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Oligonucleotide Microarrays with High Discriminating Power for the Detection of Single Nucleotide Variations in the p53 Gene

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DNA microarrays are key tools for the parallel analysis of multiple DNA target samples and can be applicable to gene expression profiling and gene mutation analysis. However, DNA microarrays have relatively poor accuracy for discrimination of perfectly-matched from single-base-mismatched duplexes; this demerit delays the diagnostic application of for genetic mutation analysis or detection of single nucleotide polymorphisms (SNPs) (Hacia, 1999; Lockhart et al., 1996; Rogler et al., 2004; Schena et al., 1995).

Poor accuracy of DNA microarrays for the detection of genetic mutation or SNPs is considered to originate partly from inherent characteristics of the solid surface for the microarray and its molecular interlayer structures. Steric hindrance by adjacent DNA probe molecules strongly affects the amount and efficiency of target probe hybridization. Therefore, the fabrication of a solid surface which can improve SNP discrimination efficiency would a breakthrough in the analysis of genetic mutations by DNA microarray technology.

Previously, we developed a space-controlled surface which was prepared by modifying a solid surface with dendrons of conical shape (Fig. 1A) (Hong et al., 2003). Dendrons on the modified surface have regular mesospacing of 3.2 nm on average (Fig. 1B) (Hong et al., 2005). We demonstrated that DNA microarrays fabricated on the dendron-modified surface could provide high discriminating power for the detection of single nucleotide variations in synthetic oligonucleotide DNA. Furthermore, the DNA microarray on the dendron-modified surface was successfully applied to the detection of single nucleotide variations in seven hotspot codons in the p53 gene (Oh et al., 2005).

The p53 gene is involved in development, differentiation and response to cellular stress and is also an important tumor suppressor in biological system. p53 protein prevents the growth of abnormal cells by activating protective mechanisms, such as cell cycle arrest and apoptosis. The role of p53 as a tumor suppressor is indicated by its high rate of mutation in human cancer cells: more than 50% of adult human tumors carry inactivating mutations or deletions in the TP53 gene. When such mutations disrupt or alter the amino acid sequence of p53, dysfunction or malfunction of the protein could initiate or fail to suppress tumorigenesis. For example, even a single mutation of p53 gene might initiate tumorigenesis (Gu et al., 2006). Consequently, detection of SNPs in the p53 gene may provide a valuable tool for use in identification of numerous cancers as another SNPs in specific gene (Chae et al., 2010).

The p53 GeneChip® (Affymetrix), detects certain mutations in the p53 gene in various cancers with reasonable accuracy (Takahashi et al., 2003). However, this chip has some miscalling point on single nucleotide mutations in the p53 gene. We have applied our DNA microarrays on the dendron-modified surface to the detection of single nucleotide variations in seven codons of the p53 gene, i.e., 154, 158, 172, 176, 182, 199, and 255, which were miscalled by the p53 GeneChip (Ahrendt et al., 1999; Chee, 1998) are missense mutational spots. SNPs in these codons have been reported in cancer patients (Oh et al., 2005). The results of our comparison experiment can implicate the effect of inherent properties of the surface on the sensitivity and selectivity of DNA microarrays. Here, we assess the sensitivity, selectivity and reliability with which our DNA microarrays on the dendron-modified surface detect SNPs in p53, and compare these metrics with those of the Affymetrix p53 GeneChip.

