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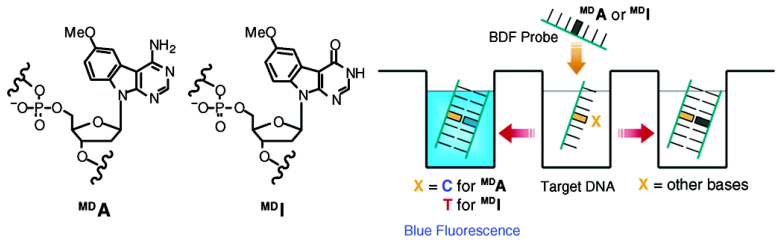

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Design of Base-Discriminating Fluorescent Nucleoside and Its Application to T/C SNP Typing

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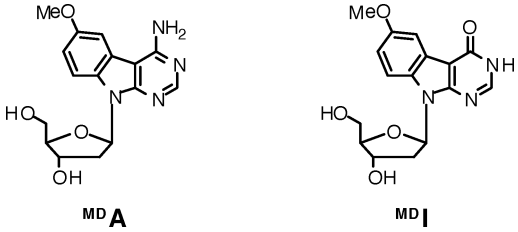
The typing of single base alterations, such as single nucleotide polymorphisms (SNPs), using DNA probes is a rapidly developing area. Most of the presently available methods utilize the difference in hybridization efficiency between the target DNA and the probe oligodeoxynucleotides (ODNs),[1] or the difference in enzymatic recognition between full-matched and mismatched duplexes.[2] However, there are still problems, such as hybridization errors, the high cost of enzymes, and the time-consuming steps required. Thus, it is highly desirable to develop an alternative method for SNP typing that can easily determine single base alterations at target sites.



Here, we report a conceptually new method for the fluorescence assay of a single base alteration (Figure 1). We devised novel fluorescent oligonucleotide probes that contain base-discriminating fluorescent (BDF) nucleosides, methoxybenzodeazaadenine ^{MD}A and methoxybenzodeazainosine ^{MD}I, which emit strong fluorescence only when the base on the complementary strand is C and T, respectively. Thus, the ^{MD}A- and ^{MD}I-containing ODNs can be used as a very effective BDF probe for the detection of single base alterations, such as SNPs and point mutations.[3]

The syntheses of the novel fluorescent nucleosides, ^{MD}A and ^{MD}I, were readily achieved in two steps from 4-chloro-6-methoxy-1*H*-pyrimido[4,5-*b*]indole.[4,5] Subsequently, nucleosides were protected and incorporated via the phosphoramidites into ODN, using a DNA synthesizer. The ODNs used in this study are summarized in Table 1.

The absorption maxima for ^{MD}A and ^{MD}I were initially observed at 327 nm (ϵ 2700) and 315 nm (ϵ 7800), respectively, where natural nucleosides have no absorption. Thus, ^{MD}A and ^{MD}I can be selectively excited with UV light above 300 nm. With excitation of ^{MD}A at 330 nm, we observed strong fluorescence ($\Phi = 0.118$) at 397 and 427 nm. For ^{MD}I, strong fluorescence was observed at 442 nm ($\Phi = 0.117$) with an excitation wavelength of 320 nm.

Next, the fluorescence of ^{MD}A-containing ODN (**ODN(^{MD}A)**) and ^{MD}I-containing ODN (**ODN(^{MD}I)**) was examined. In contrast to the strong fluorescence of ^{MD}A and ^{MD}I, the fluorescence intensities of their single-stranded ODNs and the "full-matched" duplexes with complementary strands (i.e., the strands containing T base opposite ^{MD}A and C base opposite ^{MD}I) were very weak (Figure 2a and 2b).[6] However, the fluorescence spectrum of the "mismatched" duplex **ODN(^{MD}A)/ODN(C)** showed a strong fluorescence at 424 nm ($\Phi = 0.081$). It is noteworthy that the fluorescence of **ODN(^{MD}A)/ODN(C)** was 100 times stronger than that observed for **ODN(^{MD}A)/ODN(T)**. In contrast, when **ODN(^{MD}I)** was hybridized with the complementary strand **ODN(T)**, a relatively strong fluorescence was observed at 424 nm ($\Phi = 0.011$) that was 5.5 times stronger than that observed for **ODN(^{MD}I)/ODN(C)**. For the mismatched duplexes containing purine bases opposite ^{MD}A and ^{MD}I, the fluorescence intensities were much less than those of **ODN(^{MD}A)/ODN(C)** and **ODN(^{MD}I)/ODN(T)**.

