DNA Protection by PNA from Enzymatic Digestion for Mass-spectroscopic Genotyping

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By treating single-stranded DNA with nuclease S1 in the presence of peptide nucleic acid (PNA), oligonucleotides of predetermined size were successfully obtained. Mass spectroscopy on these fragments concretely substantiated alteration of one nucleotide to another, if any, in the DNA.

Single nucleotide polymorphisms (SNPs) are common interindividual variations in human genes, and the keys for discovery of disease-responsible genes, pharmacogenetics, and others.[2] Recent completion of human genome project has further enhanced their importance. Among dozens of previous methods for SNPs detection,[3,4] mass spectroscopy has attracted much interest mainly because precise and straightforward diagnosis can be rapidly achieved.[5] However, preparation of short DNA fragments (<15 mer) containing SNP site has been difficult. Here we report a method for SNP detection in which PNA is combined with nuclease S1 (being specific to the hydrolysis of single-stranded DNA).[6–9] Designated DNA fragments are formed from the target DNA in sufficient amounts and concretely genotyped by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS).

The $PNA^{(p)}$ probe in Scheme 1 was synthesized according to the literature and a glycine residue was attached to its C-terminus.[10] The DNA substrates ($DNA^{(s)}$1–4) were prepared on an automated synthesizer and labeled with fluorescein isothiocyanate (FITC) at their 3′-termini.[11] At pH 4.6 (acetate buffer) and 10 °C, the DNA/PNA composites were digested for 30 min with nuclease S1 (from Life Technologies). Then, the mixtures were subjected to denaturing polyacrylamide gel electrophoresis and quantified with a Fuji Film FLA-3000G imaging analyzer.

$PNA^{(p)}$	H_2NCO(Gly)GCTA C TGACC-NH_2
$DNA^{(p)}$	3′- GCTA C TGACC -5′
$DNA^{(s)}$1	5′-CACTAGCGTT CGAT *G* ACTGG (FITC)-3′
$DNA^{(s)}$2	5′-CACTAGCGTT CGAT *C* ACTGG (FITC)-3′
$DNA^{(s)}$3	5′-CACTAGCGTT CGAT *A* ACTGG (FITC)-3′
$DNA^{(s)}$4	5′-CACTAGCGTT CGAT *T* ACTGG (FITC)-3′

Scheme 1. $PNA^{(p)}$ and $DNA^{(p)}$ probes as well as the substrate DNAs ($DNA^{(s)}$1–4). The nucleotide at the 15th position of $DNA^{(s)}$ (in italic) was systematically changed.

The typical electrophoresis patterns are presented in Figure 1. When $DNA^{(s)}$1 was treated with nuclease S1 in the presence of $PNA^{(p)}$, a 9-mer oligonucleotide was predominantly obtained (lane 7). The minor product was a 10-mer fragment. Here, the 3′-side portion of $DNA^{(s)}$1 is complementary with $PNA^{(p)}$, and its 5′-side portion remains single-stranded. Apparently, this single-stranded part was predominantly digested by nuclease S1, and the duplex portion was left intact. As the result, the 10-mer oligonucleotide was formed. Under the reaction conditions employed, the nucleotide in the 5′-terminus of this 10-mer (C in the present case) was further removed by the enzyme, providing the 9-mer fragment (its yield is over 50%). When $DNA^{(s)}$2, $DNA^{(s)}$3, or $DNA^{(s)}$4 was combined with $PNA^{(p)}$ and the DNA/PNA composite involves a mismatch at the 15th position (C-C, A-C, and T-C, respectively), the corresponding 9-mer fragment was also obtained in about 20% yield (lanes 8–10). It is noteworthy that $PNA^{(p)}$ must be used in order to produce the required oligonucleotides. Upon using $DNA^{(p)}$ instead of $PNA^{(p)}$ under the same reaction conditions, all of $DNA^{(s)}$1–4 substrates were completely hydrolyzed to small fragments and neither 9-mer nor 10-mer oligonucleotide was obtained (lanes 3–6). Here, the $DNA^{(p)}$ could not sufficiently protect $DNA^{(s)}$ substrates from the enzymatic digestion so that even the double-stranded portion of $DNA^{(s)}$ was cleaved by the enzyme. The DNA/DNA duplex is less stable than the corresponding PNA/DNA duplex. Furthermore, the DNA probe itself was also susceptible to the digestion by nuclease S1.