The capture probe sequences designed for the detection of seven codons were 13 to 18 nucleotides long depending on the sequences around the respective codon; their theoretical T_m values were ~55°C (Table 1). The GC content of the capture probe sequence was 44 to 92%. Four capture probes, i.e., one perfectly-matched probe, and three single-nucleotide-mismatched probes were prepared to investigate whether they can detect SNPs in the respective codon. All the probe oligonucleotides have a terminal amine group required for covalent attachment to either a di(N-succinimidyl)carbonate (DSC)-activated dendron or an epoxy surface. Target DNAs incorporating Cy3 dye were prepared by randomly priming exons 5-8 of the p53 gene which had been amplified by polymerase chain reaction (PCR) of genomic DNAs from the cell line SNU 761, which is a human carcinoma cell line deposited at the Korean Hereditary Tumor Registry. The size of random primed target DNAs

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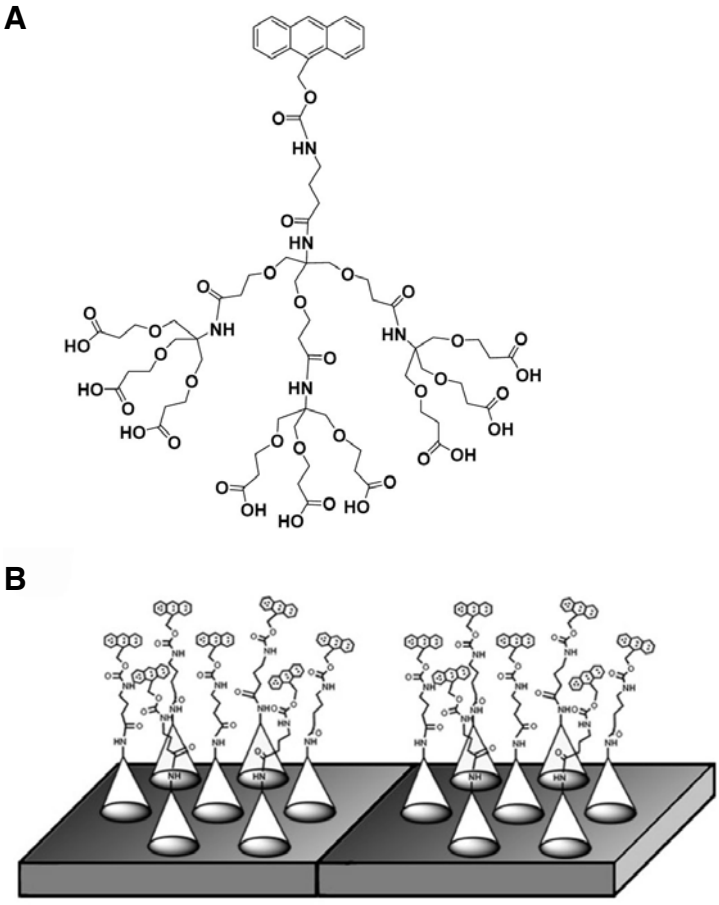

Fig. 1. (A) Structure of the dendron molecule. (B) Diagram of the dendron-modified surface.

was found to be 100 to 200 nucleotides, and the incorporation ratio was ~80 bases per dye molecule. The sequence of the p53 gene for PCR amplification was confirmed before random priming.

The dendron-modified surface was prepared as described previously (Hong et al., 2005) and modified with a DSC homobifunctional linker which has two succinimidyl groups reactive to amine. The DNA microarrays on the dendron surface were manufactured as follows. Amine-modified capture probe oligonucleotides were immobilized on the DSC-activated dendron surface of the glass slide by spotting the appropriate amine-modified oligonucleotides in a pH-8.5 buffer containing 25 mM sodium bicarbonate, 5 mM $MgCl_2$, and 10% (v/v) dimethyl sulfoxide, using a microarrayer (Cartesian Technologies, Microsys 5100) in a class 10,000 clean room. The probe oligonucleotides were spotted side-by-side in a 10 × 1 format on the microarray, which was then incubated overnight in a chamber maintained at ~85% humidity to allow sufficient reaction time for the amine-modification of DNA. Microarrays were then placed in a pH-7.4 buffer solution containing 2× SSPE and 7.0 mM sodium dodecylsulfate at 37°C for 1 h and then in boiling water for 5 min to remove oligonucleotides bound non-specifically. Finally, the DNA-functionalized microarrays were dried under a stream of N_2 gas for the subsequent hybridization.

