The molecular basis for genetic polymorphism of human deoxyribonuclease I: identification of the nucleotide substitution that generates the fourth allele

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Abstract In addition to the three alleles commonly responsible for the protein polymorphism of human deoxyribonuclease I, a mutation encoded by a fourth allele, *DNASE1*4*, was detected by isoelectric focusing. All 8 exons covering the entire open reading frame of the human DNase I gene were amplified by the polymerase chain reaction and subjected to direct sequencing. Only one nucleotide substitution, a C-to-G transition (CAG → GAG), in the codon for amino acid 9 of the mature enzyme was found. This substitution resulted in the replacement of Gln with Glu (Q9E).

Key words: Deoxyribonuclease I; Polymorphism; PCR; Genetic variation; Human

1. Introduction

Deoxyribonuclease I (DNase I; EC 3.1.21.1) has been postulated to be responsible for internucleosomal DNA degradation during apoptosis (programmed cell death) [1,2]. Genetic polymorphism of human DNase I has been demonstrated by isoenzyme pattern differences detectable by isoelectric focusing [3]. The discovery that DNase I activity is distributed in human tissues other than those of the digestive system [4,5] may indicate further in vivo function(s) of DNase I.

The structural gene encoding human DNase I has been sequenced and found to comprise 9 exons spanning approximately 3.2 kilobases [6]. Three common alleles, *DNASE1*1, *2* and **3*, the gene frequencies of which have been determined to be about 0.55, 0.44 and 0.01, respectively, occur in the Japanese population [7]. The protein polymorphism of DNase I has been found to be caused by an A-to-G transition (CAA → CGA) at nucleotide position 2317 in exon VIII, forming the second allele, and a C-to-G transversion (CCC → GCC) at position 1592 in exon VI forming the third allele. These changes are responsible for replacement of Gln by Arg at amino acid position 222 (Q222R) and of Pro by Ala at position 132 (P132A) of the mature enzyme, respectively, resulting in the different electrophoretic mobilities of each phenotype [6,8]. A rare fourth allele, *DNASE1*4*, has been observed at a mutational level (gene frequency of less than 0.01) [2,7]. However, the molecular basis of the fourth allele remains to be demonstrated.

In this study, we describe the molecular analysis of the fourth allele, using direct DNA sequencing of the specific products amplified by the polymerase chain reaction (PCR). We also present the mutation screening method which is based on mismatched PCR.

2. Materials and methods

2.1. Analysis of DNase I protein polymorphism

Urine samples, obtained from healthy Japanese individuals, were concentrated, dialyzed and finally freeze-dried, as described previously [9,10]. A 0.1% (w/v) solution of the freeze-dried material, corresponding to about 10-fold concentrated urine, was used for DNase I phenotyping. Each urine solution was treated with an equal volume of 5 U/ml *Clostridium perfringens* sialidase (Sigma, St. Louis, MO), then 5 μl digest was subjected to isoelectric focusing in a thin layer of polyacrylamide gel (IEF-PAGE) and the isoenzyme patterns of DNase I were detected by immunostaining with an anti-urinary DNase I antibody [9,11]. The enzymatic activity of serum DNase I was measured by the single radial enzyme diffusion method [3].

2.2. Primer construction

Primers for amplification and sequencing were constructed using the DNA sequence of the human DNase I gene as a base [6], as shown in Fig. 1. Each primer corresponded to one of the following regions of the DNase I genomic sequence, and the numbering system for the sequences was as described previously [6]: U1 (−355 to −336), D1 (185–204), U2 (408–427), D2 (861–880), U3 (1149–1168), D3 (1383–1402), U4 (1434–1463), D4 (1982–2001), U5 (2216–2235), D5 (2598–2617) and D6 (1586–1605). All primers were purchased from Genosys Biotechnologies (The Woodlands, TX).

