A Sensitive Peptide Nucleic Acid Probe Assay for Detection of *BRAF* V600 Mutations in Melanoma

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Abstract. *Mutated v-Raf murine sarcoma viral oncogene homolog B (BRAF) is an important biomarker for the prediction of therapeutic efficacy of several anticancer drugs. The detection of BRAF mutation faces two challenges: Firstly, there are multiple types of mutations, and secondly, tumor samples usually contain various amounts of wild-type, normal tissues. Here, we describe a newly established method for sensitive detection of multiple types of BRAF V600 mutations in excess wild-type background. The method introduced a fluorophore-tagged peptide nucleic acid (PNA) to serve as both polymerase chain reaction (PCR) clamp and sensor probe, which inhibited the amplification of wild-type templates during PCR and revealed multiple types of mutant signals during melting analysis. We demonstrated the design and optimization process of the method, and applied it in the detection of BRAF mutations in 49 melanoma samples. This PNA probe assay method detected three types of mutations in 17 samples, and was much more sensitive than conventional PCR plus Sanger sequencing.*

Mutated *v-raf* murine sarcoma viral oncogene homolog B1 (*BRAF*) is associated with several malignant diseases, such as melanoma, and colorectal, and ovarian cancer (1). It is also a predictive and prognostic marker for several therapeutic drugs that target epidermal growth factor receptor (*EGFR*) signaling pathway. For example, melanoma and papillary thyroid cancer with *BRAF* V600E mutation are sensitized to the *BRAF* inhibitors vemurafenib and dabrafenib (2); colorectal cancer with this mutation are resistant to *EGFR* inhibitors, including cetuximab, and panitumumab (3, 4). Hence, there is an increasing demand for analysis of *BRAF* mutations before treatment decisions are made.

In the analysis of *BRAF* mutations, two issues need to be considered. Firstly, there are several types of *BRAF* V600 mutations. Although the most prevalent mutations, V600E and V600K, account for the majority of *BRAF* mutations, other uncommon V600 mutations may also be important in treatment prediction and it has been suggested they be included in the test (5, 6). Secondly, the abundance of mutant alleles in a wild-type background can cause problems in mutation detection. In order to accurately detect mutations by most conventional methods, the ratio of mutant alleles must be greater than 10-20% (7). Unfortunately, the mutant ratio in some clinical samples may lower than 10% because of the genetic heterogeneity of tumor tissues and contamination of normal tissues in the samples. Therefore, an ideal assay must be sensitive enough to detect low-abundance mutations and capable of detecting multiple mutation types.

Peptide nucleic acid (PNA) is an artificial DNA analog which has high affinity to complementary DNA, but not to DNA with one or more mismatches. This superior characteristic allows it to be used in PCR to clamp wild-type amplification and enrich mutant products (8). We reported a novel design for tagging PNA oligomers with a fluorescent dye, making them both PCR clamp and sensor probes (9). Here we describe the use of this PNA probe design to establish a simple and sensitive assay for detecting multiple types of *BRAF* V600 mutations in a single tube. We demonstrate the capability of this assay method in melanoma specimens and compare the results with conventional Sanger sequencing.

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Materials and Methods

Primer and probe design. The probe pair included a 5(6)-carboxyfluorescein (FAM)-labeled PNA probe: (FAM)-OO-CGAGATTTCACTGTAGCT (Panagene, Daejeon,Korea) and a *6-Carboxyl-X-Rhodamine* (ROX)-labeled DNA probe: CCAGA CAACTGTTCAAACTGATGGGACCCACTCC-(ROX) (Purigo Biotechnology, Taipei, Taiwan). The forward primer (AGAAA TTAGATCTCTTACC) and the reverse primer (CAGTGGAA AAATAGCC) used in this PNA probe assay was designed following the guidelines described elsewhere (9). In some experiments, the sequences of PCR products from the PNA probe assay were determined using a sequencing primer CCTCAGATATAT TTCTTCATGAA.

