Original Article

POLYMORPHISM OF THE D12S66 SYSTEM IN THE JAPANESE POPULATION AND ITS DETECTION USING DEGRADED DNA

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Abstract

The short tandem repeat (STR) polymorphism of the locus D12S66 was amplified by PCR and analyzed by denaturing polyacrylamide gel electrophoresis followed by silver staining. Among 190 DNA samples from the Japanese population, six alleles were observed. The genotypic distribution meets Hardy-Weinberg expectations, and the heterozygosity was 52.7%. When sequences of the allelic products were compared, each allelic segment was 153–173 bp in size, and contained 9 to 14 GATA tetranucleotide repeat motifs. Amplification of the locus using 27 tooth and blood stain samples as sources of degraded DNA resulted in low backgrounds and reproducible patterns, suggesting the usefulness of the application of this locus for material examination.

Key words: Short tandem repeat (STR)—D12S66—Allele distribution—PCR amplification from degraded DNA

INTRODUCTION

The human genome contains a large number of STRs, and polymorphic STRs are widely used in the field of forensic science. When DNA is highly degraded, we sometimes have difficulty in amplifying PCR products with large sizes even for the amplification of STR systems. Thus it is desirable if we can use STR polymorphisms with small target sizes in such cases.

The D12S66 locus was reported by Roewer *et al.*⁸⁾ as a tetranucleotide repeat polymorphism with allelic size differences from 132–156 bp. Many polymorphic STRs used in the

field of forensic science have PCR product sizes ranged from approximately 100 bp to 400 bp. The D12S66 system belongs to the group with small target sizes among these STR systems.

In this paper we attempted to establish a database for the D12S66 locus in the Japanese population to apply this polymorphism for material examination from degraded DNAs.

MATERIALS AND METHODS

1. Samples

Genomic DNA was extracted from blood

samples of 190 healthy unrelated Japanese individuals. Informed consent was given from the blood and tooth donors. Leukocyte preparations from blood were suspended in saline-EDTA (75 mM NaCl, 24 mM EDTA) containing 1% sodium dodecyl sulfate (SDS) and digested with 0.1 mg/ml of proteinase K at 55°C overnight, followed by treatment with RNase at 55°C for 2 hrs. DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH7.6).

Samples of teeth and cotton used for hemostasis after extraction of teeth were obtained from 27 patients in a dental clinic. The enamel layer of the tooth was scraped with a dental instrument and the remaining materials were crushed into powder using a "diamond mortar", a special device that crushes hard tissues, followed by decalcification in 3 ml of 0.5 M EDTA for 7 days. Although not all of the tooth powder dissolved in the EDTA, it was centrifuged, washed in TE twice, and re-suspended in TE, followed by proteinase K digestion and DNA extraction as described above. Samples of cotton were washed in sterile physiological saline twice, centrifuged, suspended in TE containing 1% sodium dodecyl sulfate (SDS), and digested with 0.1 mg/ml of proteinase K at 55°C overnight. The solution was transferred to another tube, and DNA was extracted as described above.

2. Detection of D12S66 locus

Segments of D12S66 were enzymatically amplified from genomic DNA using the following oligonucleotides as primers: primer 1: 5'-TCATTTAAGCATTTGAGGGAA-3', primer 2: 5'-AGACTTCAAAACAGACACTT-3'⁽⁸⁾. Each amplification of D12S66 was performed in a 30 µl mixture containing 10 ng of genomic DNA, 10 mM Tris-HCl at pH8.3, 50 mM KCl, 2.25 mM MgCl₂, 0.01% gelatin, 200 µM dNTP, 400 nM of each primer and 2.5 U of AmpliTaq DNA polymerase or 1.25 U of AmpliTaq Gold (Perkin-Elmer Cetus). The following PCR conditions were used for the first mixture: 94°C for 5 min, followed by 30

cycles of denaturation at 94°C for 30 sec, annealing at 51°C for 30 sec and extension at 72°C for 90 sec. After the 30th cycle, an extra step was performed at 72°C for 10 min to extend the template completely. The PCR conditions using AmpliTaq-Gold were 95°C for 9 min, followed by 30 cycles of denaturation at 95°C for 40 sec and annealing and extension at 51°C for 75 sec. After the 40th cycle, an extra step was performed at 51°C for 5 min to extend the PCR products completely.