2.3. PCR and subcloning of PCR products

Genomic DNA was prepared from heparin-anticoagulated venous blood samples using a QIAamp blood kit (Qiagen, Chatsworth, CA). Amplification of each fragment was performed using a MiniCycler (model PTC-150, MJ Research, Watertown, MA). Approximately 200 ng genomic DNA was added to the PCR mixture (50 μl), which comprised 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.5% (w/v) glycerol, 0.01 mM dithiothreitol, 0.2 mM each deoxyribonucleotide triphosphates (dATP, dGTP, dCTP, dTTP), 4 μM each primer and 2.5 U *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, MD). After denaturation at 94°C for 3 min, amplification was carried out for 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min, followed by a further 10-min extension at 72°C. Two partially overlapping fragments, PCR-5 and -3, in which entire exons II–V and VI–IX were included, were amplified using two pairs of primers, U1 and D6, and U4 and D5, respectively. The PCR-5 fragment was purified by a QIAquick PCR purification kit (Qiagen) and subcloned directly into a TA cloning pCR II vector (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Twelve independent subclones were then selected and isolated.

2.4. DNA sequencing analysis

The PCR-amplified fragments were separated by 1.5% (w/v) agarose gel-electrophoresis and extracted with a QIAEX gel-extraction kit

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(Qiagen). The resulting double-stranded fragments were subjected to direct DNA sequencing using the corresponding sequencing primers indicated in Fig. 1. Sequencing was carried out using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions, and the products were analyzed using a DNA sequencer (model 373A, Applied Biosystems). Direct DNA sequencing of each fragment was performed at least twice. The twelve independent PCR-5 subclones, which carried exon II with a sequence aberration, were separately sequenced using D1 or universal −21M13 forward primer as the sequencing primer, as described above.

2.5. Mutation analysis with a mismatched PCR primer

In order to confirm the mutation site, the mismatched PCR method [12] was used. The exon sequences including the mutation site were amplified with a pair of primers, the sequences of which were: 5'-CCTGAAGATCGCAGCCTTCAACCTC-3' (sense primer M1) and 5'-ACCAGCCCTAGACTCCAGAG-3' (antisense primer M2). The sense primer was designed with a deliberate mismatch. This meant that the PCR product amplified from the fourth allele *DNASEI*4* would have a newly created *Xho*I site (CTCGAG) at the mutation site, whereas that from the other alleles would not contain this site. Amplification was performed as described above, and then the 5 μl PCR product was digested with 40 U *Xho*I (New England Biolabs, MA) at 37°C for 2 h. The digested products were subjected to 2.5% (w/v) Metaphore agarose (FMC BioProducts, Rockland, ME) gel-electrophoresis and detected by ethidium bromide staining.

3. Results

A rare electrophoretic mutant (Oot-c (man)) of DNase I was detected in one of the urine samples collected from more than 700 healthy Japanese individuals. The mutation was confirmed to have been transmitted from the subject's mother (Oot-m). The DNase I phenotypes of Oot-c and Oot-m were identified as 2–4 and 1–4, respectively, using a previously described method [9]. The DNase I activities in these subjects' sera were determined to be 4.16 ± 0.78 and 3.84 ± 0.10 U/l, respectively, and their specific activities did not differ significantly from those of other phenotypes 1, 1–2 and 2 [3].

Fragments PCR-5 and -3 were amplified individually, as a previous observation indicated that direct PCR amplification of genomic DNA would cover the majority of exonic sequences of the DNase I gene [6]. Since exon I contains only the non-coding region of mRNA, exon I was omitted from the PCR-5 fragment. Specific amplification of each exon using an appro-
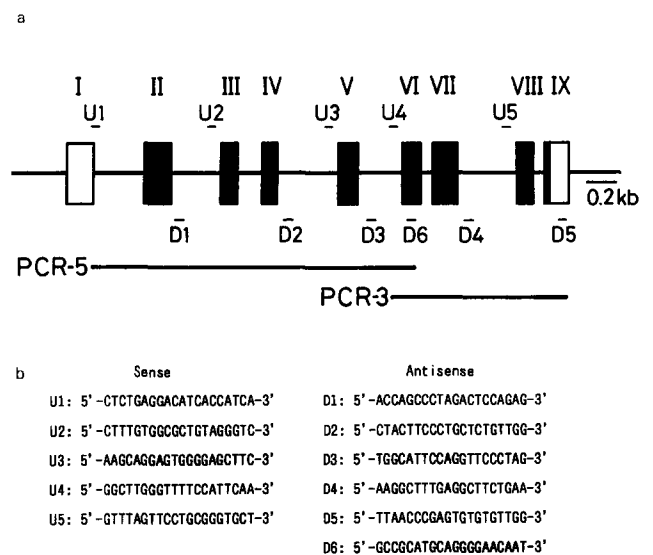