PCR and melting analysis. The PCR mixture (20 μl) contained 50 mM Tris-HCl, 3 mM $MgCl_2$, 0.05% bovine serum albumin, 200 μM of each dNTP, 500 nM forward primers, 84-500 nM reverse primer (for asymmetric PCR), 0.5 U Platinum *Taq*, 0.5 μM PNA probe, 0.2 μM DNA anchor probe, and sample templates. PCR and melting analysis were carried out on a LightCycler 2.0 (Roche Diagnostics, Taipei, Taiwan) instrument with the following thermal programs: a short preincubation at 94˚C for 2 min, a 50-cycle amplification (95˚C for 2 s; 68˚C for 5 s; 55˚C for 5 s; and 60˚C for 30 s), and a melting cycle (95˚C for 20 s, rapid cooling to 40˚C, and then slowly increasing temperature from 40˚C to 95˚C at a ramping rate of 0.7˚C/s). The fluorescence signal was measured at 610/530 nm during the melting cycle.

Sensitivity test. The sensitivity of the mutation assay was carried out by analyzing mixed reference DNA. Different amounts of mutant genomic DNA from the HT-29 cell line (with a heterozygous *BRAF* c.1799T>A mutation, correlated to p.V600E) were added to 100 ng genomic DNA from the TSGH cell line (with wild-type *BRAF*), generating mutant percentages ranging from 0.05% to 50%. PCR was performed as described above.

Clinical specimens and analysis. A total of 49 formalin-fixed and paraffin-embedded tissues (FFPET) of melanoma were analyzed. The study was approved by Chang Gung Medical Foundation Institutional Review Board (IRB no.102-3677A3). Genomic DNA from the tissues was extracted by Blood and Tissue Extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Fifty nanograms of sample DNA was analyzed by the PNA probe assay. After PCR and melting analysis, mutation was identified by the appearance of a mutant melting peak. If the mutant sequences needed to be determined, the PCR product was sent for Sanger sequencing using the primer described above.

Results

Design of PNA probe assay. The PNA probe was used as both a PCR clamp and sensor probe. The PNA probe complements the wild-type allele and inhibits its amplification, but has a mismatch with the mutant allele which allows its amplification (Figure 1A). In addition, the PNA was labeled with a donor fluorophore, which can undergo fluorescent resonance energy transfer with an acceptor fluorophore on a DNA anchor probe (Figure 1B).

