allele frequencies were determined for 112 unrelated Japanese population. The estimated allele frequency was allele 2=0.87 and allele 1=0.13, and the heterozygosity was 0.226. The genotype frequencies were in good accordance with the Hardy-Weinberg equilibrium.

Key words: polymorphism — variable number of tandem repeat (VNTR) — nitric oxide synthase

Over the last decade, the polymorphism of nuclear DNA has been used as a new genetic marker in addition to conventional markers such as protein polymorphism and blood groups. The genomes of eukaryotes including humans are known to be interspersed with segments of directly repeated DNA and some of these loci show variable numbers of the tandem repeats which have been designated VNTR sequences.[1] These repeat sequences have been applied to the forensic sciences, including paternity testing and individual identification.[2-5] The VNTR loci can be successfully amplified by the polymerase chain reaction (PCR) using specific primers.

Nitric oxide has been recently implicated in a number of diverse physiological processes, including smooth muscle relaxation, inhibition of platelet aggregation, neurotransmission and immune regulation.[6-9] There appear to exist at least three distinct isoforms of nitric oxide synthase (NOS), and these can be classified into two categories, constitutive and

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inducible NOSs. The constitutive NOS enzymes are continually present in such cells as endothelial cells (eNOS) and neurons (nNOS). Recently, Miyahara *et al*[10] reported the complete nucleotide sequence of the human eNOS gene, consisting of 26 exons with a total size of 21 kb. The VNTR region that we have examined here is included in intron 4 of the gene and a 27-bp direct repeat is located five times in tandem. In the present paper, we report VNTR polymorphism at intron 4 of the human eNOS gene among 112 Japanese subjects.

MATERIALS AND METHODS

DNA samples

Peripheral blood samples were obtained from 112 Japanese individuals. DNA was prepared using a nucleic acid extraction kit (IsoQuick, MicroProbe, CA, USA) and estimated with a spectrophotometer (Beckman DU640).

Polymerase chain reaction (PCR)

Primers were synthesized on an Applied Biosystems Model 391 DNA synthesizer as follows: 5'-TTGGCTGGAGGAGGGGAAAGAA-3' (sense) and 5'-TTGGGGGAGAAGCAGCAGCCA-3' (antisense). PCR amplifications were performed in a total volume of 50 μl containing 100 ng of DNA template, 25 pmoles of each primer, 2.5 units of Taq DNA polymerase (Takara Shuzo, Otsu, Japan) and 10 nmoles each of the four deoxyribonucleotide 5'-triphosphates (dATP, dCTP, dGTP and dTTP) in PCR buffer that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$. PCR were carried out using an automated Thermal Cycler (Perkin-Elmer Cetus) for 35 cycles, with each cycle being performed for 1.5 min at 94℃ (denaturing), 20 sec at 67℃ (annealing) and 5 min at 72℃ (extension). After the last cycle, the samples were incubated for an additional 5 min at 72℃.

Agarose gel electrophoresis

The PCR products were electrophoresed on 4% agarose gels (Seakem/Nusieve=1/3) and stained with ethidium bromide for 20 min.

Nucleotide sequencing

DNA fragments of the PCR products were isolated using an extraction kit (Qiaex II, Qiagen) and ligated to the *Sma* I-digested pGEM7Z vector. Plasmid DNA was prepared and confirmed by electrophoresis after digestion with a restriction enzyme, *Pvu* II. For nucleotide sequences, we further purified the DNA using polyethylene glycol. Nucleotide sequences were determined by the dideoxynucleotide chain termination method using a dye primer cycle sequencing kit (Applied Biosystems) with a DNA sequencer (model 373A, Applied Biosystems).

Southern-blot analysis[11]

Based on the nucleotide sequences, the Band-2 DNA fragment (Fig 2-A) was selected as a specific probe. The probe was labelled with digoxigenin (DIG)-dUTP by the PCR method using the same primers described

above. The reaction mixture contained 25 pmoles of each primer, 2.5 units of Taq DNA polymerase, 4 nmoles each of three deoxyribonucleotide 5'-triphosphates (dATP, dCTP and dGTP) and 2.6 nmoles dTTP containing 1.4 nmoles DIG-dUTP in a total volume of 50 μl. The DNA samples examined were amplified by the PCR, separated by gel electrophoresis and transferred to nylon membranes (Hybond-N, Amersham). Hybridization was carried out using the DIG-labelled probe and a detection kit (Boehringer Mannheim).

