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DNA microarrays on a dendron-modified surface improve significantly the detection of single nucleotide variations in the p53 gene

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ABSTRACT

Selectivity and sensitivity in the detection of single nucleotide polymorphisms (SNPs) are among most important attributes to determine the performance of DNA microarrays. We previously reported the generation of a novel mesospaced surface prepared by applying dendron molecules on the solid surface. DNA microarrays that were fabricated on the dendron-modified surface exhibited outstanding performance for the detection of single nucleotide variation in the synthetic oligonucleotide DNA. DNA microarrays on the dendron-modified surface were subjected to the detection of single nucleotide variations in the exons 5–8 of the p53 gene in genomic DNAs from cancer cell lines. DNA microarrays on the dendron-modified surface clearly discriminated single nucleotide variations in hotspot codons with high selectivity and sensitivity. The ratio between the fluorescence intensity of perfectly matched duplexes and that of single nucleotide mismatched duplexes was >5–100 without sacrificing signal intensity. Our results showed that the outstanding performance of DNA microarrays fabricated on the dendron-modified surface is strongly related to novel properties of the dendron molecule, which has the conical structure allowing mesospacing between the capture probes. Our microarrays on the dendron-modified surface can reduce the steric hindrance not only between the solid surface and target DNA, but also among immobilized capture probes enabling the hybridization process on the surface to be very effective. Our DNA microarrays on the dendron-modified surface could be applied to various analyses that require accurate detection of SNPs.

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are distributed throughout the human genome and implicated in genetic disorders and disease susceptibility (1). DNA microarray techniques allow parallel analysis of multiple DNA target samples and can be applicable to gene expression profiling and gene mutation analysis. Even if DNA microarray technology is relatively well established for gene expression profiling (2–4), accurate analyses of genetic mutation by DNA microarrays (5) are still in an early stage because even SNPs that are the most suitable targets for DNA microarray analysis are detected with relatively poor accuracy.

Poor accuracy of DNA microarray for the detection of SNPs or single base mutation is considered to originate from inherent properties of the surface and molecular interlayer structures that are not well characterized. A mixed monolayer self-assembled on gold surface increased the hybridization efficiency significantly (6) and a space-controlled gold surface improved the efficiency of an alpha-helix formation for immobilized oligopeptides (7). Therefore, surface characteristics is one of the critical major elements to determine the performance of microarray. Fabricating solid surface that can provide excellent SNPs discrimination efficiency could be a breakthrough in gene mutation analysis by DNA microarray technology.

Previously, we reported the preparation of surface materials allowing the mesospacing between capture probes by introducing a conical-shaped dendron that could provide enough space for unhindered interactions between biomolecules (8). We also observed that a glass substrate modified with the dendron could significantly reduce non-specific binding and enhance selectivity of DNA microarrays: The observed selectivity was equivalent to that observed in solution for detecting single point mutation in synthetic oligonucleotide target DNA (9). This high SNPs discrimination efficiency was found to be largely related to the characteristics of the

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dendron-modified surface that guaranteed mesospacing among the probe DNAs.

In biological system, the p53 gene plays key roles as a tumor suppressor in various biological events, such as cell cycle regulation, gene transcription, DNA repair, genomic stability, chromosomal segregation and apoptosis (10–12). The loss of wild-type function of the p53 gene could lead to cancers and was the most frequently mutated gene in a variety of human cancers (12–14). For example, 70 and 60% of lung and colon cancers were found to be related to p53 mutations. The p53 GeneChip®, which was developed by Affymetrix, showed reasonable accuracy for detecting certain mutations in the p53 gene in various cancers (15–19). However, the p53 Gene Chip® still needs improvement in order to be widely adopted for the accurate and reliable analysis of single nucleotide mutations in the p53 gene. Creating new surface is essential to improve the detection accuracy because the accuracy seems to be strongly related to the properties of surface on which DNA microarrays are fabricated.

In order to investigate the performance of the dendron-modified surface characteristics for the detection capability of DNA microarrays in real biological samples, we applied our dendron-modified surface methodology for the detection of single nucleotide variations in the p53 gene from cancer cell lines. DNA microarrays on the dendron-modified surface showed high selectivity and sensitivity, confirming that optimization of surface characteristics could be very important to fabricate DNA microarrays with enhanced performance.

MATERIALS AND METHODS

All chemicals were reagent grade or of better quality than reagent grade and used as received unless mentioned. Ultrapure water (18 MΩ/cm) was obtained from a Milli-Q purification system (Millipore, USA). Aldehyde-coated glass slides were purchased from Telechem International (USA). Bare glass slides were obtained from Corning (USA).