Hybridization with Cy3-labeled target DNA was performed in a hybridization buffer solution 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 rinsed in a series of buffer solutions containing 1× SSC and 0.1% SDS, 0.1× SSC and 0.1% SDS, and 1× SSC sequentially at room temperature using a hybridization station (GeneTAC™, Genomic solutions Inc.). Fluorescence images were obtained using a confocal laser scanner (ScanArray Lite, GSI Lumonics), and the intensity of each spot was analyzed using microarray analysis software (ImaGene, BioDiscovery Inc.). DNA microarrays on the epoxy plates (SuperEpoxy, Telechem International, Inc.) were prepared according to the protocol provided by the supplier.

The 28 kinds of capture probes with all possible perfectly-matched or single-mismatched nucleotide sequences for seven codons were spotted in a 10 × 1 format on a single microarray with the dendron-modified surface, then 30 nM target DNA of the p53 gene from SNU 761 was hybridized. The matched sequences of each codon in SNU 761 was G<u>G</u>C, C<u>G</u>C, G<u>T</u>T, <u>T</u>GC, <u>T</u>GC, <u>G</u>GA, and A<u>T</u>C for codons 154, 158, 172, 176, 182, 199, and 255, respectively; the mismatched nucleotides are underlined. Fluorescence images of all seven codons were obtained after hybridization (Fig. 2A). Although the fluorescence intensity differed from codon to codon, all matched sequences of the seven codons could be clearly detected because the intensity of the single-base-mismatched pair (I_{MM}) was much lower than that of perfectly-matched pairs (I_{PM}). When the relative intensities were assessed from ten independent measurements of the fluorescence intensities, $0.001 \leq I_{MM}/I_{PM} \leq 0.18$, which is sufficiently low to detect all the matched sequences correctly (Fig. 2B). Given that the matched sequences of these codons were miscalled by the Affymetrix p53 GeneChip, those signal ratios obtained by the DNA microarrays on the dendron-modified surface demonstrated high selectivity for p53 genes

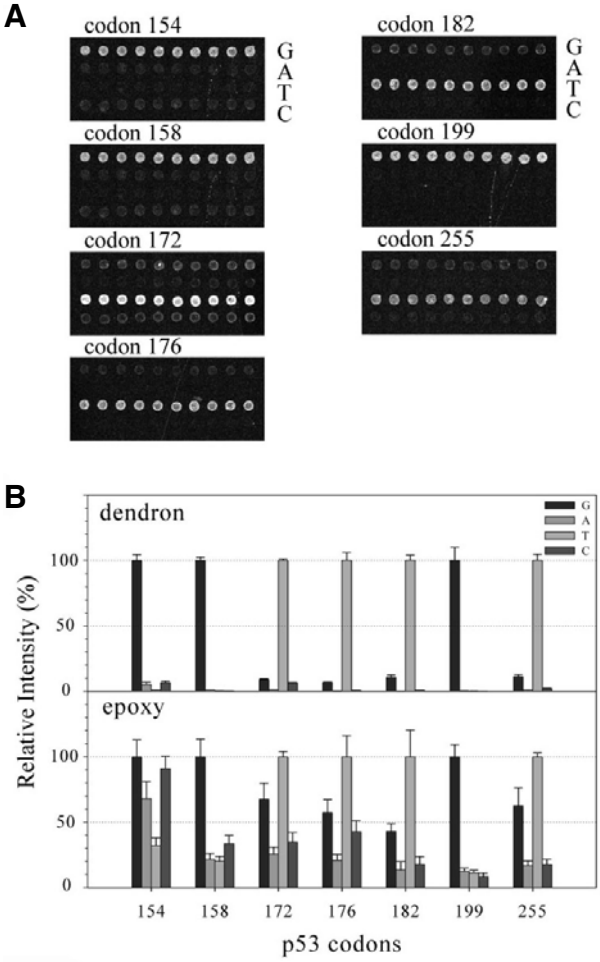

Fig. 2. (A) Fluorescence images after hybridization for the detection of single nucleotide variations in seven codons of p53 gene on the dendron-modified surface. All capture probes were spotted in a 10 × 1 format. (B) Relative fluorescent intensity obtained in DNA microarrays on the dendron-modified surface (upper) and the epoxy surface (lower). The fluorescent intensity of the perfectly-matched sequence of each codon was set to 100%.