RESULTS AND DISCUSSION

The 27-bp tandem repeat observed at intron 4 of the human eNOS gene is shown in Fig 1. The five repeat region can be divided into two groups from 5' to 3' side, three and two repeats each. The difference between the groups is substitution of a single base at position 19 in the 27-bp core, being A in the three groups, but G in the two groups (Fig 1). Under our PCR conditions, the amplified product was predicted to be 239 bp. However, as shown in Fig 2A, gel electrophoresis of the PCR products showed reproducible patterns of one or three bands, which corresponded to those detected by Southern hybridization analysis (Fig 2B). We designated these bands as Band-1, Band-2 and Band-3 from smaller to larger fragments (Fig 2A). As shown in Fig 3, the sequencing analysis revealed that the nucleotide sequence for Band-2 (m.w.=239) was consistent with that reported by Miyahara *et al.*[10] The allele size of Band-1 was 27-bp smaller than that of Band-2. Although we did not analyze the sequences of all Band-1 samples examined, the sequence analysis of Band-1 from 5 persons showed that the allele lacked one 27-bp core containing A at position 19, resulting in four tandem repeat (Fig 3). On the contrary, we could not obtain the clone showing the nucleotide sequence that was compatible with the repeat region for Band-3. It is well-known that there often exist extra bands in the typing of MCT118, one of the VNTRs of human genome, which has been used most frequently in the field of forensic medicine.[12–17] Watanabe *et al*[18] reported that the extra bands were DNA heteroduplex caused by mutual cross-annealing and that the extra bands could easily take the high-order structure due to a non-complimentary sequence of bases. We extracted each band allele from agarose gels and re-amplified these DNA fragments as

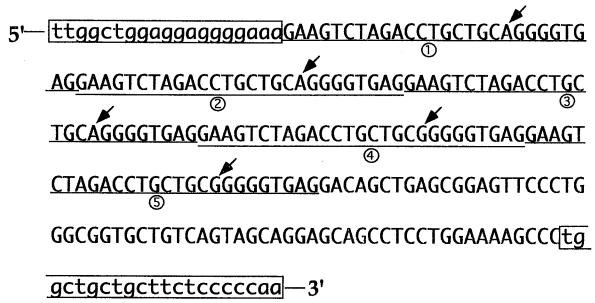

Fig 1. Nucleotide sequences of the VNTR region examined at intron 4 of the human eNOS gene by Miyahara *et al.*[10] The oligonucleotide primers used are boxed, and each 27-bp repeat core unit is underlined. Arrow shows the position 19 in each repeat core.

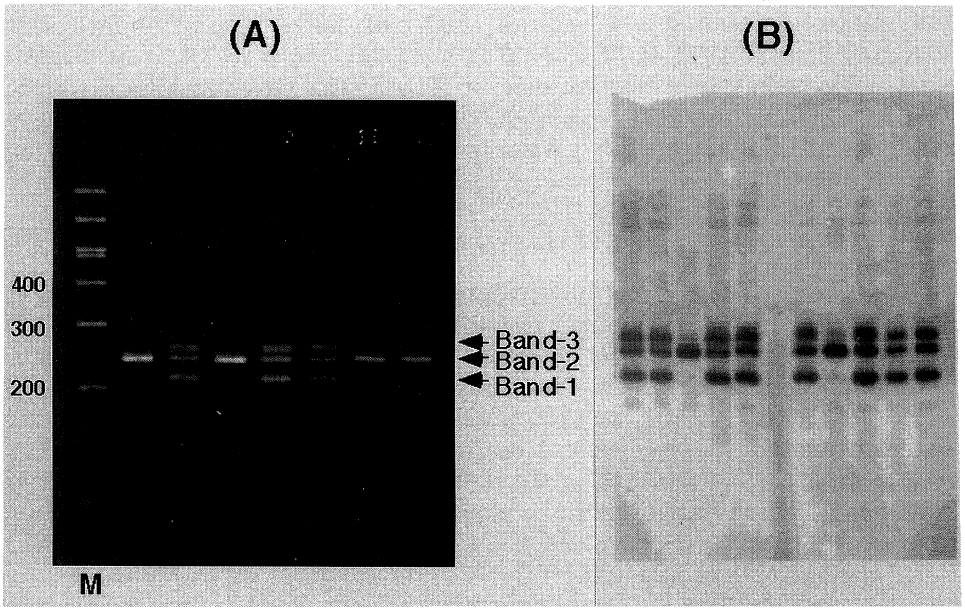

Fig. 2 (A) Agarose gel electrophoretic pattern of amplified products of genome DNA. M; size-marker (BioMarker™Low). (B) Southern hybridization results of amplified products of genome DNA. See text in detail.