Capture probe oligonucleotide

The capture probe sequences were designed by the use of the Array Designer software (PREMIER Biosoft International, USA). Amine-modified capture probe oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany) and Bionics (Korea). Capture oligonucleotides used in this study are listed in Table 1.

Preparation of the dendron-modified surface

The di(*N*-succinimidyl)carbonate (DSC)-activated dendron-modified surface was prepared according to the procedure as described previously (9).

Preparing DNA microarrays

Amine-tethered capture probe oligonucleotides were immobilized on the DSC-activated dendron-modified surface of the glass slide by spotting the solution in a buffer containing 25 mM sodium bicarbonate, 5 mM $MgCl_2$ and 10% (v/v) dimethyl sulfoxide at pH 8.5 using a microarrayer (Cartesian Technologies, Microsys 5100) in a clean room (class 10 000). After spotting the probe oligonucleotides side by side in a 10×1 format, the microarray was incubated in a chamber maintained at ~85% humidity for overnight to give the amine-tethered DNA sufficient reaction time. Slides were then stirred in a buffer solution containing 2× SSPE (0.30 M sodium chloride, 0.020 M sodium hydrogen phosphate and 2.0 mM EDTA), pH 7.4 and 7.0 mM SDS at 37°C for 1 h and then in boiling water for 5 min to remove non-specifically bound oligonucleotides. Finally, the DNA-functionalized microarray was dried under a stream of nitrogen for the subsequent hybridization. Different kinds of probes were spotted in a single plate. DNA microarrays on the aldehyde plates (Telechem International, SMA) were prepared according to the supplier's protocol.

Genomic DNA samples

Genomic DNAs of SNU-cell lines (SNU-475 and 761) were kindly provided by Prof. Ja-Lok Ku at Korean Hereditary Tumor Registry, Seoul National University. SNU-cell lines are human carcinoma cell lines deposited at the Korean Hereditary Tumor Registry.

Subcloning and sequencing

The DNA segment from the exon 5 to the exon 8 in the p53 gene of each cell line was amplified by PCR with two kinds of primer pairs: a forward primer, Fwd I (5′-CTG ACT TTC AAC TCT GTC TCC T-3′) or Fwd II (5′-TAC TCC CCT GCC CTC AAC AA-3′) and a reverse primer, Rev I (5′-TGC ACC CTT GGT CTC CTC CAC-3′) or Rev II (5′-CTC GCT TAG TGC TCC CGG G-3′). Each genomic DNA was amplified in the 20 µl solution containing 10 pmol of the primer pair, Fwd I and Rev I, 250 µM dNTP mixture, 2.5 U *Taq* polymerase

Table 1. Capture oligonucleotides used in this study

No	Probe name	Exon	Sequence[a] (5′→3′)	Nucleotide
1	175	5	GTTGTGAGGCNCTGCCCC N = G (wt), A, T, C (mt)	18
2	215	6	TTTCGACATANTGTGGTGGTG N = G (wt), A, T, C (mt)	21
3	216	6	TCGACATAGTNTGGTGGTGCC N = G (wt), A, T, C (mt)	21
4	239	7	CATGTGTNACAGTTCCTGCA N = A (wt), G, T, C (mt)	20
5	248	7	CATGAACNGGAGGCCCATC N = C (wt), A, T, G (mt)	19
6	273	8	TTGAGGTGCNTGTTTGTGC N = G (wt), A, T, C (mt)	19
7	282	8	GAGAGACNGGCGCACAG N = C (wt), A, T, G (mt)	17
8	175-T30	5	$(T)_{30}$-GTTGTGAGGCNCTGCCCC N = G (wt), A, T, C (mt)	48
9	239-T30	7	$(T)_{30}$-CATGTGTNACAGTTCCTGCA N = A (wt), G, T, C (mt)	50

The sequences underlined represent the codons as numbered under 'Probe name'. wt, wild type; mt, mutant type.
[a]The oligonucleotides have an amino group at the 5′ end.

(Takara, Japan) in 1× buffer (supplemented with *Taq* polymerase) in a thermocycler (Hybaid, Multiblock System, UK). The reaction cycles were programmed as follows: an initiation activation of the polymerase at 95°C for 1 min, subsequent 20 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 90 s, followed by a final elongation step at 72°C for 5 min. The amplified PCR products of genomic DNA were diluted 20-fold and subjected to the second nested PCR with the same reaction conditions using the second primer pair, Fwd II and Rev II. After separated on agarose gel and purified by a gel extraction kit (Qiagen, USA), the amplified DNAs were subcloned into pGEM T-easy vector (Promega) and used to transform *Escherichia coli* DH5α. The subcloned plasmid was purified by the use of a Plasmid Mini kit (Qiagen) for the determination of nucleotide sequences. Bidirectional sequencing was performed using pUC/M13 sequencing primers with the sequences, 5′-GTT TTC CCA GTC ACG ACG TTG-3′ and 5′-TGA GCG GAT AAC AAT TTC ACA CAG-3′, respectively.