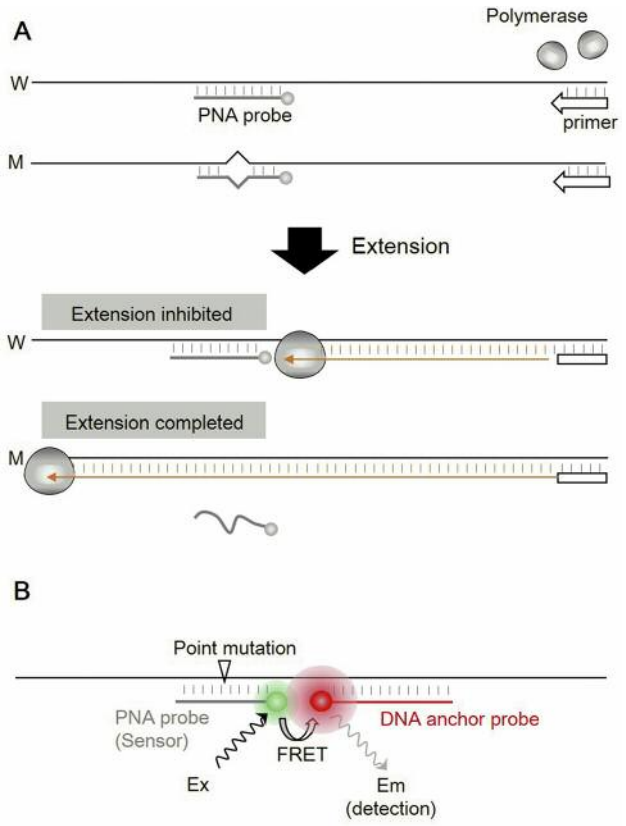

Figure 1. *Principle and design of the peptide nucleic acid (PNA) probe assay. A: The PNA probe serves as a polymerase chain reaction (PCR) clamp to inhibit wild-type amplification but allow the amplification of mutants in PCR. The PNA probe complements perfectly to the wild-type template but has a mismatch to the mutant template (upper panel). During extension, progression of polymerase is hindered by the PNA probe on the wild-type template but not on the mutant template (lower). B: The PNA probe serves as a sensor probe, which couples with a DNA anchor probe to form a pair of hybridization probes for the detection of V600 mutations. Fluorescent resonance energy transfer (FRET) occurs between the fluorophores (green and red circles) labeled on the two probes. W, Wild-type; M, mutant; Ex, excitation; Em, emission.*

The PNA sensor and the DNA anchor, thus form a pair of hybridization probes that can generate characteristic melting peaks for mutant genotypes in melting analysis. Furthermore, in order to accomplish better clamping efficiency, the primer opposite the PNA probe should be farther than 50 nucleotides from the PNA binding site (9). Here, we designed a forward primer at a distance of 137 nucleotides from the PNA-binding site.

Optimization of PNA probe assay. The extension temperature during thermal cycling is one of the key parameters for efficiently clamping wild-type amplification. If PCR were carried out under an extension temperature higher than the