Fig. 1. (a) Schematic representation of the genomic structure of human DNase I [6] and the design of the primers for amplification and direct sequencing. Exons are shown by boxes with Roman numerals, and protein-coding exons are indicated by a solid box. The positions and direction of each primer are indicated by U1–U5 and D1–D6. 'U' and 'D' denote the sense and antisense primers, respectively. Two PCR-amplified fragments, PCR-3 and -5, used as templates for sequencing analysis, are presented underneath. (b) Sequences of the primers used for PCR-amplification and direct sequencing.

priate pair of PCR primers (shown in Fig. 1) demonstrated no difference between mutant and normal samples with regard to the intensity and length of the PCR-amplified fragment band resolved on agarose gel (data not shown). We were able to determine the nucleotide sequences of each exon from individuals with phenotypes 1, 2, 1–4 and 2–4. Exon II revealed only one heterozygous nucleotide substitution: C-to-G replacement occurred at position 91 in exon II (Fig. 2). The other exons of both the mutants were also sequenced to exclude other possibilities. The C91G mutation was found in the heterozygous state in the propositus. In order to confirm this substitution, the amplified PCR-5 fragment containing exon II from the propos-
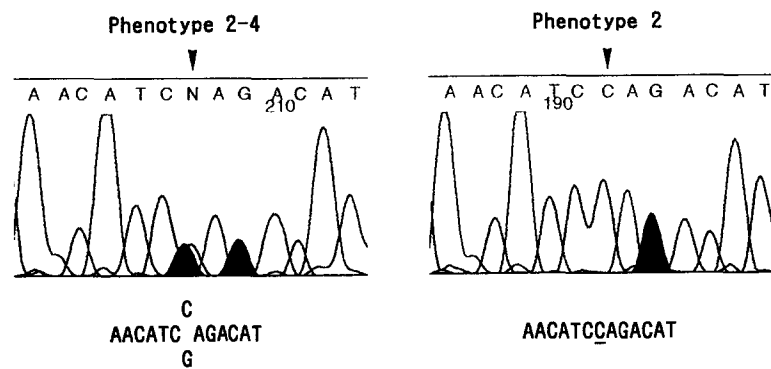

Fig. 2. Sequencing results showing the mutation site in the propositus (Oot-c; phenotype 2–4) compared with the corresponding sequence of phenotype 2. The PCR-amplified product, PCR-5, in which exon II was included, was subjected to direct sequencing analysis using the U1 sequencing primer. The peaks derived from G are marked with solid black. Arrowheads show the mutation site at position 91. The nucleotides are numbered from 5' to 3' starting from A at the translation initiation codon. The corresponding sequences are written underneath and the nucleotide substitutions are underlined. The results indicate that phenotype 2–4 is heterozygous for the C-to-G transition.
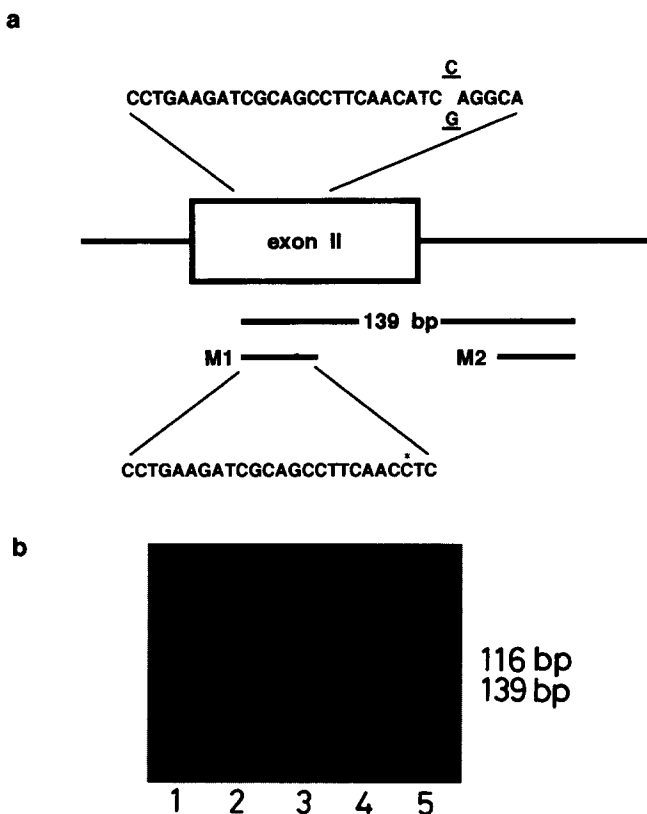
a