The above-mentioned reaction mixtures were overlaid with one drop of mineral oil (SIGMA), and amplification was performed in a Perkin-Elmer Cetus DNA Thermal Cycler apparatus PJ 2000. The PCR products were electrophoresed in 6% denaturing polyacrylamide gels, followed by silver staining⁽¹⁾.

3. Sequencing analysis

Each amplified DNA fragment was eluted from silver stained gels using the "crush and soak" method⁽⁹⁾, re-amplified by PCR, and directly sequenced using Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Cetus); sequence analysis was performed on an ABI 373A DNA Sequencer.

RESULTS

1. D12S66 typing and sequencing

Six kinds of different allelic products with different sizes were identified at the D12S66 locus among 190 Japanese individuals (Fig. 1). PCR products regarded as having the same sizes were loaded side by side on a denaturing gel and were compared for typing. All of the same allelic products showed the same migration distance, suggesting that each allele represented a 4 bp difference. The genotype distribution of the D12S66 locus is shown in Table 1. The genotypic distribution meets Hardy-Weinberg expectations ($\chi^2 = 5.23$, df = 3, $0.02 > P > 0.01$). The allelic frequencies are shown in Table 2. The heterozygosity was 52.7%. The most predominant allele was allele 11. Although Gomolka *et al.*⁽³⁾ have examined the D12S66 locus in Germans and

Fig. 1 Electrophoresis of D12S66 in a 6% denaturing gel followed by silver staining. PCR amplification was conducted by using AmpliTaq. The allelic fragments showed doublet patterns probably resulting from A-addition in the 3' side during PCR amplification. This phenomenon is usually observed in denaturing polyacrylamide gel electrophoresis of STRs. The sizes of the fragments were indicated on the right, and allele designations were indicated on the left.

Lane 1: ladder marker, Lane 2: genotype 9/10,
Lane 3: genotype 9/11, Lane 4: genotype 11/12,
Lane 5: genotype 10/13, Lane 6: genotype 12/14,
Lane 7: ladder marker

Table 1 Distribution of D12S66 genotype frequencies

Genotype	Observed	Expected	χ^2 values
10/11	26	25.94	0.00
10/12	12	7.78	2.29
11/11	77	80.28	0.13
11/12	50	48.17	0.07
11/13	10	10.37	0.01
12/12	6	7.22	0.21
12/13	5	3.11	1.15
Others	4	7.13	1.37
Total	190	190.00	5.23
	df= 3	0.02>P>0.01	

* All classes with less than 5 events were pooled.

Table 2 Comparison of the allele frequencies. Total number of samples examined are shown in parentheses

Allele	Japanese* (190)	Caucasians (30)
9	0.005	0.100
10	0.105	0.017
11	0.650	0.550
12	0.195	0.200
13	0.042	0.083
14	0.003	0.050

* present study

eight Eurasian populations, they did not show actual values of allelic frequencies. Thus we cited the values of Caucasians reported by Roewer *et al.*⁸⁾ for comparison. They also found six alleles designated from A to F in the D12S66 locus. The size of each allele was first shown to be 132–156 bp⁸⁾, which was later corrected to be 153–173 bp³⁾. The distribution of the allelic frequencies of Caucasians were not

so different from ours except for the frequencies of infrequent alleles 9, 10, 13, and 14.

Six different kinds of allelic products were sequenced from both strands. Each allelic segment contained 9 to 14 GATA tetranucleotide repeats in the sequences of the 153 bp, 157 bp, 161 bp, 165 bp, 169 bp and 173 bp fragments, respectively (Fig. 2).

TCATTTAAGCATTTGAGGGAAATGGATAGGTGGACAAATTA (GATA)₉₋₁₄
GAGATAATAGACA (GATA)₃ ATTAGAGAGTTTAGANNCCTTGAAGACCN
AAGTGTCTGTTTTGAAGTCT

Fig. 2 Sequence of the D12S66 locus. Sequences corresponding to the primers were underlined. Allelic differences depend on the number of GATA tetranucleotide repeats in a variable region.

Fig. 3 Electrophoresis of the D12S66 amplified from blood stain (B) and tooth samples (T) of five individuals. Amplification was conducted by using AmpliTaq Gold in the samples of blood stains and using AmpliTaq in the samples of teeth. Some samples showed single bands and others showed doublet patterns. However, these patterns can be judged as identical alleles when single band corresponded to one of the doublet bands. When each band was re-amplified, it usually shows the same migration distances in an electrophoresis on a denaturing gel, and the same nucleotide sequences are usually obtained.

