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Analysis of Genetic Polymorphism of Deoxyribonuclease I in Japanese From Shimane Prefecture Using Genotyping Method

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A deoxyribonuclease I (DNase I)-genotyping method using polymerase chain reaction (PCR) has been developed, underlying the molecular basis of the genetic polymorphism controlled by four codominant alleles, *DNASE1*1, *2, *3* and **4*. Two new alleles, *DNASE1*5* and *DNASE1*6*, were recently discovered. In this study, we have added a new DNase I-genotyping method based upon both allele-specific amplification and mismatched PCR followed by *Bam* HI digestion. Using this method, the genotype distribution of DNase I in unrelated individuals from Shimane Prefecture in Japan was examined and compared with the results of other Japanese and/or ethnic population studies. The *DNASE1* allele frequencies for the Japanese individuals from Shimane Prefecture were determined to be *DNASE1*1* 0.5763, *DNASE 1*2* 0.4153, and *DNASE1*4* 0.0084 (χ^2=1.8304, 0.75> p >0.60). The existence of a decreasing north-to-south gradient in the *DNASE1*2* allele was revealed from the study of 10 Japanese populations containing Shimane prefecture. The allele frequency of *DNASE1*2* of the Shimane Prefecture population was relatively lower than those of the German and Turkish populations but higher than that of the Ovambos population. These findings suggest the presence of a different mechanism in the chance fluctuations of *DNASE1* alleles among different populations.

Key words: deoxyribonuclease I, genetic polymorphism, Shimane Prefecture, PCR

INTRODUCTION

The genetic polymorphism of human deoxyribonuclease I (DNase I, EC 3.1.21.1) has been demonstrated by differences in the pI values of type-specific isozymes (1).

Due to its favorable distribution of gene frequencies in a Japanese population, the *DNASE1* polymorphism is a valuable marker in genetic, forensic, and clinical investigations (2-10). However, when conventional isoelectric focusing (IEF) is used, the extremely small differences between the pI values of the isozyme bands produce a slightly lower resolution for rare heterozygotes, such as 1-3 or 2-4, than for other heterozygotes (11). DNase I is controlled by six codominant alleles, *DNASE1*1, *2, *3, *4, *5,* and **6* (12-16), at a single autosomal locus assigned to chromosome 16p13.3 (17). Our prior studies (12-16) have confirmed the nucleotide substitutions involved in the protein polymorphism of DNase I, which have been used to develop the DNase I genotyping method using PCR (18,19). Using this method, the allele frequencies of the DNase I polymorphism in a Shimane Prefecture population were determined.

MATERIALS AND METHODS

Biological samples

Blood samples were collected with written informed consent from each participant from Japanese individuals living in Shimane Prefecture. The genomic DNA was prepared from the buffy coat using a QIAamp DNA mini kit (QIAGEN Inc., Chatsworth, CA).

DNase I genotyping method

A genotyping assay for the detection of the DNase I polymorphism for *DNASE1*1, DNASE1*2, DNASE 1*3,* and *DNASE1*4* from the DNA samples was performed as previously described (18). The primer sets for the detection of *DNASE1*5* and *DNASE1*6* based upon the nucleotide sequence of the human DNase I

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gene (12, 16, 17) and other primers are shown in Table 1. Genomic DNA samples were added to the PCR mixtures (25 μl) consisting of a 1× PCR buffer, 1.5 mM $MgCl_2$, 0.4 μM of each primer, 250 μM dNTPs, and 1 U of Takara EX Taq DNA polymerase from Takara (Kyoto, Japan). After initial denaturation at 94 ℃ for 5 min, amplification was performed by denaturation at 94 ℃ for 1 min, annealing at 60 ℃ for 1 min, and extension at 72 ℃ for 1 min for 30 cycles, followed by a final extension at 72 ℃ for 5 min.

To detect the *DNASE1*5* allele, an amplification refractory mutation system (ARMS) (20-22) was used. The sense primer V-22 differed from primer V-21 by a single 3'-end nucleotide. A pair of primers, V-22 and D-2, was used for *DNASE1*5* allele amplification; a separate pair of primers, V-21 and D-2, was used for all other alleles. As an internal positive control, all PCR reaction mixtures contained an additional primer set, C-1 and C-2, to detect the other part of the DNase I gene that has a structure common to alleles. Co-amplification using two different set of primers and the C-1 and C-2 primer set was carried out by PCR using a previously described method (18).