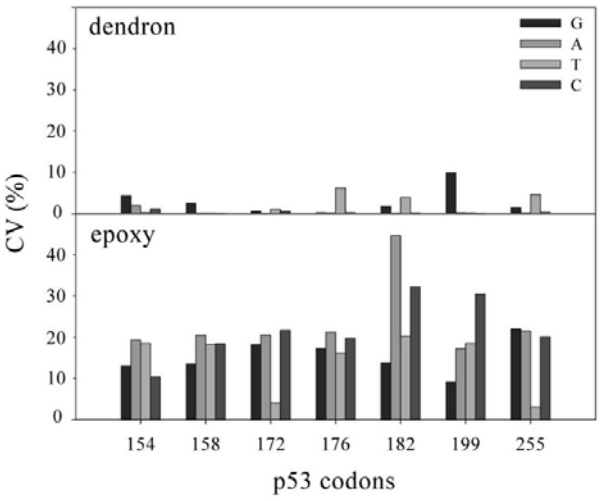

Fig. 3. Coefficients of variation on the dendron-modified surface (upper) and that on the epoxy surface (lower).

that differed by a single nucleotide. Also, these values are very similar to those ($0.007 \leq I_{MM}/I_{PM} \leq 0.16$) obtained for the detection of single nucleotide variations in seven hotspots in the p53 gene (Oh et al., 2005). Our results indicate that the DNA microarrays on the dendron-modified surface can discriminate perfectly-matched duplexes from single-base-mismatched duplexes with high selectivity for the codons miscalled previously.

For comparison of the sensitivity and selectivity of DNA microarrays on the dendron-modified surface with those on another type of surface, we also fabricated the same DNA microarrays on the epoxy surface; when these microarrays were used, $0.1 \leq I_{MM}/I_{PM} \leq 0.9$. Only three out of seven codons were selectively detected if the cut-off value was set to $I_{MM}/I_{PM} = 0.5$ (Fig. 2B). These results indicate that the sensitivity and selectivity of DNA microarrays could be strongly dependent on inherent properties of the surface, and that DNA microarrays on the dendron-modified surface exhibited much better sensitivity and selectivity than those on the epoxy surface.

A probe set on a single microarray can often be restricted to oligonucleotides with similar GC content to ensure their comparable sensitivity because of the lesser stability and lower melting temperature of the A:T pair compared with those of G:C pair. Therefore, this limits the diversity of sequences that can be used as capture probes in a single DNA microarray. The melting temperatures of capture probes can be made similar by adjusting the size of each capture probe (Fotin et al., 1998; Vainrub and Pettitt, 2003), but this approach cannot guarantee that each probe's selectivity will be high enough to discriminate perfectly-matched duplexes from single-base-mismatched duplexes because hybridization efficiencies can be different from probe to probe if different lengths of probes are employed in one microarray experiment. The GC content of the capture probe DNA used in this work varied from 44% (codon 199) to 92% (codon 154). To adjust the melting temperature of each capture probe to ~55°C, capture probes 13 to 18 oligonucleotides long were designed (Table 1). Despite the different lengths of capture probe DNA, high sensitivity and selectivity were obtained in our DNA microarrays on the dendron-modified surface for all seven codons, demonstrating that DNA microarrays on the dendron-modified surface could employ capture probe DNA with diverse sequences without sacrificing sensitivity and selectivity.