Preparation of target DNA

The amplified plasmid DNA was digested with EcoRI to release the exons 5–8 region of the p53 gene. The insert DNA was separated on agarose gel and purified by the use of a gel extraction kit (Qiagen). Target DNAs spanning SNPs sites were random primed and labeled in the 20 μl solution containing 50 ng of template DNA with 50 U Klenow enzyme (NEB), 1× EcoPol buffer supplemented with Klenow enzyme, 6 μg of random octamer (Bionics, Korea), low dT dNTP mixture (100 μM dATP, 100 μM dGTP, 100 μM dCTP and 50 μM dTTP) and 50 μM Cyanine3-dUTP (NEN) at 37°C for 2 h. Unincorporated nucleotides were removed using a QIAquick PCR purification kit (Qiagen). After assessing the specific activity and the number of nucleotides per incorporated fluorescent dye by UV spectrophotometry, aliquots of the labeled DNA were subjected to hybridization.

Hybridization

Hybridization was performed in the hybridization buffer containing 30 nM target DNA tagged with Cy3 fluorescent dye for 1 h at 50°C, 1 h at 47°C and 2 h at 45°C and then washed at room temperature in a buffer containing 1× SSC and 0.1% SDS, 0.1× SSC and 0.1% SDS, and 1× SSC respectively, in a hybridization station (Genomic solutions, GeneTAC).

Fluorescence scanning and signal quantitation

The image acquisition and the fluorescence intensity analysis were carried out by the use of a confocal laser scanner (GSI Lumonics, ScanArray Lite) and a quantitative microarray analysis software (BioDiscovery, ImaGene).

RESULTS AND DISCUSSION

Design of capture probes and preparation of target DNAs

Codons, 175, 215, 216, 239, 248, 273 and 282 in the p53 gene, which have been known as missense mutational hotspots among cancer patients, were selected for this study. Codons 175, 248, 273 and 282 were chosen from the international IARC TP53 mutation database (20), and other codons 215, 216 and 239 found very frequently among Korean cancer patients were selected from the p53 mutational hotspot database in the Korean Hereditary Tumor Registry. The amino acid encoded by the codon 175 occurs in the L2 loop in the vicinity of the zinc binding site and plays a critical role in stabilizing the L2 and L3 loops. The two amino acids encoded by the codons 248 and 273 interact directly with the minor groove and the backbone phosphate of DNA, respectively. The codon 282 encodes a residue that plays a structural role in the loop–sheet–helix motif (21).

The capture probe sequences designed for the detection of seven codons were 18–21 nt long depending on the sequences around the respective codon and their calculated T_m values were ~55°C (Table 1). Four capture probes, one perfectly matched and three single-nucleotide-mismatched probes were prepared to investigate the selective detection of each codon. All the probe oligonucleotides have a terminal amine group required for the covalent attachment to the DSC-activated dendron surface or the aldehyde surface. Cy3-incorporated target DNAs were prepared by the random priming (22) of the exons 5–8 region in the p53 gene, which had been amplified by PCR of the genomic DNA obtained from cancer cell lines, SNU 761 and 475. The size of random primed target DNAs was found to be 100–200 nt and the incorporation ratio was ~80 bases per dye molecule. SNU 761 has no mutation in exons 5–8, and SNU 475 has a single point mutation in the codon 239 (AAC→GAC). The nucleotide sequence of each PCR product was confirmed before random priming.

Preparation of the dendron-modified surface

The structure of the dendron, which is a second generation dendrimer with reactive functional groups at the branch termini, is shown in Figure 1. The dendron-modified surface was prepared by self-assembling dendron molecules on a slide (9). Each reaction step was confirmed by both UV spectrophotometry and atomic force microscopy. High-resolution scanning

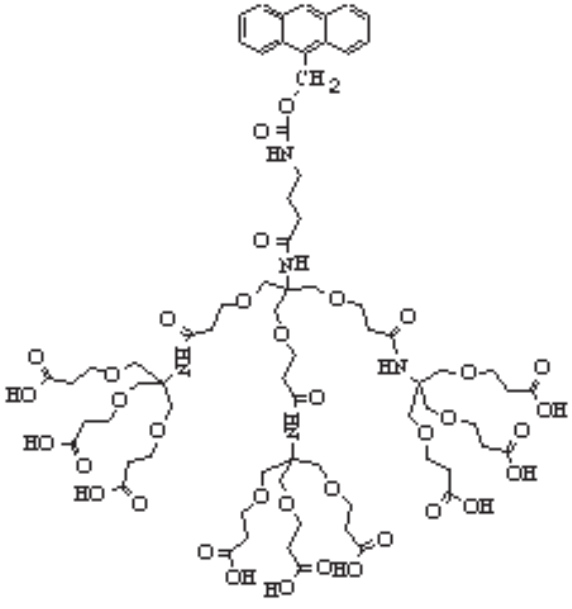

Figure 1. Structure of the dendron molecule.