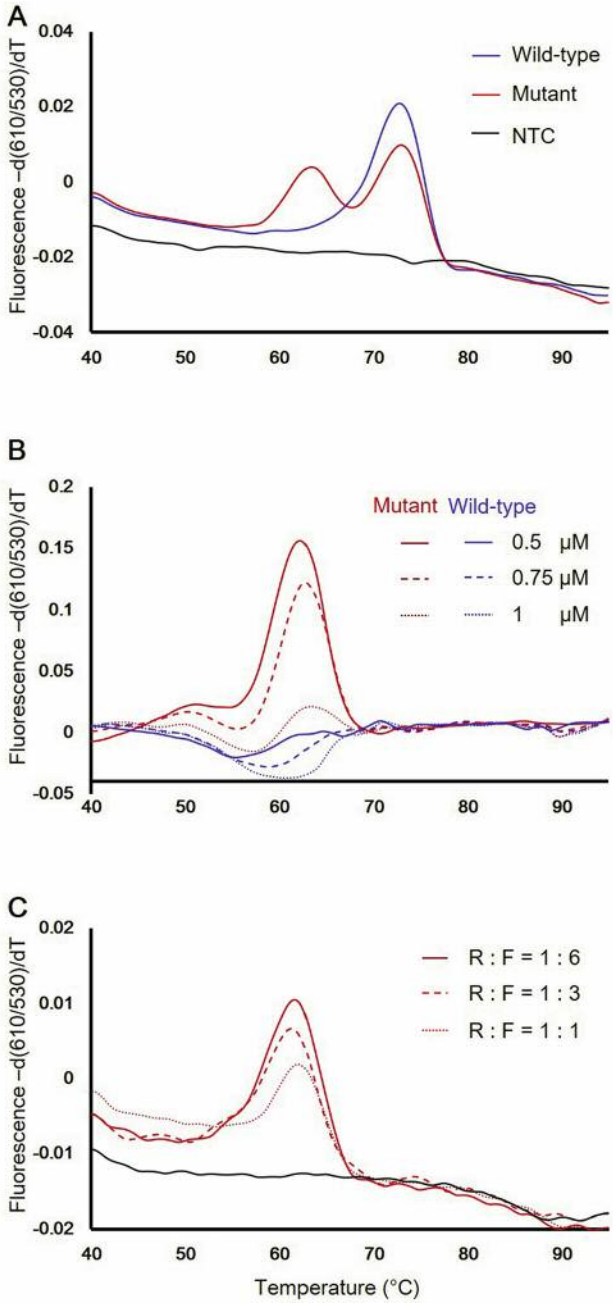

Figure 2. *Optimization of peptide nucleic acid (PNA) probe assay using the control templates from genomic DNAs of TSGH (wild-type BRAF) and HT-29 (heterozygous mutant BRAF) cell lines. A: Non-clamping polymerase chain reaction (PCR) was carried out to allow the amplification of both wild-type and mutant templates. The PCR product from the wild-type template exhibits a melting peak with a melting temperature (Tm) at 73˚C (blue line); that from a heterozygous mutant exhibits two peaks, one with a Tm at 73˚C and the other at 63˚C (red line). B: PNA probe at concentrations of 0.5, 0.75, and 1 μM were tested for their efficiency of wild-type clamping and mutant enrichment under the clamping PCR condition (extension at 60˚C). C: Different ratios of the forward and reverse primers were tested to determine the effect on the melting signal. NTC, No template control.*

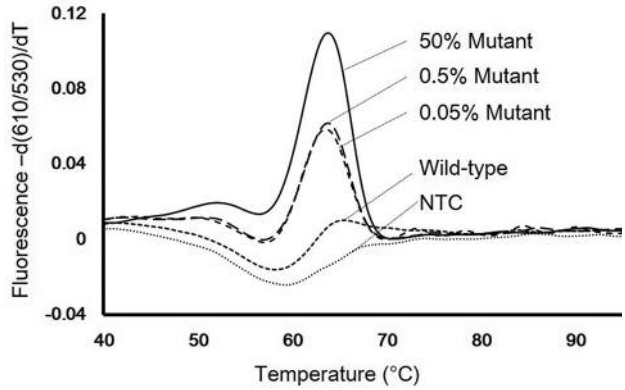

Figure 3. *Sensitivity of peptide nucleic acid (PNA) probe assay for the detection of BRAF V600E mutation. Mixed templates containing different proportions of mutant template in a wild-type background were analyzed.*

optimal condition (*e.g.* 72˚C), both mutant and wild-type templates would be amplified. This non-clamping reaction can be used to observe the association–dissociation status of PNA to different templates. Through the non-clamping reaction with control templates, we found that the PNA probe and the wild-type template had 50% dissociation at 73˚C [the wild-type melting temperature (*T*m), at the peak of the wild-type curve] and maximum association at approximately 62˚C (at the left bottom of the wild-type peak), whereas the PNA probe and the mutant template had 50% dissociation at 63˚C (the *T*m of mutant) (Figure 2A). Therefore, the optimal extension temperature should be around 62˚C. In the later experiments, we set the extension temperature at 60˚C, which allows efficient clamping of extension for wild-type templates but not for mutant templates. Next, different PNA concentrations were tested. We found that PNA probe at 0.5, 0.75, and 1 μM was able to clamp wild-type amplification efficiently, but the mutant signal was reduced when the PNA was higher than 0.5 μM (Figure 2B). Thirdly, asymmetric PCR was tested to determine if it could increase single-stranded templates for probe binding and thus enhance the melting signal. We found that the mutant had the strongest melting peak when using forward and reverse primers at a ratio of 6:1 (Figure 2C).

Sensitivity of the PNA probe assay. To demonstrate the sensitivity of our method, a control experiment was conducted using mixed templates consisting of different mutant DNA ratios in a wild-type background. Under optimal PCR conditions, the wild-type DNA amplification was completely inhibited, and only the reactions containing detectable mutant templates generated a melting peak. The result indicated that the assays for *BRAF* V600E detected as

Table I. *Comparison of the results of detection of BRAF V600 mutations using peptide nucleic acid (PNA) probe assay and conventional method (traditional polymerase chain reaction plus Sanger sequencing).*

Genotype	PNA probe assay, n=49	Conventional method, n=49
V600E	13	10
V600K	1	1
V600M	3	0
Wild-type	32	38

few as 0.05% mutants, which generated marked mutant melting peaks. In contrast, the reaction containing only the wild-type template generated no peaks (Figure 3).