b

Fig. 3. Detection of the Q9E mutation in exon II. (a) Mismatched amplification of genomic DNA by PCR using a pair of primers, M1 and M2. The asterisk indicates a mismatched C residue in primer M1. (b) Agarose gel-electrophoresis of PCR-amplified products with XhoI digestion. Lane 1, phenotype 1; lane 2, the mother of the propositus (phenotype 1–4); lane 3, the propositus (phenotype 2–4); lane 4, the plasmid inserted with DNASE1*4; lane 5, the father of the propositus (phenotype 2).

itus (phenotype 2–4) was subcloned, then 12 independent clones were selected and subjected separately to DNA sequencing: five clones exhibited G at position 91, whereas the rest had C, thus corresponding to the DNASE1*2 allele. These findings supported the sequencing result. This mutation resulted in the substitution of Gln (codon CAG) by Glu (GAG) at position 9 of the mature enzyme. We have already determined that an A-to-G substitution at position 2317 and a C-to-G substitution at position 1592 occur in DNASE1*2 and *3 alleles, respectively [6,8]. Sequence analysis of the amplified PCR-3 fragment from the propositus enabled identification of G at position 2317 (corresponding to the DNASE1*2 allele) and C at position 1592 (corresponding to DNASE1*1 and *2 alleles). Analysis of amplified fragments PCR-3 and -5 from Oot-m (phenotype 1–4) gave the same results as in the propositus. Thus, the nucleotide sequences in a coding region of the mutant were identical to those of phenotype 2, except for a single nucleotide at position 91, or of phenotype 1, except for two nucleotides at positions 91 and 2317.

This C91G mutation neither suppressed nor created any known restriction enzyme recognition site in the DNase I gene. Therefore, we used mismatched PCR amplification followed by XhoI digestion to detect the missense mutation [12,13]. A 139-bp DNA fragment containing the mutation site was amplified

using the primers M1 and M2 as shown in Fig. 3. If the C91G mutation is present, the mismatched nucleotide provides a new XhoI recognition site. This mismatched PCR-amplified product was therefore expected to be degraded by XhoI to 23- and 116-bp fragments. However, no new XhoI site is yielded if the DNASE1*1, *2 or *3 allele is present. In fact, an amplified product of the plasmid into which the DNASE1*4 allele was inserted was completely digested by XhoI to yield only a 116-bp fragment, whereas those derived from the other alleles were not. The mismatched PCR method was applied to genomic DNAs from the propositus and his family (Fig. 3). Both the amplified DNAs derived from the propositus and his mother had two bands (116 and 139 bp), and were therefore heterozygous for the C91G mutation. These results were consistent with those of the phenotyping of their enzymes by isoelectric focusing. The specificity with this method was satisfactory, and enabled us to detect the C91G mutation from samples without DNase I enzyme protein.