electron microscope (HRSEM) image showed that the surface manifests the mesospacing of 3.2 nm in average between the dendrons. Tapping mode AFM image also showed that the resulting layer was smooth and homogeneous without any aggregates or holes (9).

Selectivity of the DNA microarrays on the dendron-modified surface

The selectivity indicating the capability of discriminating single nucleotide variation is one of the most important factors to determine the performance of DNA microarrays. In order to investigate the selectivity of DNA microarrays on the dendron-modified surface, single nucleotide variations in seven hotspot codons of the p53 gene were simultaneously examined.

After 28 kinds of capture probes that have all the possible nucleotide sequences at seven codons were spotted on a single slide, 30 nM target DNA for SNU 761 was hybridized. The matched sequence of each codon in SNU 761 was CGC, AGT, GTG, AAC, CGG, CGT and CGG for the codons 175, 215, 216, 239, 248, 273 and 282, respectively; underlined: the mismatched nucleotides. The fluorescence images of all seven codons after hybridization are shown in Figure 2. Although the fluorescence intensity was different from codon to codon, all matched sequences of seven codons exhibited invariably strong fluorescence intensity. There is a drastic difference in the intensity between the matched and mismatched pairs. The normalized fluorescence signal ratio, i.e. intensity for one base mismatched pair to that for the perfectly matched pair, MM/PM, ranged from 0.007 to 0.16 when the relative intensities were assessed from 10 independent measurements of the fluorescence intensities (Table 2). We also observed that the DNA microarrays on the dendron-modified surface discriminated single nucleotide variations with high selectivity factor of <0.01 in synthetic oligonucleotide target DNAs (9). These discriminating efficiencies obtained in both synthetic and real target DNAs are unprecedentedly high because selectivity factors of 0.19–0.57 were observed previously for microarrays fabricated on various amine surfaces, including a mixed self-assembled monolayer for the detection of single nucleotide variations even in short synthetic oligonucleotide target DNAs (23).

We also investigated the dependence of discrimination efficiency for the detection of single nucleotide variations on the concentrations of target DNAs. Interestingly, it was observed that high discrimination efficiency (<0.10) as judged by the signal intensity ratio (MM/PM) of codon 273 was maintained in the broad range of target concentration from 3 to 100 nM (Figure 3). For other codons, similar results were obtained