The ability of DNA microarrays to distinguish consistently among probes is another critical measure of the reliability of DNA microarrays. To assess the effect of surface on the repeatability of results, the coefficients of variation (CV = 100× Variance/Mean) were obtained from 10 spots of the same capture probes and compared between DNA microarrays on the dendron-modified surface and those on the epoxy surface. DNA microarrays on the dendron-modified surface showed CVs < 10% for all probes, whereas $10 \leq CV \leq 40\%$ were obtained for DNA microarrays on the epoxy surface (Fig. 3). These results indicate that the data from DNA microarrays on the dendron-modified surface are much more consistent than those from DNA microarrays on the epoxy surface.

Thus, the dendron-modified surface as substrate of DNA microarrays confers several advantages over the epoxy surface as follows. [1] It makes DNA microarrays very selective because the DNA microarrays on the dendron-modified surface could discriminate perfectly-matched duplexes from single-nucleotide-mismatched duplexes with high selectivity. [2] It makes DNA microarrays much more reliable because the data

Table 1. Capture oligonucleotides for the detection of single nucleotide variations in the p53 Gene

codon	Sequence[a] (5′ – 3′)	Nucleotide	Tm (°C)	GC (%)
154	CCCGCCCGNCACC N = G (wt), A, T, C (mt)	13	58	92
158	CGCGTCCNCGCCAT N = G (wt), A, T, C (mt)	14	58	78
172	ACGGAGGTNGTGAGGC N = T (wt), A, G, C (mt)	16	56	62
176	TTGTGAGGCGCNGCC N = T (wt), A, G, C (mt)	15	56	66
182	ATGAGCGCNGCTCAGATA N = T (wt), A, G, C (mt)	18	55	50
199	ATCCGAGTGGAANGAAAT N = G (wt), A, T, C (mt)	18	52	44
255	CACCATCANCACACTGGA N = T (wt), A, G, C (mt)	18	55	50

[a]The oligonucleotides have an amino group at the 5′-end. The underlined sequences represent the codons with a mismatched nucleotide (N) for the mutant type of the capture probe. wt, wild type; mt, mutant type

obtained from experiments of DNA microarrays on the dendron-modified surface were less variable than those on the epoxy surface. [3] Diverse sequences of capture probes can be employed in one microarray experiment. Therefore, oligonucleotides that have > 90% GC content and that differ from each other in length by > 40% can be employed as capture probes in a single DNA microarray.

We believe that all those advantages are consequences of the inherent characteristics of the dendron-modified surface, which allows regular mesospacing (~3 nm) between functional groups, resulting in the reduction of the steric hindrance among immobilized capture probes, and among target DNAs hybridized to immobilized capture probes. Because steric hindrance and surface effects could be reduced significantly enough to mimic the hybridization in the solution phase, increased DNA hybridization efficiency with high selectivity and signal intensity could be achieved using the DNA microarrays on the nanoscale controlled surface. This is consistent with previous reports that steric hindrance by adjacent DNA probe molecules has an important effect on the amount of hybridization, and on the efficiency of hybridization (Afanassiev et al., 2000; Peterson et al., 2001; 2002; Shchepinov et al., 1997; Southern et al., 1999). Our observations suggest that properties of the solid surface on which DNA microarrays fabricated have important effects on the sensitivity and selectivity of DNA microarrays.

In conclusion, we have shown that DNA microarrays fabricated on the dendron-modified surface could unambiguously and simultaneously detect single nucleotide variations in seven codons of the p53 tumor-suppressor gene from a cancer cell line, which were miscalled previously by the Affymetrix p53 GeneChip, and that these arrays can do so with high selectivity and reliability. The outstanding sensitivity and selectivity of DNA microarrays on the dendron-modified surface seems to be a consequence of the characteristics of the dendron molecule on the surface, which has a conical structure and allows mesospacing between immobilized capture probes. Future investigations should examine whether DNA microarrays on a dendron-modified surface can be applied to other diagnostic analyses which require accurate and precise detection of single nucleotide polymorphisms related to disease.

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