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Detection of Fragment Length Polymorphism of the VNTR Loci D1S80 and D2S123 by PCR Amplification, PAGE and Silver Staining

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Abstract: The highly polymorphic variable number of tandem repeat (VNTR) loci in the human genome are informative markers for the genetic characterization of individuals in the paternity test and forensic science as well as for the study of human disease. In this study, VNTR loci D1S80 and D2S123 have been amplified by PCR and the amplified length polymorphic alleles were detected with a discontinuous vertical PAGE system and silver staining. For explicit DNA typing, PCR optimization, in which amplification efficiencies are similar over a wide range of allele sizes, non-specific amplifications are minimal, and new longer alleles have high amplification efficiency, has been performed by changing the PCR reaction buffer composition and thermal cycling conditions. It turned out that adding an appropriate amount of Tween 20 and NP40 to the PCR reaction buffer and raising the annealing temperature to 68℃ in thermal cycling made it possible for optimal VNTR loci amplification. A modified PAGE system for VNTR separation was established. Under these conditions, new longer alleles in the D1S80 locus were discovered and D2S123 pattern changes in colorectal tumors were observed. These technical tips are valuable for detecting various amplified fragment length polymorphisms.

Key words: Amp-FLP, D1S80, PAGE, PCR, variable number of tandem repeat.

A subclass of eukaryote tandemly repeated DNA contains a very short simple sequence repeat $(CA)_n$ (Weber and May, 1989). These tandem repeat units are termed variable number of tandem repeats (VNTRs). In the human genome there are 50,000~100,000 interspersed $(CA)_n$ blocks (Braaten *et al.*, 1988). However, the function of the $(CA)_n$ blocks is unknown, but it has been proposed that they serve as hot spots for recombination (Slightom *et al.*, 1980) or participate in gene regulation (Hamada *et al.*, 1980). The extent of $(CA)_n$ block repeat varies considerably within the human species. Therefore VNTRs are considered as "length polymorphic" rather than "sequence polymorphic". For this reason, VNTRs serve as personal identification markers in the field of forensic science and as susceptibility markers for certain diseases in the field of medicine. New VNTR loci are continually being reported (Horn *et al.*, 1989; Boerwinkle *et al.*, 1989; Ludwig *et al.*, 1989; Kasai *et al.*, 1990; Allitto *et al.*, 1991).

One of the VNTR markers, D1S80, consists of repeat units of 16 base pairs (bp) in length and has been localized by multipoint linkage analysis to the distal end of chromosome 1p (Nakamura *et al.*, 1988). The marker shows at least 29 alleles which could determine 435 genotypes with a discrimination power of 95~98 % depending on the population (Nam *et al.*, 1994). Lately, remarkable and unexpected pattern changes at the D2S123 locus were observed in the majority of the herditary nonpolyposis colorectal cancer-derived tumors (Peltomake *et al.*, 1993; Aaltonen *et al.*, 1993). These changes consisted of shifts in the electrophoretic mobility of $(CA)_n$ dinucleotide repeat fragments (Peltomake *et al.*, 1993; Aaltonen *et al.*, 1993). However, characteristics of these changes have not been demonstrated. We search for changes at the D2S123 locus by studying DNA extracted from colorectal tumor tissues and comparing it with matched normal DNA from the same individuals.

For the VNTR analysis, the RFLP method via Southern blotting is laborius and time consuming. The RFLP could not resolve most VNTR loci (Budowle *et al.*, 1991). Thus recent studies have tended to adopt PCR technology to obviate the need for isotopic detection. The amplified fragment length polymorphism (Amp-FLP) technique is a new convenient method to

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analyze VNTR loci. In this study, we established the optimal PCR conditions for explicit typing by raising the amplification efficiencies over a wide size range of DNA fragments and reducing the non-specific amplifications, and employed as a detection system high resolution PAGE. With this method, we discovered new longer alleles in D1S80 and a mutation of the D2S123 locus in colorectal tumor tissues of Korean patients.