4. Discussion

Sequence analysis of exon II from DNase I phenotypes 1–4 and 2–4 demonstrated that the substitution of C by G at nucleotide position 91 resulted in replacement of Gln by Glu at amino acid position 9 (Q9E) in the fourth allele, DNASE1*4. All the nucleotide substitutions involved in the genetic polymorphism of human DNase I, which is controlled by four alleles in the Japanese population, could be determined. The nucleotide/amino acid substitutions underlying the protein polymorphism of DNase I are summarized in Table 1. The results of this study demonstrated that the automated, direct cycle sequencing of double-stranded PCR products provides a highly reproducible method for identification of DNA sequence variations [14]. The isoenzyme of phenotype 4 is focused to the IEF-PAGE gel region between those of phenotypes 1 and 2. In comparison with phenotype 2 enzyme, the substitution of Gln by Glu at amino acid position 9 in phenotype 4 led to a change in the charge state of the enzyme. The pI values of the phenotype 1, 2 and 4 enzymes were predicted using GENE-TYX-MAX (Software Development Co., Tokyo, Japan) and calculated to be 4.37, 4.43 and 4.40, respectively. These findings account for their differences in electrophoretic mobility. The enzymatic activities of phenotypes 2–4 and 1–4 were similar to those of phenotypes 1, 1–2 and 2. Furthermore, position 9 may not be part of any putative functional site of human DNase I, as deduced from the bovine enzyme [16]. The amino acid resi-

Table 1
Nucleotide (amino acid) substitutions generating genetic polymorphism of human DNase I

Phenotype	Exon II	Exon VI	Exon VIII
	91 (9)	1592 (132)	2317 (222)
1	CAG (Gln)	CCC (Pro)	CAA (Gln)
2	CAG (Gln)	CCC (Pro)	CGA (Arg)
3	CAG (Gln)	GCC (Ala)	CAA (Gln)
4	GAG (Glu)	CCC (Pro)	CGA (Arg)

The nucleotide residues are numbered starting from adenine at the translation initiation codon according to the previous paper [6]. The amino acid residues are also numbered, in parentheses, starting with the N-terminal residue determined in our previous study involving protein sequencing of the enzyme [15].

	4	9	14	127	132	137	217	222	227
Human									
phenotype 1	AAFNIQTFGET			EFAIVPLHAAP			AGMLIQGAVVP		
phenotype 2	AAFNIQTFGET			EFAIVPLHAAP			AGMLIRGAVVP		
phenotype 3	AAFNIQTFGET			EFAIVALHAAP			AGMLIQGAVVP		
phenotype 4	AAFNIETFGET			EFAIVPLHAAP			AGMLIRGAVVP		
Bovine	AAFNIRTFGET			EFAIVALHAAP			AGSLLQSSVVG		
Ovine	AAFNIRTTGET			AFAIVPLHASP			AGPLLQSSVVG		
Porcine	AAFNIRTTGET			EFAIVPLHAAP			AGPLLQRAVVP		
Rat	AAFNIRTFGDT			EFAIVPLHSAP			AGPLLQAAVVP		

Fig. 4. Comparison of the partial amino acid sequences, including the polymorphic (mutational) sites, between human, bovine [17], ovine [17], porcine [17] and rat [18] DNase I's. The amino acid residues are numbered starting with the NH$_2$-terminal residue. The amino acid residues responsible for the genetic polymorphism of human DNase I are underlined.

due at this position is not conserved between human and other mammalian enzymes; the bovine, ovine, porcine [17] and rat [18] counterparts have Arg at the corresponding position (Fig. 4). Therefore, it is reasonable to conclude that the amino acid substitution at position 9 does not affect any specific function of DNase I.

Elucidation of the mutation site in phenotype 4 allows us to postulate the complete molecular basis of the genesis of polymorphism in human DNase I. Considering that *DNASE1*1* and **2* alleles occur at high frequencies in the Japanese population, and that Gln at position 222 is conserved in other mammals, it is plausible to assume that the *DNASE1*1* allele is an ancestral monomorphic gene. A point mutation in exon VIII of the *DNASE1*1* allele may have produced *DNASE1*2*. The *DNASE1*3* and **4* alleles must have been generated by a point mutation in exon VI of *DNASE1*1* and one in exon II of *DNASE1*2*, respectively.

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