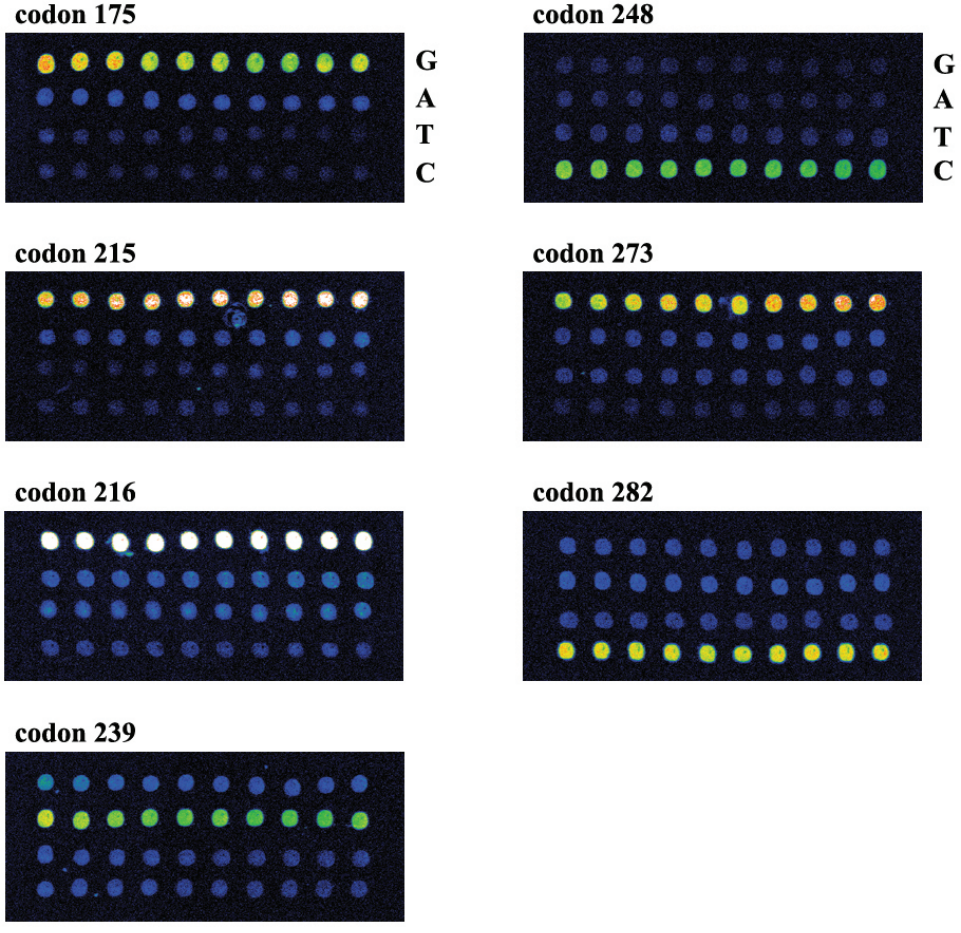

Figure 2. Fluorescence images after hybridization for the detection of single nucleotide variations in seven hotspot codons of p53 gene on the dendron-modified surface. All the capture probes were spotted in a 10 × 1 format.

Table 2. Relative fluorescence intensity of seven hotspot codons in p53 gene on dendron-modified surface

Codon	Relative intensity[a] (%)			
	G	A	T	C
175	**100** (±3.4)	10.1 (±0.9)	3.1 (±0.3)	1.7 (±0.1)
215	**100** (±7.1)	4.9 (±0.7)	3.3 (±0.4)	2.1 (±0.2)
216	**100** (±1.7)	8.2 (±0.9)	7.4 (±0.6)	3.4 (±0.2)
239	15.9 (±2.4)	**100** (±3.4)	9.4 (±1.1)	9.2 (±0.8)
248	2.1 (±0.4)	4.9 (±1.0)	5.9 (±0.5)	**100** (±6.9)
273	**100** (±8.4)	5.5 (±0.7)	4.5 (±0.2)	1.3 (±0.1)
282	8.1 (±0.3)	13.4 (±1.0)	6.8 (±0.5)	**100** (±4.9)

[a]The fluorescence intensity of perfectly matched sequence of each codon was set to 100 and shown in bold. Numbers in parenthesis represent relative standard deviation for each capture probe based on 10 independent measurements.

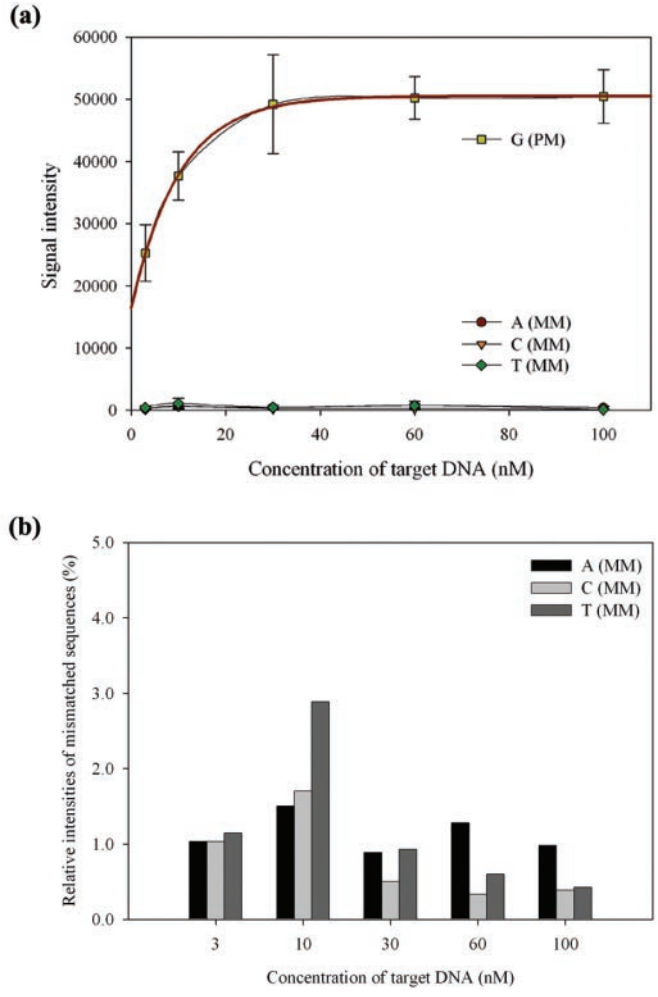

Figure 3. (**a**) Dependence of fluorescence signal intensity and (**b**) relative fluorescence intensity depending on the concentration of target DNAs for the detection of codon 273. PM, perfectly matched; MM, mismatched. The fluorescence intensity of perfectly matched sequence of each concentration of target DNA was set to 100.