The mismatched PCR method (22-24) was employed to discriminate the *DNASE1*6* allele from the *DNASE1*2* allele. The sequence including the nucleotide substitution that generated the mutation was amplified using a pair of primers, U-4 and D-2. The U-4 primer was designed with a deliberate mismatch at the first and second residue from the 3'-end in order to create a new *Bam* HI site at the mutation site in the *DNASE1*6* allele. After amplification with PCR, 5 μL PCR-product was digested with 50 U *Bam* HI in a 20 μL reaction volume at 37 ℃ for 2 h.

The PCR products or digests (5 μL) were separated in an 8% polyacrylamide gel in a x 1 TBE buffer at a constant voltage (200 V). The patterns on the gels were visualized by silver staining as described previously (25).

RESULTS AND DISCUSSION

In the present study, we have added two new primer sets to the DNase I-genotyping method for the detection of recently identified alleles, *DNASE1*5* and *DNASE1*6* (15-17).

The nucleotide sequence of *DNASE1*5* is identical to that of *DNASE1*2* except for a single nucleotide at position 1227 and to that of *DNASE1*1* except for two nucleotides at positions 1227 and 2317 (15). Therefore, it was necessary to distinguish the *DNASE 1*5* allele from *DNASE1*2*. An ARMS (20-22) was employed to detect *DNASE1*5* (Fig. 1). Two allele-specific primers, V-21 and V-22, corresponded to the sequences of phenotypes 1, 2, 3, 4, and 6 and to that of phenotype 5, respectively, and differed only in their terminal 3'-nucleotide. As an internal control fragment, a 403-bp fragment was co-amplified to detect a different region of the DNase I gene using a pair of primers, C-1 and C-2, in each reaction, thus helping to avoid false-negative results due to the failure of PCR (18). As shown in Fig. 1, the 796-bp ARMS fragments from a heterozygote carrying the

Table 1. Characterization of PCR-oligonucleotide primers for DNase I-genotyping

Primer	Sense/ antisense	Sequence[a]	Corresponding region in DNase I gene[b]	Comment
U-1	Sense	5'-ATCGTGGTTGCAGGGATGCTGCCTC-3' (**)	2292-2316	Used for discrimination between *DNASE1*1* (*3) and *2 (*4, *5 and *6); incorporated with two mismatched residues
D-1	Antisense	5'-AGTTCAACAGGTGTGGGGAG-3'	2533-2552	Used for discrimination between *DNASE1*3* and *2 (*4, *5* and *6)
U-21	Sense	5'-GTCAGGGAGTTTGCCATTGTT(C)-3'	1572-1593	Used for ARMS; specific to *DNASE1*1, *2, *4, *5* and *6
U-22	Sense	5'-GTCAGGGAGTTTGCCATTGTT(G)-3'	1572-1593	Used for ARMS; specific to *DNASE1*3*
D-2	Antisense	5'-AAGGCTTTGAGGCTTCTGAA-3'	1982-2001	Used for ARMS and detection of *DNASE1*6*; common to all alleles
C-1	Sense	5'-GTTTAGTTCCTGCGGGTGCT-3'	2216-2235	Used for amplification of an internal control positive fragment in ARMS analysis
C-2	Antisense	5'-TTAACCCGAGTGTGCGTTGG-3'	2598-2617	Used for amplification of an internal control positive fragment in ARMS analysis
U-3	Sense	5'-CCTGAAGATCGCTGCCTTCAACTC-3' (*)	66-89	Used for detection of *DNASE1*4*; incorporated with one mismatched residues
D-3	Antisense	5'-ACCAGCCCTAGACTCCAGAG-3'	185-204	Used for detection of *DNASE1*4*
V-21	Sense	5'-AGGCCTGACCAGGTGTCTGCG(G)-3'	1206-1227	Used for ARMS; specific to *DNASE1*1, *2, *3, *4* and *6
V-22	Sense	5'-AGGCCTGACCAGGTGTCTGCG(A)-3'	1206-1227	Used for ARMS; specific to *DNASE1*5*
U-4	Sense	5'-GTGAGACCCTCCCAGTGGTCATGG-3' (**)	1799-1822	Used for detection of *DNASE1*6*; incorporated with two mismatched residues

[a] Residues with an asterisk indicate the mismatched nucleotide in each primer. Residue in parentheses corresponds to the polymorphic site underlying *DNASE1*3* or *DNASE1*5* alleles.

[b] Nucleotide residues are numbered starting from A at the translation initiation codon according to the previous study (12).

*DNASE1*5* allele were amplified separately by PCR with both primers, V-21 and V-22, whereas the fragments carrying no *DNASE1*5* allele were amplified with primer V-21 alone.