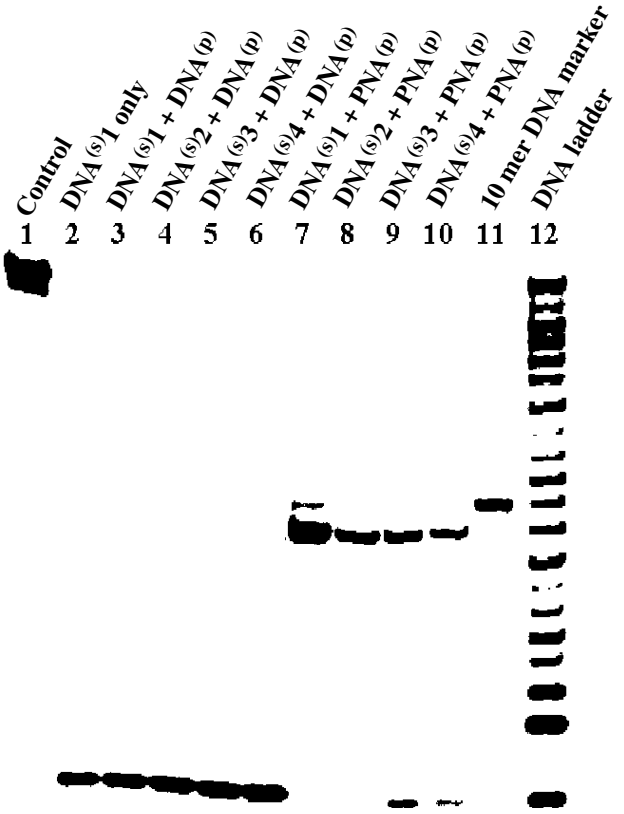

Figure 1. Electrophoresis patterns for the nuclease S1 digestion of $DNA^{(s)}$1–4 in the presence of $PNA^{(p)}$ or $DNA^{(p)}$. Reaction conditions: [$DNA^{(s)}$1–4] = 10 and [$PNA^{(p)}$] = [$DNA^{(p)}$] = 12 μmol/L, and [nuclease S1] = 0.25 unit/μL at pH 4.6 and 10 °C for 30 min (L = dm^3).

The main DNA fragments obtained by the enzymatic digestion were further analyzed by MALDI-TOF MS (see Figure 2).[12] As shown in Table 1, the experimentally determined mass numbers of these fragments were in fair agreements with the values calculated for the corresponding 9-mer fragments which bear the FITC derivative at their 3′-ends. The change of one nucleotide in these oligonucleotides resulted in mass-difference of 9–40, which was exactly identical with the difference of molecular weights of A, G, C, and T. Accordingly, the kind of nucleotide at the 15th position in the substrate DNA was unanimously pinned down by this method, and a single-base discrimination was successfully accomplished. Other kinds of mismatches could be analyzed in a similar way.

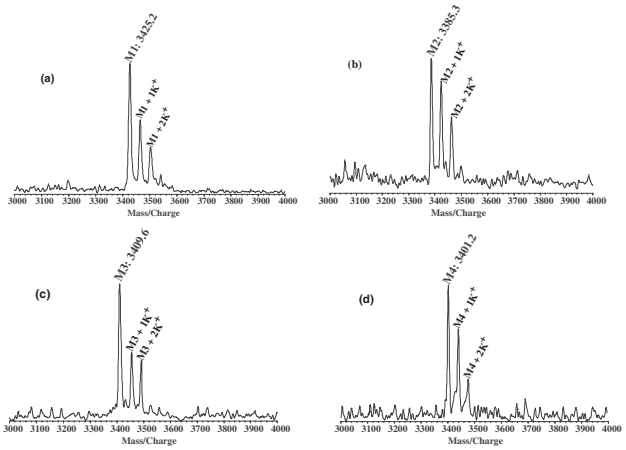

Figure 2. MALDI-TOF MS spectra of the main products of nuclease S1 digestion of $DNA^{(s)}$ in the presence of $PNA^{(p)}$. (a) $DNA^{(s)}1+PNA^{(p)}$ (G-C full-match); (b) $DNA^{(s)}2+PNA^{(p)}$ (C-C mismatch); (c) $DNA^{(s)}3+PNA^{(p)}$ (A-C mismatch); (d) $DNA^{(s)}4+PNA^{(p)}$ (T-C mismatch). Mass spectra were recorded in a negative ion mode. The small peaks next to the major peak correspond to the adducts with K^+ ions.