(data not shown). The fluorescence signal intensity of the matched duplex was increased with the concentration of the target DNAs and saturated at ~30 nM target DNA as shown in Figure 3a. These results demonstrated that DNA microarrays fabricated on the dendron-modified surface could detect target DNA of nanomolar concentration with high discriminating ability in the broad range of target DNA concentration.

Sensitivity of the DNA microarrays on the dendron-modified surface

The sensitivity of DNA microarrays is another important factor to determine the performance of DNA microarrays. Previously, we reported that the signal intensity was strongly dependent on the density of functional groups on the surface (23). In order to compare the signal intensity of our DNA microarrays on the dendron-modified surface with that on the aldehyde surface, we also fabricated the DNA microarray on the aldehyde surface for the detection of the codons 175 and 239. The signal intensity on the dendron-modified surface (Figure 4a, left panel) was found to be similar to or higher than that on the aldehyde surface (Figure 4b, left panel). This is an interesting result since it was reported that the surface density of reactive aldehyde groups was 5×10^{12}/mm^2 (www.arrayit.com), whereas that of reactive amine groups of the dendron-modified surface was 1×10^{11}/mm^2 (9). This result suggests that the hybridization efficiency of DNA microarrays on the dendron-modified surface is much higher than that on the aldehyde surface.

To investigate the effect of the linker in the capture probe on hybridized signal intensity and/or discrimination efficiency, a T30 spacer composed of 30 thymidine molecules was placed between the 5′ ends of capture probes and terminal amine groups. Spacers were reported to increase the hybridization efficiency in the detection of single nucleotide variations in synthetic oligonucleotide target DNAs because spacers kept capture probes away from the solid surface minimizing the influence of the surface (24,25). As shown in Figure 4, DNA microarrays on the dendron-modified and aldehyde surfaces showed different effect of the T30 spacer on the signal intensity and the discrimination efficiency of SNPs after the hybridization of DNA. In the case of DNA microarrays on the dendron-modified surface, the T30 spacer could enhance the hybridization signal intensity by a factor of ~3 for both codons 175 and 239 without any loss of SNPs discrimination efficiency (Figure 4a). Therefore, synergistic effects were observed when the vertical separation from the surface was combined with optimum lateral spacing between the probes. In contrast, T30 spacer on the aldehyde surface could not enhance the signal intensity (Figure 4b), implying that T30 spacer alone was not effective in enhancing the hybridization of long target DNAs. These results again support that precise control of the optimum spacing of capture probes is utmost important to give high selectivity and sensitivity of DNA microarrays.