The nucleotide sequence of *DNASE1*6* was identical to that of *DNASE1*2* except for a single nucleotide at position 1826 and to that of *DNASE1*1* except for two nucleotides at positions 1826 and 2317 (16). Therefore, it was necessary to distinguish the *DNASE 1*6* allele from *DNASE1*2*. A C-to-T transition was identified in exon VII of the *DNASE1*6* allele; the other 6 alleles had C at the corresponding position (16). We used mismatched PCR amplification followed by *Bam* HI digestion to detect the *DNASE1*6* allele. A 203-bp DNA fragment, containing the mutation site in exon VII, was amplified using a pair of D-2 and mismatched U-4 primers. The U-4 primer reflects the region adjacent to the mutation site and has two mismatched C residues instead of G at the first and second positions from the 3'-end compared with the other alleles. When the *DNASE1*6* allele containing the C185T substitution was amplified, the mismatched nucleotide created a new *Bam* HI site at the mutation site that is not present in other alleles. As shown in Fig. 2, the amplified product carrying no *DNASE1*6* allele was completely digested by *Bam* HI to yield only a 180-bp fragment. Therefore, the appearance of a 180-bp fragment produced by *Bam* HI digestion on the agarose gel indicates the absence of the *DNASE1*6* allele. Both amplified products

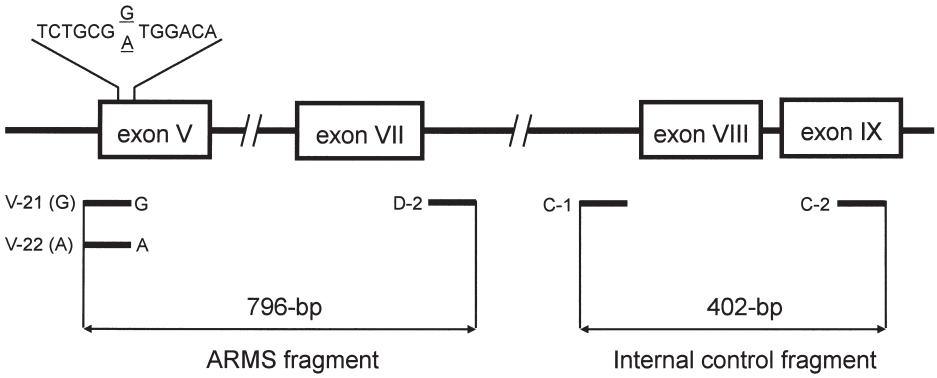


Fig. 1. Detection of the *DNASE1*5* allele by ARMS for G1227A. Relative positions of ARMS primers, V-21, V-22, and D-2, and other primers (C-1 and C-2) for amplification of an internal positive control fragment in DNase I gene. The nucleotide sequences of these primers are presented in Table 1. Two allele-specific primers, V-21 and V-22, differ only in their terminal 3'-nucleotide.

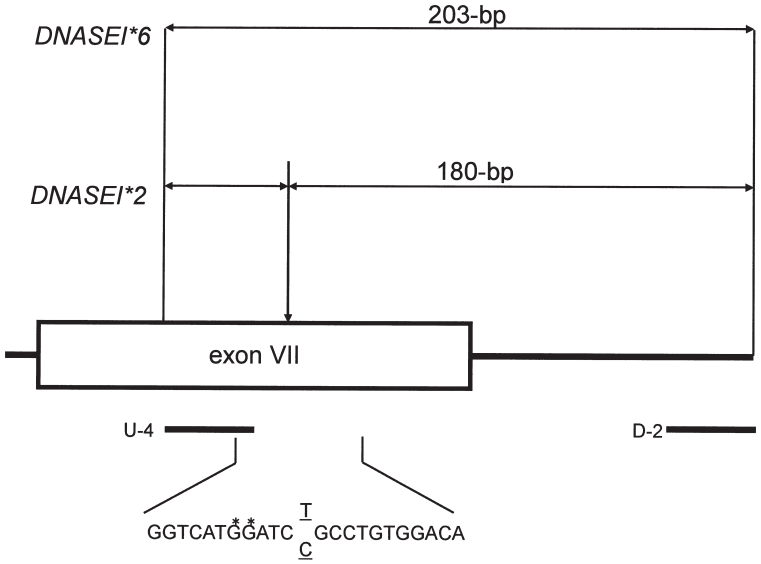
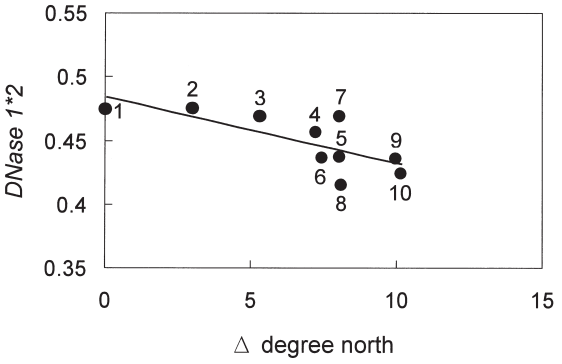