Table 1. Mass numbers of the fragments obtained by digesting various DNA substrates with nuclease S1 in the presence of $PNA^{(p)a}$

DNA substrate	Calcd	Found[b]
$DNA^{(s)}1$	3424.7	3425.2
$DNA^{(s)}2$	3384.1	3385.3
$DNA^{(s)}3$	3408.2	3409.6
$DNA^{(s)}4$	3399.1	3401.2

[a]The conditions of the enzymatic digestion are presented in the text.
[b]Experimental error in these values is around 2.

In conclusion, oligonucleotides of predetermined size in the target DNA were obtained by combining PNA and nuclease S1. By analyzing these fragments with mass spectroscopy, alteration of only one nucleoside to another was straightforwardly substantiated. Application of this technique to the detection of SNPs in human genomes is currently under way in our laboratory.

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References and Notes

1 S. Ye and X. Liang. equally contributed to this work.
2 D. Altshuler, V. J. Pollara, C. R. Cowles, W. J. Van Etten, J. Baldwin, L. Linton, and E. S. Lander, *Nature*, **407**, 513 (2000).
3 A.-C. Syvänen, *Nat. Rev. Genet.*, **2**, 930 (2001).
4 C. A. Mein, B. J. Barratt, M. G. Dunn, T. Siegmund, A. N. Smith, L. Esposito, S. Nutland, H. E. Stevens, A. J. Wilson, M. S. Phillips, N. Jarvis, S. Law, M. de Arruda, and J. A. Todd, *Genome Res.*, **10**, 330 (2000), and references cited therein.
5 a) L. A. Haff and I. P. Smirnov, *Genome Res.*, **7**, 378 (1997). b) T. J. Griffin, W. Tang, and L. M. Smith, *Nat. Biotechnol.*, **15**, 1368 (1997). c) P. Ross, L. Hall, I. Smirnov, and L. Haff, *Nat. Biotechnol.*, **16**, 1347 (1998). d) T. J. Griffin and L. M. Smith, *Anal. Chem.*, **72**, 3298 (2000). e) X. Y. Sun, H. Ding, K. Hung, and B. C. Guo, *Nucleic Acids Res.*, **28**, e68 (2000). f) M. S. Bray, E. Boerwinkle, and P. A. Doris, *Hum. Mutat.*, **17**, 296 (2001). g) X. Y. Sun, K. Hung, L. Wu, D. Sidransky, and B. C. Guo, *Nat. Biotechnol.*, **20**, 186 (2002). h) J. Stoerker, J. D. Mayo, C. N. Tetzlaff, D. A. Sarracino, I. Schwope, and C. Richert, *Nat. Biotechnol.*, **18**, 1213 (2000).
6 P. E. Nielsen, *Curr. Opin. Struct. Biol.*, **9**, 353 (1999), and references cited therein.
7 A pioneering work on the enzymatic digestion of DNA/PNA composites: V. Demidov, M. D. Frank-Kamenetskii, M. Egholm, O. Buchardt, and P. E. Nielsen, *Nucleic Acids Res.*, **21**, 2103 (1993).
8 Other applications of PNA to SNPs detection: a) P. L. Ross, K. Lee, and P. Belgrader, *Anal. Chem.*, **69**, 4197 (1997). b) P. Jiang-Bancom and J. E. Girard, *Anal. Chem.*, **69**, 4894 (1997).
9 A. Ray and B. Nordén, *FASEB J.*, **14**, 1041 (2000).
10 a) T. Takahashi, K. Hamasaki, A. Ueno, and H. Mihara, *Bioorg. Med. Chem.*, **9**, 991 (2001). b) W. C. Chan and P. D. White, in ''Fmoc Solid Phase Peptide Synthesis: A Practical Approach,'' ed. by W. C. Chan and P. D. White, Oxford University Press, New York (2000), p 41.
11 The 3′-labeled oligonucleotide was synthesized by using 3′-(6-FAM) CPG column (from Glen Research Co.) according to standard phosphoramidite chemistry. All the synthesized PNA and DNA were purified by reversed-phase HPLC and characterized by MALDI-TOF MS.
12 Prior to the mass spectroscopy, the reaction mixtures were desalted by a reversed-phase column (Merck LiChrospher 100 RP-18(e)).