Materials and Methods

DNA sample

Fresh human blood, which was withdrawn with anticoagulant 3.8% sodium citrate, was subjected to a Ficoll gradient in order to collect white blood cells. White blood cells were lysed with SDS and EDTA. Surgically resected colorectal tumor specimens and normal mucosals were obtained, and dissected for DNA extraction. Subsequently DNA was treated with proteinase K and RNase, and extracted with phenol/chloroform. D1S80 and D2S123 were amplified by PCR and the products were detected for the individual samples. PCR amplification DNA amplification was performed using the primers for D1S80 5′-GAAACTGGCCTCCAAACACTG-CCCGCCG-3′ and 5′-GTCTTGTTGGAGATGCACGT-GCCCTTGC-3′ desribed by Kasai *et al.* (1990), and those for D2S123, 5′-AAACAGGATGCCTGCCTTTA-3′ and 5′-GGACTTTCCACCTATGGGAC-3′ obtained from Dr. Vogelstein (Johns Hopkins University by personal communication). Each sample was amplified in the 50 μL reaction volume containing 100 ng of human genomic DNA, 1 μM of each primer, 200 μM of each dNTP and 2.5 U Taq DNA polymerase. The buffer composition and pH was varied to achieve optimum amplification by using the Perkin-Elmer Cetus Cycler model #9600 (USA). Each cycle consisted of denaturation at 94℃ and 95℃ for 1 min, primer annealing at 55, 60, 65 or 75℃ for 1 min and primer extension at 70 or 72℃ for 1 or 4 min.

Polyacrylamide gel electrophoresis and silver staining

To resolve the amplified DNA fragments, a discontinuous PAGE system was employed as described by Sajantilla *et al.* (1992) with some modification. Vertical, 0.75 mm polyacrylamide gel consisted of the stacking gel (3% acrylamide, 0.1% bisacrylamide and 7% glycerol in 33 mM Tris-sulfate, pH 9.0) and resolving gel (6% acrylamide, 0.1% bisacrylamide and 7% glycerol in 33 mM Tris-sulfate, pH 9.0). 5 μL of the PCR products and 2 μL of sample loading buffer (20% sucrose and 0.2% bromophenol blue) were loaded in each lane. Electrophoresis was carried out using a running buffer (9 mM Tris base, 90 mM boric acid and 2 mM EDTA, pH 9.0) at constant voltage (200 V). The separated alleles in polyacrylamide gel were detected using silver staining (Bassam *et al.*, 1991) with some modification.

Allele marker

To determine the explicit allele types, D1S80 allele marker was purchased from Cetus (USA) and reamplified to serve as a reproducible allele marker for explicit typing (Sajantila *et al.*, 1992).

Results

Comparison between EtBr staining of agarose gel electrophoresis and silver staining of PAGE

In order to test sensitivity of detection and allele resolution power, an agarose gel system followed by EtBr staining and a PAGE system followed by silver staining were compared. The PAGE followed by silver staining was superior to the agarose gel system with EtBr staining both in sensitivity of detection and in resolution of different size D1S80 alleles. Because the agarose gel system was less laborious than the PAGE system, the possibility of using agarose gel system as a typing tool was seriously considered. In our judgement the agarose gel system, however, seemed not quite satisfactory for the purpose of D1S80 allele typing. Therefore the PAGE system was adopted.

Optimization of PCR amplification

Human genomic DNAs from genetically unrelated Koreans were amplified with various reaction mix compositions. Amplified length polymorphic fragments (Amp-FLP) were separated with the vertical PAGE system and visualized with silver staining as described in Materials and Methods. Non-specific amplification should be minimum and the amplification efficiency of longer alleles be maximum for explicit allele typing. Optimization of PCR amplification was performed by changing the reaction buffer composition and thermocycling temperature. Optimum amplification conditions for clean and efficient band products were obtained by adding KCl (50 mM), β-ME (5 mM), NP40 (0.001%) and Tween 20 (0.001%) in Tris buffer (10 mM, pH 8.5) (Fig. 1b) and raising the annealing temperature to 68℃ (Fig. 1a).