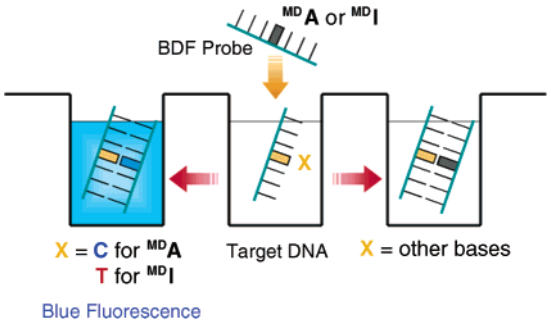


Figure 1. Schematic illustration of a new homogeneous SNP typing method using ^{MD}A- and ^{MD}I-containing base-discriminating fluorescent (BDF) probes.

Table 1. Oligodeoxynucleotides (ODNs) Used in This Study

	sequences
ODN(^{MD}A)	5′-d(CGCAAT**^{MD}A**TAACGC)-3′
ODN(^{MD}I)	5′-d(CGCAAT**^{MD}I**TAACGC)-3′
ODN(T)	5′-d(GCGTTA**T**ATTGCG)-3′
ODN(C)	5′-d(GCGTTA**C**ATTGCG)-3′
ODN(A)	5′-d(GCGTTA**A**ATTGCG)-3′
ODN(G)	5′-d(GCGTTA**G**ATTGCG)-3′
ODN$_{BRCA1}$(^{MD}A)	5′-d(GGTACCA**^{MD}A**TGAAATA)-3′
ODN$_{BRCA1}$(^{MD}I)	5′-d(GGTACCA**^{MD}I**TGAAATA)-3′
ODN$_{BRCA1}$(T)	5′-d(TATTTCA**T**TGGTACC)-3′
ODN$_{BRCA1}$(C)	5′-d(TATTTCA**C**TGGTACC)-3′

The fluorescence spectra of **ODN(^{MD}A)/ODN(C)** and **ODN(^{MD}I)/ODN(T)** extended to 550 nm, as is shown in Figure 2a and 2b. Thus, the fluorescence emission from these solutions was visible to the human eye and clearly distinguishable from the solution of duplexes containing other base pairs (Figure 2c and 2d). The hybridization of BDF probes, **ODN(^{MD}A)** and **ODN(^{MD}I)**, with a target DNA facilitates the clear discrimination with the naked eye of C and T, respectively, located at a specific site of the target DNA.

Having established the fluorescence character of the ^{MD}A- and ^{MD}I-containing BDF probes, we tested the SNP detection of the T/C SNP sequence of the human breast cancer 1 gene (BRCA1)[7] by means of BDF probe hybridization. BDF probe, **ODN$_{BRCA1}$(^{MD}A)** or **ODN$_{BRCA1}$(^{MD}I)**, was mixed with a sample solution of the target sequence, **ODN$_{BRCA1}$(T)**, **ODN$_{BRCA1}$(C)**, or a 1:1 mixture

10.1021/ja035408l CCC: $25.00

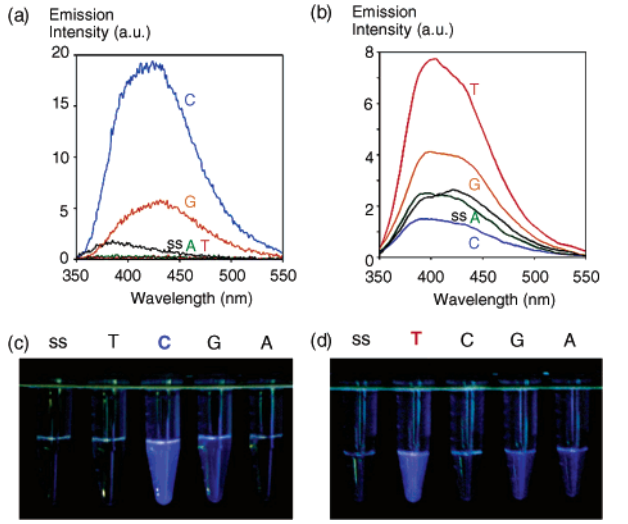


Figure 2. (a) Fluorescence spectra of 25 μM **ODN(^{MD}A)** hybridized with 25 μM **ODN(T)**, **ODN(C)**, **ODN(G)**, or **ODN(A)** (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). Excitation was at 330 nm. (b) Fluorescence spectra of 25 μM **ODN(^{MD}I)** hybridized with 25 μM **ODN(T)**, **ODN(C)**, **ODN(G)**, or **ODN(A)** (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). Excitation was at 330 nm. (c and d) Comparison of the fluorescence for the bases opposite ^{MD}A and ^{MD}I, respectively (25 μM strand concentration, 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). "ss" denotes a single-stranded BDF probe. The sample solutions were illuminated with a 312 nm transilluminator.