2. Detection of the D12S66 locus from degraded DNA

The D12S66 locus was amplified from DNA samples obtained from 27 tooth and 27 blood stain samples from the same donors. PCR amplification was successful in 26 (96%) and 25 (93%) of the tooth and blood stain samples, respectively. Although faint bands resulting from slippage artifact were observed, they did not affect genotyping (Fig. 3). The patterns obtained from teeth and blood stains were identical and showed comparatively low background despite using degraded DNAs as sources of materials.

DISCUSSION

In comparison of the allelic frequencies

between Japanese and Caucasians, the allelic distribution of the D12S66 locus was not so different in the prominent alleles, allele 11 and 12. Allelic frequencies of the D12S66 locus reported by Gomolka *et al.*³⁾ in Germans ($n=267$) and seven Eurasian populations, Ahom ($n=32$), Hindu ($n=43$), Kachari ($n=42$), Thais ($n=45$), So ($n=25$), Kampuchea ($n=42$), and South Korea ($n=51$), also distributed unimodally. Kayser *et al.*⁴⁾ found allelic size differences of 4bp in the D12S66 locus among the samples of non-human primates (*Macaca mulatta*). It is possible that the D12S66 locus was polymorphic before the divergence of human and non-human primates. This could in part be the reason for the similarity of the allelic distributions in several ethnic groups.

Although the heterozygosity of the D12S66

locus in various populations was not very high, the amplification efficiency of the locus from degraded DNA was fairly high. The bands resulting from slippage artifact usually appeared in lower molecular weight regions, and no samples showed faint bands in molecular weight region larger than the migrating positions of the largest target DNA bands. In another experiment using the same degraded samples, we also tried to amplify the VWA locus, a tetranucleotide repeat polymorphism within intron 40 of the von Willebrand factor (vWF) gene⁹. The sizes of the VWA locus ranged from 126 bp to 170 bp, which are not so different from those of the D12S66 locus. However in the amplification of VWA, faint bands also appeared in the larger molecular weight regions of some samples¹¹. Since the sequence organization of the VWA locus is more complicated, with two variable regions of tetranucleotide repeat motifs (TCTG)₃₋₅(TCTA)₁₀₋₁₅ in the common alleles⁶, one of the reasons for the origin of these bands is probably heteroduplex formation during PCR amplification. In the amplification of the D12S66 locus, we did not observe such artifacts, which made interpretation of the band patterns simpler.

In the amplification of the CD4 locus using the same degraded DNA samples, unequal amplification efficiency of the alleles with different sizes was observed¹¹. Some samples showed higher amplification efficiency in the smaller alleles than in the larger ones. The sizes of the fragments of the CD4 locus was 86–121 bp including simple sequence differences in the number of pentanucleotide repeat motifs (CTTTT and CCTTT). In comparison between the CD4 locus and the VWA locus using the same degraded DNA samples, amplification of the VWA locus was difficult in 7% of the 80 samples in which typing of the CD4 locus was possible. In these experiments, since we used teeth and blood stain samples from the same donor, some samples were concluded to be correctly typed in spite of the unbalanced amplification of the alleles of the CD4 locus. These unbalanced patterns will cause difficulty in typing when there were

no other samples derived from the same donor. Although the amplification of the CD4 locus seems more efficient than that of the VWA locus, amplification efficiency of the alleles of the locus from degraded DNAs seemed to be affected more easily than that of other STR loci with larger allele sizes; such as VWA (126–170 bp)¹¹, D12S66(153–173 bp), D21S11 (213–239 bp)¹⁰, and D12S67 (233–273 bp)⁸ (unpublished observation). We seldom had difficulty in typing these polymorphisms. The DYS389 locus contains two adjacent polymorphic regions, which can be simultaneously amplified using one primer pair². Application of this locus for detection using degraded DNAs also showed a similar tendency in that the allele determination of a larger-sized fragment was more reliable than that of a smaller-sized fragment⁷. These results suggested that, in an amplification using degraded DNAs, successful amplification depends in part on the sizes of the target DNAs.

In conclusion, the present results indicated that the D12S66 locus is a useful marker for the detection of polymorphism from degraded DNAs. However, more information is necessary to establish a reliable protocol for PCR amplification of STRs using highly degraded DNAs.

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