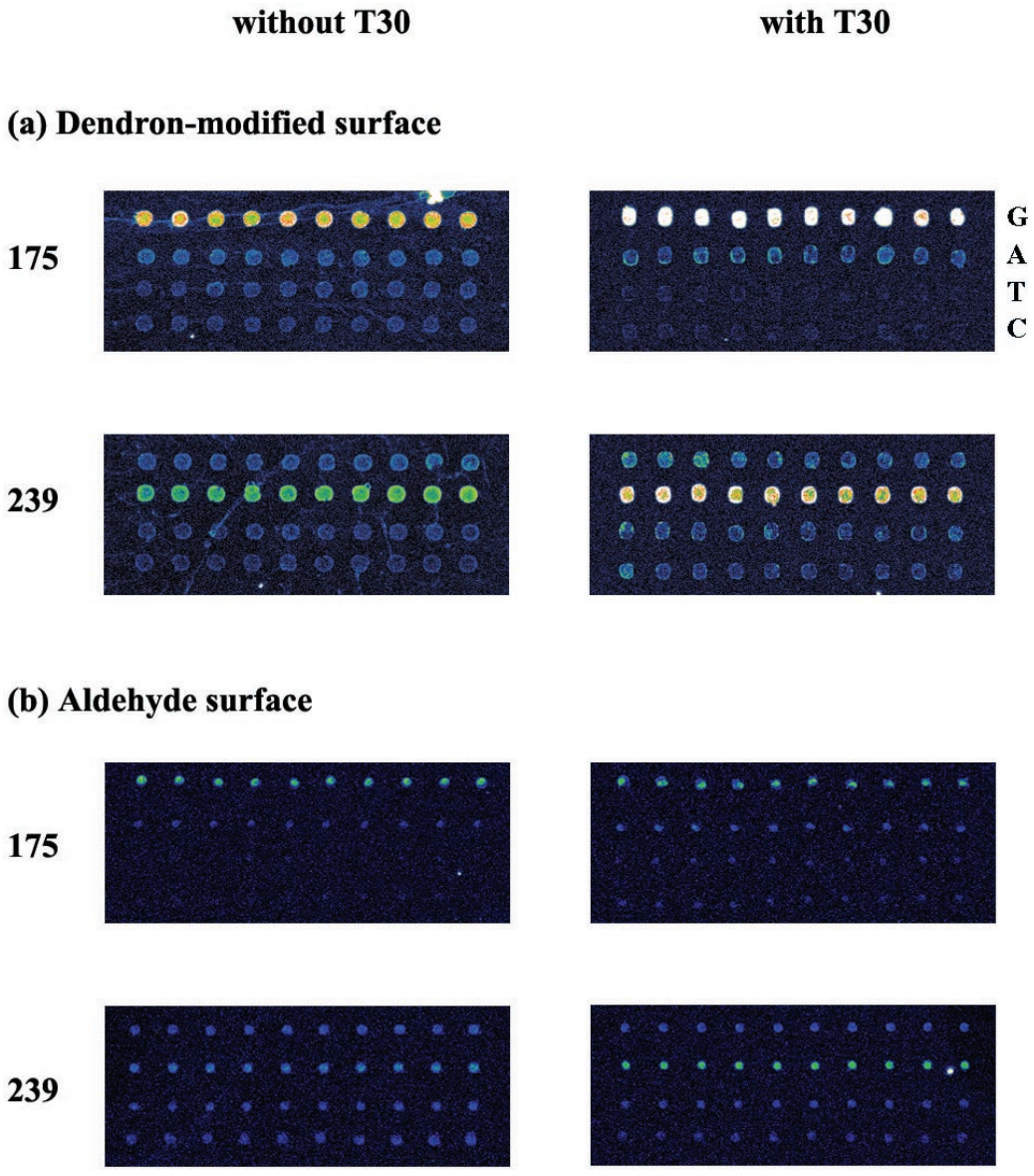

Figure 4. Fluorescence image for the detection of codons 175 and 239 on the (**a**) dendron-modified surface and (**b**) aldehyde surface using capture probes without and with T30 spacer.

Detection of heterozygous mutation

In order to identify the heterozygous mutations, we analyzed a 1:1 mixture of the target DNA from SNU 761 and 475 that have wild (AAC) and mutant (GAC) sequence at the codon 239, respectively. The 1:1 mixture (in terms of concentration) of target DNA was prepared by the random priming of the 1:1 mixture of genomic DNA templates that were extracted from both cancer cell lines. A representative fluorescence image and quantitative results are shown in Figure 5a and b, respectively. When 100% of DNA template from SNU 761 was used as a target DNA, the ratio between the fluorescence intensity of wild-type AAC and that of GAC, TAC or CAC mutant was <0.2, while 100% of DNA template from SNU 475 as a target DNA also exhibited the same ratio of <0.2 between the fluorescence intensity of GAC mutant and that of AAC wild-type, TAC or CAC mutant. For the 1:1 mixture of target DNA (50% DNA template from SNU-761 + 50% DNA template from SNU-475), the observed ratio was 1.0:1.0:<0.2 (wild-type AAC: GAC mutant: TAC or CAC mutant). The quantitative signal intensity ratio of 1.0:1.0 between wild-type AAC and GAC mutant demonstrates again high selectivity of DNA microarrays on the dendron-modified surface. These results indicate that DNA microarrays on the dendron-modified surface were capable of detecting heterozygous mutations reliably.

We believe that the high performance of DNA microarrays on the dendron-modified surface is attributed to the inherent characteristics of the surface, which allows mesospacing between functional groups resulting in the reduction of the steric hindrance not only among immobilized capture probes, but also between immobilized capture probes and target DNAs. Because steric hindrance and surface effect could be