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Band-1:  TTGGCTGGAGGAGGGGAAAGAAGTCTAGANCTGCTGCAGGGGTGAGGAAGTCTAGACCTGCTGCAGGGGTGAG//////////////////////////
         |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
eNOS:    TTGGCTGGAGGAGGGGAAAGAAGTCTAGACCTGCTGCAGGGGTGAGGAAGTCTAGACCTGCTGCAGGGGTGAGGAAGTCTAGACCTGCTGCAGGGGTGAG
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Band-2:  TTGGCTGGAGGAGGGGAAAGAAGTCTAGACCTGCTGCAGGGGTGAGGAAGTCTAGACCTGCTGCAGGGGTGAGGAAGTCTAGACCTGCTGCAGGGGTGAG

Band-1:  GAAGTCTAGACCTGCTGCGGGGGTGAGGAAGTCTAGACCTGCTGCGGGGGTGAGGACAGCTGAGCGGAGTTCCCTGGGCGGTGCTGTCAGTAGCAGGAGC
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eNOS:    GAAGTCTAGACCTGCTGCGGGGGTGAGGAAGTCTAGACCTGCTGCGGGGGTGAGGACAGCTGAGCGGAGTTCCCTGGGCGGTGCTGTCAGTAGCAGGAGC
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Band-2:  GAAGTCTAGACCTGCTGCGGGGGTGAGGAAGTCTAGACCTGCTGCGGGGGTGAGGACAGCTGAGCGGAGTTCCCTGGGCGGTGCTGTCAGTAGCAGGAGC

Band-1:  AGCCTCCTGGAAAAGCCCTGGCTGCTGCTTCTCCCCCAA
         |||||||||||||||||||||||||||||||||||||
eNOS:    AGCCTCCTGGAAAAGCCCTGGCTGCTGCTTCTCCCCCAA
         |||||||||||||||||||||||||||||||||||||
Band-2:  AGCCTCCTGGAAAAGCCCTGGCTGCTGCTTCTCCCCCAA
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Fig 3. Comparison of nucleotide sequences of Band-1, Band-2 and the VNTR region of the human eNOS gene. Each repeat core unit is underlined and the arrow shows the position 19 in each repeat core. The sequence of Band-2 was consistent with that of the VNTR region. Band-1 lacked one 27-bp core containing A at position 19, resulting in four tandem repeat.

templates under our PCR conditions (Fig 4). Agarose gel electrophoresis of the PCR products using Band-1 or Band-2 as a template showed only a single band equivalent to each band, respectively. On the contrary, when a mixture of Band-1 and Band-2 was used as a template for PCR, three bands, Bands-1, -2 and -3, were acquired. Similar results were also obtained when Band-3 was used as a template. In addition, incubation of Band-1 and Band-2 at 94℃ for 5 min and then at 67℃ for 2 min resulted in the appearance of Band-3 (data

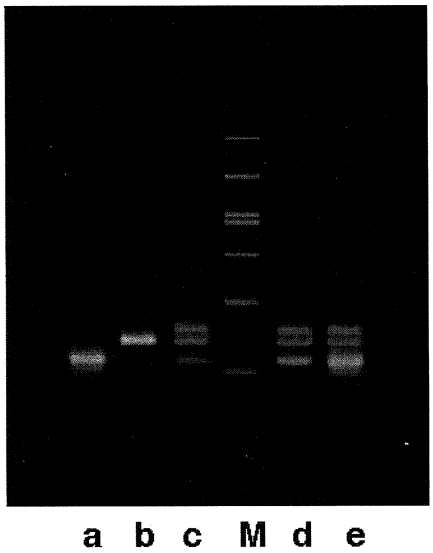

Fig 4. Agarose gel electrophoretic pattern of amplified products using (a) Band-1, (b) Band-2, (c) Band-3, (d) heterozygote DNA and (e) a mixture of Band-1 and Band-2 as a template for PCR. M; size-marker (BioMarker™Low)

not shown). These results suggested that the extra band, Band-3, would be formed by binding Band-1 with Band-2. The mismatch would occur during annealing when the two alleles having similar sequences co-existed, resulting in the production of the extra band with a double hetero structure, as reported elsewhere.[18)]

The genotype and allele frequencies observed at the VNTR are summarized in Table 1. Allele 1 is coincident with band-1 at gel electrophoresis and allele 2 is band-2. Therefore, there logically exist three genotypes, genotype 1-1, 2-1 and 2-2. In this population study, two different genotypes were observed, genotype 2-2 and 2-1. Genotype 2-2, being homozygote, was detected in 83 out of the 112 Japanese samples and showed a frequency of 74.1%. On the other hand, heterozygote, genotype 2-1, was detected in 25 samples (25.9%).

TABLE 1. Distribution of genotype and allele frequencies

Genotype	Number observed(%)	Number expected*(%)	Allele frequency**
2-2	83 (74.1)	85 (75.7)	2=0.87
2-1	29 (25.9)	25 (22.6)	1=0.13
1-1	0 (0.0)	2 (1.7)	$x^2=2.687$***
			0.10<P<0.12 (d.f.=1)
	Total 112 (Japanese)		

*The number expected of genotype frequencies was calculated from allele frequency data on the basis of Hardy-Weiberg equilibrium.