Clinical application to melanoma specimens. The feasibility of our PNA probe assay was tested on 49 FFPET samples from melanoma. *BRAF* mutations in the samples were analyzed by a conventional method (traditional PCR plus Sanger sequencing) and by our PNA probe assay. The conventional method detected mutations in 11 samples, including 10 of V600E, and one of V600K. Our PNA probe assay detected mutations in 17 samples, including all those detected by the conventional method plus an additional six mutations (Table I). To verify the results of the PNA probe assay, PCR products of the assay were sent for Sanger sequencing. Since the mutant templates were enriched after PNA probe assay, the Sanger sequencing clearly revealed the accurate mutation signals. The results confirmed the mutations identified by the PNA probe assay, which included 13 of V600E, three of V600M, and one of V600K. The results indicated that our PNA probe assay was more sensitive than the conventional method, and that the assay was able to successfully detect at least three types of V600 mutations. Typical results of the melting curve of the PNA probe assay and their confirmation sequences are shown in Figure 4.

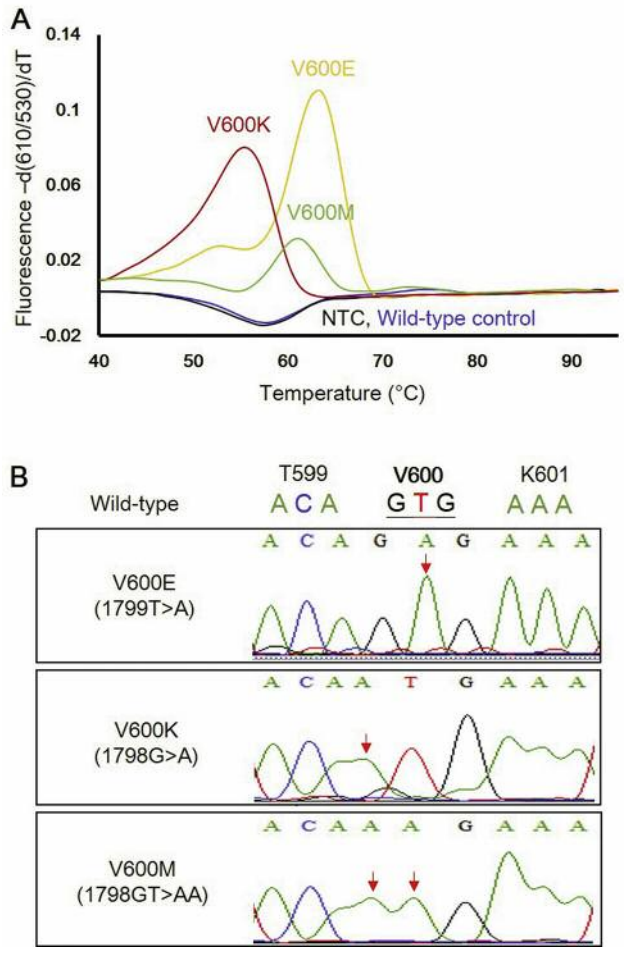

Figure 4. *Typical results of analysis of BRAF in three melanoma samples with different V600 mutations. A: The melting analysis. B: Confirmation by Sanger sequencing of polymerase chain reaction products from the peptide nucleic acid probe assay. The red arrows indicate the mutation nucleotides.*

Discussion

We demonstrated the development of a PNA probe assay for *BRAF* V600 mutation and applied it to melanoma samples. The assay detected multiple mutation types, and was found to be more sensitive at detecting mutations in a wild-type background than conventional methods. In addition, the assay has a simple design, with a single primer and probe set, which reduces cost and simplifies the optimization procedures.