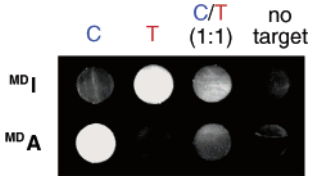


Figure 3. Determination of the T/C allele type of BRCA1 using the fluorescence change of BDF probes, **ODN$_{BRCA1}$(^{MD}A)** and **ODN$_{BRCA1}$(^{MD}I)**. A volume of 25 μM **ODN$_{BRCA1}$(C)** or **ODN$_{BRCA1}$(T)** was hybridized with 25 μM BDF probes (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). Fluorescence was observed using a fluorescence imager Versa Doc Imaging System (BioRad) equipped with a 290−365 nm transilluminator. The image was taken through a 380 nm long pass emission filter.

of **ODN$_{BRCA1}$(T)** and **ODN$_{BRCA1}$(C)**, to mimic the heterozygous state, and the fluorescence of the mixture was immediately read at room temperature with a fluorescence imaging instrument (Figure 3). As a result of the hybridization of BDF probes with **ODN$_{BRCA1}$(T)**, a strong emission was obtained for the addition of **ODN$_{BRCA1}$(^{MD}I)**, whereas the emission from the **ODN$_{BRCA1}$(^{MD}A)/ODN$_{BRCA1}$(T)** duplex was negligible. In contrast, for a sample solution containing **ODN$_{BRCA1}$(C)**, the addition of **ODN$_{BRCA1}$(^{MD}A)** showed a strong fluorescence, whereas a very weak fluorescence was observed for **ODN$_{BRCA1}$(^{MD}I)**. When BDF probes were added to a 1:1 mixture of **ODN$_{BRCA1}$(T)** and **ODN$_{BRCA1}$(C)**, a weak fluorescence emission was observed for both BDF probes and was clearly distinguishable from those of homozygous samples. Therefore, the present method using a combination of ^{MD}A- and ^{MD}I-containing BDF probes constitutes a very powerful tool for T/C SNP typing, although the general utility of our method is limited by the flanking base pairs of ^{MD}A and ^{MD}I.[8]

In summary, we have devised a simple method for the detection of single nucleotide alteration by exploiting novel BDF probes. We designed new base-discriminating fluorescent nucleosides ^{MD}A and ^{MD}I, which can distinguish C and T, respectively, from other bases opposite the fluorescent base. The present SNP typing method using ^{MD}A- and ^{MD}I-containing BDF probes is a very powerful homogeneous assay that does not require enzymes or time-consuming steps, and avoids hybridization errors. In addition, a combination of these ^{MD}A- and ^{MD}I-containing BDF probes facilitates the T/C SNP typing of a heterozygous sample. Further work on the mechanistic aspects and more effective BDF probes is in progress in our laboratory.

Acknowledgment. We thank Drs. M. Iida and T. Kato (Otsuka Pharmaceutical Co., Ltd.) for valuable discussions on the SNP studies.

Supporting Information Available: Detailed experimental data on ^{MD}A, ^{MD}I, and the related ODNs (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) Fluorescence quantum yields (Φ). A single-stranded **ODN(^{MD}A)**, 0.005; **ODN(^{MD}A)/ODN(T)**, <0.0005; **ODN(^{MD}A)/ODN(C)**, 0.081; **ODN(^{MD}A)/ODN(G)**, 0.020; **ODN(^{MD}A)/ODN(A)**, 0.0006; a single-stranded **ODN(^{MD}I)**, 0.006; **ODN(^{MD}I)/ODN(C)**, 0.002; **ODN(^{MD}I)/ODN(T)**, 0.011; **ODN(^{MD}I)/ODN(G)**, 0.007; **ODN(^{MD}I)/ODN(A)**, 0.003. The fluorescence quantum yields were calculated according to: Morris, J. V.; Mahaney, M. A.; Huber, J. R. *J. Phys. Chem.* **1976**, *80*, 969−974.

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(8) When the flanking base pair was a G/C base pair, then the fluorescences of ^{MD}A and ^{MD}I were considerably suppressed. Thus, the SNP typing method would be inaccurate for the sequence containing a G/C base pair flanking SNP site.

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