**Allelic designations are the same as those of bands obtained at agarose gel electrophoresis.

***Chi^2 test for Hardy-Weinberg equilibrium of the VNTR allele frequencies
d.f.=degree of freedom.

Genotype 1-1 was not detected in the present study. As shown in Table 1, the estimated frequency of allele 2 was 0.87, whereas that of allele 1 was 0.13. The genotype frequencies were in good accordance with the Hardy-Weinberg expectations[19] and the index of heterozygosity was estimated to be 0.226.

REFERENCES

1) Nakamura Y, Leppert M, O'Connel P, Wolff R, Holm T, Culver M, Martin C, Fujimoto E, Hoff M, Kumlin E, White R : Variable number of tandem repeat (VNTR) markers for human gene mapping. Science **235** : 1616-1622, 1987
2) Boerwinkle E, Xiong W, Fourest E, Chan L : Rapid typing of tandemly repeat hypervariable loci by the polymerase chain reaction : Application to the apolipoprotein B3' hypervariable region. Proc Natl Acad Sci USA **86** : 212-216, 1989
3) Wu S, Seino S, Bell GI : Human collagen type II, alpha 21, (COL2A1) gene : VNTR polymorphism detected by gene amplification. Nucleic Acids Res **18** : 3102, 1990
4) Deka R, DeCroo S, Yu LM, Ferrell RE : Variable number of tandem repeat (VNTR) polymorphism at locus D17S5 (YNZ) in four ethnically defined human populations. Hum Genet **90** : 86-90, 1992
5) Katsuyama Y, Harashima N, Tsuchikane A, Ota M, Liu C, Zenda H, Fukushima H : Allele distribution of a VNTR polymorphism at D4S43 locus in the Japanese population. Jpn J Legal Med **50** : 6-9, 1996
6) Garthwaite J, Charles SJ, Chess-Williams R : Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature **336** : 385-388, 1988
7) Schmidt HHH, Walter U : NO at work. Cell **78** : 919-925, 1994
8) Asano K, Chee CBE, Gaston B, Lilly CM, Gerard C, Drazen JM, Stamler JS : Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. Proc Natl Acad Sci USA **91** : 10089 - 10093, 1994
9) Guo FH, DeRaeve HR, Rice TW, Stuehr DJ, Thunnissen FBJM, Erzurum SC : Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium *in vivo*. Proc Natl Acad Sci USA **92** : 7809-7813, 1995
10) Miyahara K, Kawamoto T, Sase K, Yui Y, Toda K, Yang L-X, Hattori R, Aoyama T, Yamamoto Y, Doi Y, Ogoshi S, Hashimoto K, Kawai C, Sasayama S, Shizuta Y : Cloning and structural characterization of the human endotherial nitric-oxide synthase gene. Eur J Biochem **223** : 719-726, 1994
11) Sambrook J, Fritsch EF, Maniatis T : Molecular Cloning. 2nd ed, New York, Cold Spring Harbor Laboratory Press. 1989
12) Kasai K, Nakamura Y, White R : Amplification of a variable number of tandem repeats (VNTR) locus (pMCT118) by the polymerase chain reaction (PCR) and its application to forensic science. J Forensic Sci **35** : 1196-1200, 1990
13) Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC : Analysis of the VNTR locus D1S80 by the PCR followed by high-resolution PAGE. Am J Hum Genet **48** : 137-144, 1991
14) Skowasch K, Wiegand P, Brinkmann B : pMCT118 (D1S80) : a new allelic ladder and an improved electrophoretic separation lead to the demonstration of 28 alleles. Int J Leg Med **105** : 165-168, 1992
15) Kloosterman AD, Budowle B, Daselaar P : PCR-amplification and detection of the human D1S80 VNTR locus. Int J Leg Med **105** : 257-264, 1993
16) Sugiyama E, Honda K, Katsuyama Y, Uchiyama H, Tsuchikane A, Ota M, Fukushima H : Allele frequency distribution of the D1S80 (pMCT118) locus polymorphism in the Japanese population by the polymerase chain reaction. Int J Leg Med **106** : 111-114, 1993
17) Nagai A, Yamada S, Bunai Y, Ohya I : Analysis of the VNTR locus D1S80 in a Japanese population. Int J Leg Med **106** : 268-270, 1994
18) Watanabe G, Umetsu K, Suzuki T : On the origin of extra bands at MCT118 typing. Jpn J Legal Med **50** : 10-16, 1996
19) Tamarin RH : Principles of Genetics. 2nd ed, Boston, USA, Willard Grant Press/PWS Publishers. 1986