Several types of *BRAF* mutation were reported, mostly having single or double nucleotide changes. Among them, *BRAF* V600E and V600K mutations account for the highest prevalence of all *BRAF* mutations and are the common targets of molecular tests (10). However, some less common mutations, such as V600R and V600M, are also important in treatment selection (5, 6). Methods using specific antibodies (*e.g.* immunohistochemistry) (11), specific primers (12), or specific probes (*e.g.* allele-specific, hydrolysis probes) (13) usually need multiple reactions to detect different types of mutations. Although multiplex antibodies, primers and probes can be added in a single reaction, they may interfere with each other and lead to complications in optimization. In comparison, our PNA probe assay is the simplest design and easy to set up. Since the PNA probe is complementary to the wild-type sequence, any variation in the PNA region covered can be enriched and revealed in the melting analysis.

In the melanoma samples, our PNA probe assay detected more mutants than did conventional PCR plus Sanger sequencing. Similar results have been reported using other sensitive detection methods in several types of cancer (14-17). This is because traditional PCR plus Sanger sequencing can only detect mutant alleles with a presence greater than 10-20% in the wild-type background (18, 19); however, clinical specimens may contain a mutant allele at a lower frequency. The reason for this is that cancer tissues are genetically heterogeneous and contaminated with various amounts of normal tissue (20, 21). A previous study has shown that in different dissections of FFPET samples, the mutant DNA percentage ranged from 4.9% to 81.2% (22). These facts suggest that a sensitive method is required for mutation detection in melanoma and other types of cancer.

The sensitivity of the assay depends on the clamping efficiency during PCR. The factors determine clamping efficiency include primer position and extension temperature (9, 23). In the current study, we demonstrated how to use melting analysis to optimize extension temperature. Through the melting analysis, the association–dissociation status of the PNA probe with different templates can be observed. Figure 2A shows the melting peak of the wild-type template had a peak at 73˚C and a trough (left) at 62˚C. This means at a temperature below 62˚C, the PNA probe has maximal binding with the wild-type template and conducts maximal clamping of wild-type amplification. In contrast, the mutant had a peak at 63˚C (the mutant *T*m), indicating that only half of the templates were associated with PNA. Loosely associated PNA can be repelled easily by the *Taq* polymerase. Therefore, the extension temperature at around 62˚C allows amplification of mutant templates but not wild-type templates. In addition to extension temperature, we found that asymmetric PCR enhance the melting signals. This may be because the excess forward primers generate single-strand templates, which allow more probes to bind to the templates without the competition from the antisense strand. We found that an ideal ratio of forward to reverse primers was 6:1. Further increase of the ratio reduced PCR efficiency and led to a decrease of mutant signal.

The PNA probe assay can be completed in a single tube on a real-time PCR instrument. Mutations can be identified through the appearance of melting peaks without the need for further manipulation. Although different mutations have different melting profiles, the resolution of melting curves is generally not good enough to distinguish each specific mutation type. Fortunately, the PNA probe assay only enriches the mutant templates but does not change them. The mutant PCR products can be further sequenced to determine their mutation types, although it is not necessary for the purpose of differentiating mutants from wild-type. In the present study, we demonstrated the sequencing results of three mutant PCR products. The sequencing curves are clear, as the wild-type background has been eliminated in the clamping PCR.

Recently, detection of gene mutations in body fluids has attracted attention, as obtaining specimens from body fluids, in so-called liquid biopsies, is relatively non-invasive and can be used to monitor disease progression or treatment efficacy (24). In body fluids, disease-derived nucleic acid is rare, compared to normal DNA. The presented PNA probe assay has good sensitivity and may serve as a powerful tool in the application of liquid biopsies.

Acknowledgements

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