Amplification of VNTR locus D1S80 and D2S123 under optimum conditions

Over 100 genetically unrelated Koreans were typed for D1S80 allele (the Korean allele distribution data has been presented by Nam *et al.*, 1995) after optimum PCR amplification. One example of D1S80 allele typing is demonstrated in Fig. 2. High molecular weight

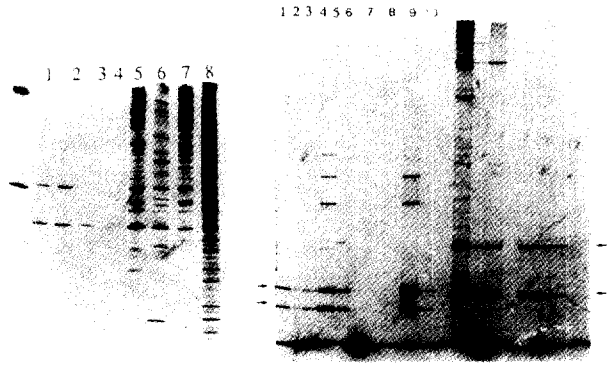

Fig. 1. (a) Effect of thermal cycling condition on PCR amplification of D1S80 locus in reaction buffer same as that of lane 5 in Fig. 1b. PCR products were separated in PAGE and detected with silver staining. Lane 1 and 2, denaturation at 95℃ for 1 min, annealing at 68℃ for 1 min and extension at 70℃ for 4 min (lane 1) or 1 min (lane 2): lane 3 and 4, 94℃ for 1 min, annealing at 65℃ for 1 min and extension at 72℃ for 4 min (lane 3) or 1 min (lane 4): lane 5 and 6, 94℃ for 1 min, annealing at 60℃ for 1 min and extension at 72℃ for 4 min (lane 5) or 1 min (lane 6): lane 7 and 8, 94℃ for 1min, annealing at 55℃ for 1 min and extension at 72℃ for 4 min (lane 7) or 1 min (lane 8). (b) Effect of reaction mix composition on PCR amplification of D1S80 locus. PCR thermal cycling condition was denaturation at 95℃ for 1 min, annealing at 65℃ for 1 min, elongation at 72℃ for 1 min and final extention at 72℃ for 10 min. Reaction buffer (lane #1~5) contains 10 mM Tris (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, except additive concentrations: lane 1, 0.001% gelatin: lane 2, 5 mM β-ME, 0.01% gelatin, 0.01% NP40 and 0.01% Tween 20: lane 3, 0.001% gelatin, 0.01% NP40 and 0.01% Tween20: lane 4, 5 mM β-ME and 0.001% gelatin: lane 5, 5 mM β-ME, 0.001% gelatin, 0.001% NP40 and 0.001% Tween 20. Reaction buffer of lane #6-10 contains 300 mM Tris (pH 8.0) and 1.5 mM $MgCl_2$ except additive concentration: lane 6, 5 mM β-ME, 0.01% gelatin, 0.01% NP40 and 0.01% Tween 20: lane 7, 0.01% gelatin, 0.01% NP40 and 0.01% Tween 20: lane 8, 5 mM β-ME, 0.001% gelatin, 0.001 % NP40 and 0.001% Tween 20: lane 9, 5 mM β-ME: lane 10, 5 mM β-ME, 0.01% gelatin.

new alleles were detected and the longer alleles were evenly amplified as low molecular weight alleles under the optimized conditions. Precise typing of D1S80 alleles could be possible by using standard allele markers lane by lane. Standard allele markers were provided by amplifying the serially diluted allele mixture under optimum PCR conditions.

Mutations of the VNTR D2S123 locus were detected in colorectal tumor tissues by amplifying under bands in optimum conditions and separating the bands in a high resolution PAGE system. We compared the amplified D2S123 pattern of tumor tissues with matched normal DNA from the same individuals (Fig. 3). D2S 123 pattern change were observed in two of 16 colorectal tumor patients.