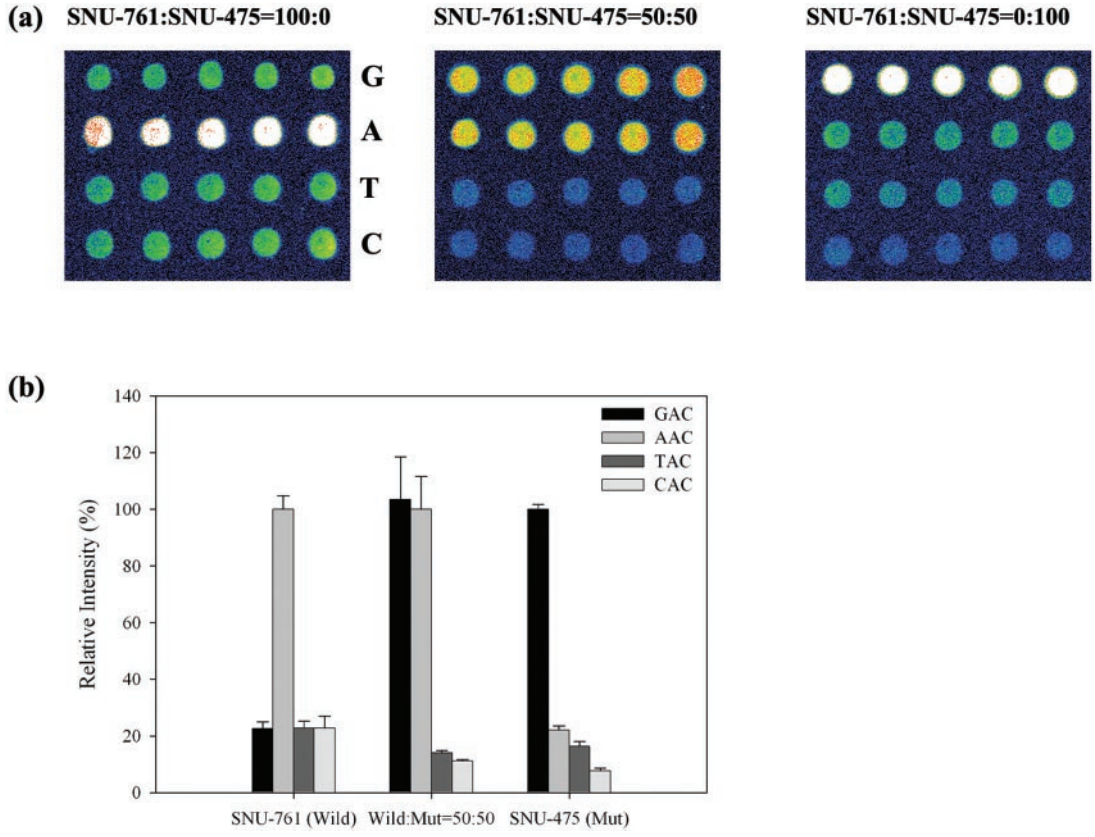

Figure 5. Detection of heterozygous mutations by DNA microarrays on the dendron-modified surface. Wild-type and mutant target DNAs were prepared by random priming of PCR products, which were generated by PCR amplification of exons 5–8 of p53 gene in genomic DNA from SNU-761 (wild-type) and SNU-475 (mutant type), respectively. A 1:1 mixed target DNA was prepared by random priming of 1:1 mixture of PCR products from SNU-761 and SNU-475. (**a**) Fluorescence images of the array. (**b**) Relative intensity of signals for wild-type (SNU-761), 1:1 mixed, and mutant type (SNU-475) target DNAs. The error bars were obtained from quintuplicate runs of the examination.

reduced significantly to mimic the solution phase phenomena, enhanced DNA hybridization efficiency with high selectivity and signal intensity was achieved for the DNA microarrays on the nano-scale controlled surface. This is well coincident with previous reports that the steric hindrance by the adjacent DNA probe molecules plays an important role in governing the amount of hybridization and hybridization efficiency (6,25–28). For example, Georgiadis *et al*. (6,26) showed that hybridization on the surface was strongly dependent on the density of capture probes when the hybridization on two kinds of slides with different probe density was analyzed by surface plasmon resonance spectroscopy. Shchepinov *et al*. (25) also showed that high hybridization yield could be achieved by adjusting the capture probe density on the surface using a combination of cleavable and stable linkers. Moreover, enhanced hybridization efficiency in the detection of M13 phage DNA was achieved on a mixed self-assembled monolayer boasting reduced hindrance among capture probes (28). Our observation again supports that the properties of surface on which DNA microarrays fabricated play critical roles to determine the performance of DNA microarrays.

In conclusion, we have shown that the DNA microarrays fabricated on the dendron-modified surface could unambiguously detect single nucleotide variations in seven hotspot codons of the p53 tumor-suppressor gene simultaneously with high selectivity and sensitivity. The outstanding performance of DNA microarrays on the dendron-modified surface seems to be attributed to the inherent characteristics of the dendron molecule on the surface, which has conical structure, rendering mesospacing between immobilized capture probes. It remains to be investigated that our DNA microarrays on the dendron-modified surface can be applied to various analyses that require accurate and precise detection of SNPs.

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