Fig. 2. Mismatched PCR for the discrimination of the nucleotide substitution C1826T underlying the *DNASE1*2* and *DNASE1*6* alleles. Nucleotide sequences of D-2 and mismatched U-4 primers are shown in Table 1. Nucleotide marked with an asterisk indicates the mismatch incorporated into the U-4 primer to create a new *Bam* HI site (GGATCC) at the polymorphic site. Underlined nucleotides correspond to substitution site.

from heterozygotes carrying the *DNASE1*6* allele were partially digested to yield two bands representing 203- and 180-bp fragments derived from the *DNASE1*6* and the *DNASE1*1, *2, *3, *4, or *5* alleles.

Using the above method, DNase I-genotyping of Japanese individuals from Shimane Prefecture was carried out. Four common phenotypes were found in the Japanese individuals from Shimane Prefecture. The gene frequencies of the alleles were tentatively estimated for the population, and the phenotype distribution was found to have Hardy-Weinberg equilibrium. The *DNASE1* allele frequencies for the Japanese individuals from Shimane Prefecture were determined to be *DNASE1*1* 0.5763, *DNASE1*2* 0.4153, and *DNASE1*4* 0.0084 (χ^2 =1.8303, 0.75> p >0.60). Differences in the genotype distribution among nine other prefectures and three German, Turkish, and Ovambos populations are shown in Table 2. In a previous study (4), nine phenotypes, including six rare ones, were reported in a Japanese population. In the German, Turkish, and Ovambos populations, only three common phenotypes were found (19). We have reported the existence of a decreasing north-to-south gradient in the *DNASE1*2* allele in Japanese populations from Hokkaido, Aomori, Miyagi, Gunma, Tokyo, Fukui, Tottori, Ehime, and Fukuoka (4). Such a tendency was also recognized in data including Shimane Prefecture (Fig. 3).



1: Hokkaido 2: Aomori 3: Miyagi 4: Gunma 5: Tokyo
6: Fukui 7: Tottori 8: Shimane 9: Ehime 10: Fukuoka

Fig. 3. The cline of *DNASE1*2* allele frequencies in the ten Japanese populations. We obtained the equation: y = -0.0052x + 0.4843

Table 2. Allele frequencies of *DNASE1* polymorphism in Shimane and other Japanese Populations

Population	Group Size	*Allele Frequencies*					
		*DNASE1*1*	*DNASE1*2*	*DNASE1*3*	*DNASE1*4*	*DNASE1*5*	*DNASE1*6*
Japanese							
Shimane (Izumo)[a]	118	0.5763	0.4153		0.0084		
Hokkaido (Asahikawa)[b]	99	0.5253	0.4747				
Aomori (Aomori)[b]	100	0.525	0.475				
Miyagi (Sendai)[b]	96	0.5313	0.4687				
Gunma (Maebashi)[b]	275	0.5309	0.4564	0.0091	0.0018		0.0018
Tokyo[b]	200	0.5600	0.4375	0.0025			
Fukui (Fukui)[c]	1213	0.5501	0.4367	0.0116	0.0012	0.0004	
Tottori (Yonago)[b]	100	0.5313	0.4687				
Ehime (Matsuyama)[b]	101	0.5644	0.4356				
Fukuoka (Kurume)[b]	125	0.5760	0.4240		0.0010		
Total	2427	0.5481	0.4430	0.0069	0.0016	0.0002	0.0002
German							
Münster[b]	204	0.2524	0.7476				
Munich[b]	195	0.3385	0.6615				
Total	399	0.2945	0.7055				
Turk (Adana)[d]	136	0.2206	0.7794				
Ovambos (Namibia)[d]	176	0.8722	0.1278				

[a]This study
[b]From Takeshita et al. (4)
[c]From Yasuda et al. (7)
[d]From Fujihara et al. (19)

When compared to those of the German and Turkish populations, the allele frequency of *DNASE1*2* was relatively lower for the Japanese population. In contrast, the allele frequency of *DNASE1*1* in the Ovambos population was much higher than that for the Japanese and other populations. These results suggest the presence of a different mechanism in the chance fluctuations of *DNASE1* alleles in different populations. There is a significant association between *DNASE1*2* and liver disease, gastric carcinoma, colorectal carcinoma, and myocardial infarction in Japanese populations (7-10). Therefore, it is worth conducting further research of the worldwide distributions of *DNASE1* alleles.

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