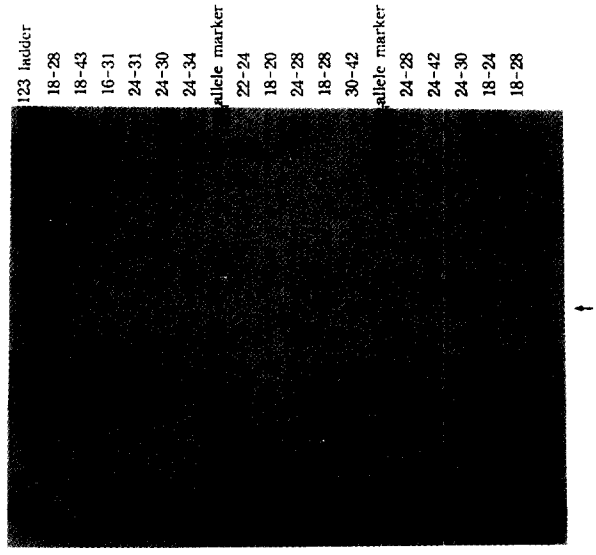

Fig. 2. Examples of D1S80 typing in unrelated Korean population. Arrows indicated the newly discovered longer alleles. Arrow indicates the newly discovered longer alleles.

Discussion

Application of the PCR to type a length polymorphic DNA marker such as a $(CA)_n$ block is valuable because it consumes less DNA and is faster than standard blotting and hybridization. VNTR D1S80 is a very informative locus for genetic characterization of individuals in the field of forensic science and human genetics. Accurate D1S80 typing is required for the routine analysis of forensic samples. The PCR typing system that is based on the analysis of length polymorphisms (VNTRs) has several problems to be solved; first, the preferential amplification of the shorter allele PCR product from a heterozygote or non-specific amplification; second, proper resolution of each allele and sensitive detection of PCR products; third, the confirmation of the alleles by standard allele markers. In this study, we have demonstrated the technical tips for best performance of typing. Optimum PCR conditions to avoid some preferential or non-specific amplification were investigated by altering the composition of the PCR mix and thermal cycling temperatures. Efficient and specific amplification products were obtained using a reaction buffer including detergents such as NP40 and Tween 20 (Fig. 1b). Non-specific amplification was dramatically decreased by raising the annealing temperature (Fig. 1a).

The analysis of the D1S80 locus by using Amp-FLP techniques has been studied previously in population genetics; Boudowle *et al.* (1991) reported 10 alleles in 99 unrelated Caucasians; Sajantila *et al.* (1992) reported 15 alleles in 140 unrelated Finnish individuals;

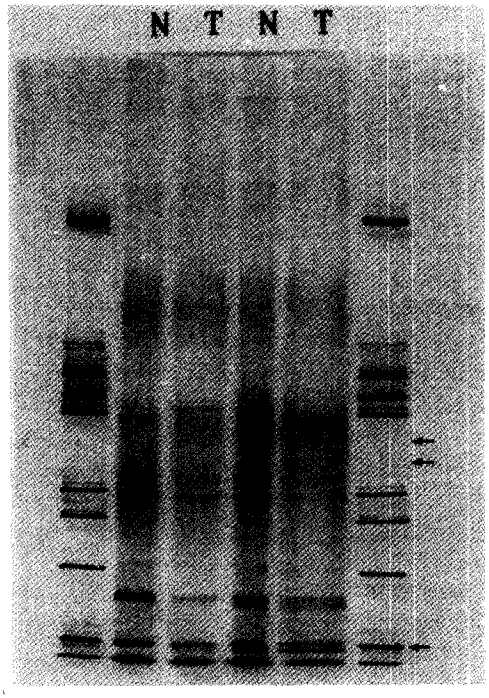

Fig. 3. Amplification of D2S123 locus using DNA extracted from colorectal tumor tissue (T) and normal mucosal membrane (N). Arrows indicate the changes of D2S123 locus in tumor sample.

Hong *et al.* (1993) present 19 alleles in 78 Koreans. In the present study, 22 alleles with 48 genotypes in 111 unrelated Koreans were identified, demonstrating that PCR optimization makes it possible to detect the longer allele type 42 and 43 without sacrificing the amplification of shorter alleles (Fig. 2). Considering the importance of the D1S80 locus in forensics, the fact that our system detected the larger number of alleles deserves serious attention.

Each allele was adequately resolved and detected by PAGE separation with some modification as described in Materials and Methods. PCR products should be compared with standard allele markers for precise typing because it is not possible to decide small size differences among alleles without them. For this purpose, we used the diluted standard marker as template DNA and reamplified it at optimum PCR conditions. This strategy was applied for detection of changes in the D2S123 VNTR locus which can undergo change in tumor tissue (Fig. 3). This method can be convenient for the testing of various VNTR loci related to diseases or personal identification.

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References

Aaltonen, L. A., Peltomak, P., Leach, F. S., Sistonen, P., Pylkkanen, L., Mecklin, J. P., Jarvinen, H., Powell, S. M., Jen, J., Hamilton, S. R., Petersen, G. M., Kinzler, K. W., Vogelstein, B. and de la Chapelle, A. (1993) *Science* **260**, 812.

Allito, B. A., Horn, G. T., Altherr, M. R., Richards, B., McClatchey, A. I., Wasmuth, J. J. and Gusella, J. F., (1991) *Nucleic Acids Res.* **19**, 4015.

Bassam, B. J., Caetano-Anolles, G. and Gresshoff, P. M. (1991) *Anal. Biochem.* **196**, 80.

Boerwinkle, E., Xiong, W., Fourest, E. and Chan, L. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 212.

Braaten, D. C., Thomas, J. R., Little, R. D., Dickson, K. R., Goldberg, I., Schlessinger, D., Ciccodicola, A. and D'Urso, M. (1988) *Nucleic Acids Res.* **16**, 865.

Budowle. B., Chakraborty, R., Giusti, A. M., Eisenberg, A. J. and Allens, R. C. (1991) *Am. J. Hum. Genet.* **48**, 137.

Gecz, J. (1991) *Nucleic Acids Res.* **19**, 5806.

Hamada, H., Siedman, M., Howard, B. H. and Gorman, C. M. (1984) *Mol. Cell. Biol.* **4**, 2610.

Hong, S. S., Suh, J. A., Chae, J. J., Goh, S. H., Kim, U. K. and Lee, C. C. (1993) *Mol. Cell* **3**, 457.

Horn, G. T., Richards, B. and Klinger, K. W. (1989) *Nucleic Acids Res.* **17**, 2140.

Kasai, K., Nakamura, Y. and White, R. (1990) *J. Forensic Sci.* **35**, 1196.

Ludwig, E. H., Friedl, W. and McCarthy, B. J. (1989) *Am. J. Hum. Genet.* **45**, 458.

Nakamura, Y., Carlso, M., Karpcho, K. and white, R. (1988) *Nucleic Acids Res.* **16**, 9349.

Nam, H. S., Kim, E.-H. and Lee, K.-J. (1994) *Korean J. Genetics*, **16**, 343.

Petomaki, P., Aaltonen, L. A., Sistonen, P., Pylkkanen, L., Mecklin, J.-P., Jarvinen, H., Green, J. S., Jass, J. R., Weber, J. R., Leach, F. S., Petersen, G. M., Hamilton, S. R., de la Chapelle, A. and Vogelstein, B. (1993) *Science* **260**, 810.

Sajantila, A., Budowle, B., Strom, M., Johnsson, V., Lukka, M., Peltonen, L. and Ehnholm, C. (1992) *Am. J. Hum. Genet.* **50**, 816.

Sajantila, A., Puomilahti, S., Johnsson, V. and Ehnholm, C. (1992) *Bio Techniques* **12**, 16.

Slightom, J. L., Blrchl, A. E. and Smithies, O. (1980) *Cell* **21**, 627.

Weber, J. A. and May, P. E. (1989) *Am. J. Hum. Genet.* **44**, 388.