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A DNA microarray-based methylation-sensitive (MS)-AFLP hybridization method for genetic and epigenetic analyses

Abstract We previously developed a PCR-based DNA fingerprinting technique named the Methylation Sensitive (MS)-AFLP method, which permits comparative genome-wide scanning of methylation status with a manageable number of fingerprinting experiments. The technique uses the methylation sensitive restriction enzyme *Not* I in the context of the existing Amplified Fragment Length Polymorphism (AFLP) method. Here we report the successful conversion of this gel electrophoresis-based DNA fingerprinting technique into a DNA microarray hybridization technique (DNA Microarray MS-AFLP). By performing a total of 30 (15×2 reciprocal labeling) DNA Microarray MS-AFLP hybridization experiments on genomic DNA from two breast and three prostate cancer cell lines in all pairwise combinations, and Southern hybridization experiments using more than 100 different probes, we have demonstrated that the DNA Microarray MS-AFLP is a reliable method for genetic and epigenetic analyses. No statistically significant differences were observed in the number of differences between the breast-prostate hybridization experiments and the breast-breast or prostate-prostate comparisons.

Keywords Methylation-sensitive amplified fragment length polymorphism (MS-AFLP) · DNA Microarray MS-AFLP · DNA methylation · Copy number

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Introduction

Cytosine methylation of the CpG dinucleotide sequence in DNA is associated with a variety of physiological phenomena, including genomic imprinting and X chromosome inactivation (Holliday and Pugh 1975; Riggs 1975; Bird 2002). DNA methyltransferases (DNMTs) catalyze this methylation reaction. The methylation patterns are maintained through DNA replication by maintenance methylation (Wigler 1981), primarily catalyzed by DNMT1 (Bestor et al. 1988). The enzymes DNMT3A and B are responsible for de novo methylation, and mutations in the *DNMT3B* gene have been found to cause the immunodeficiency syndrome ICF (Hansen et al. 1999; Okano et al. 1999; Xu et al. 1999).

Hypermethylation and hypomethylation—alterations in DNA methylation—are frequently found in pathological states, most evidently in cancer (Feinberg and Vogelstein 1983; Jones and Baylin 2002), although the causative role of methylation abnormalities in tumorigenesis has not been genetically demonstrated (see Baylin and Bestor 2002 for the controversy surrounding this topic). Regional hypermethylation has been observed in the promoters of certain tumor suppressor genes (Herman et al. 1994, 1998; Kane et al. 1997), whereas hypomethylation was found mainly in repeated sequences (Feinberg and Vogelstein 1983; Ji et al. 1997; Narayan et al. 1998; Qu et al. 1999; Yamamoto et al. 2001).

In order to examine methylation patterns, various methods have been developed (see the review by Laird 2003). Some methods utilize chemical reactions to discriminate unmethylated cytosine residues from methylated ones, while others utilize differential susceptibility toward methylation-sensitive restriction enzymes. The most commonly used chemical method is the sodium bisulfite modification (Clark et al. 1994). This is often combined with PCR, restriction enzyme digestion, or DNA sequencing (Herman et al. 1996; Xiong and Laird 1997). The methylation-sensitive restriction enzymes

have been used in conjunction with hybridization or PCR (Bird and Southern 1978; McGrew and Rosenthal 1993). In addition to the specific gene sequence approach, genome-wide approaches have also been described. For example, the Restriction Landmark Gel Scanning (RLGS) method (Hayashizaki et al. 1994), the Methylation-Sensitive Restriction Fingerprinting (MSRF) method (Huang et al. 1997), Methylated CpG island Amplification-Representational Difference Analysis (MCA-RDA; Toyota et al. 1999), and Differential Methylation Hybridization (DMH; Huang et al. 1999) represent the genome-wide approach.

Over the past several years, we have also developed a technique for the efficient detection of alterations in DNA methylation on a genome-wide scale. We combined a methylation-sensitive restriction enzyme, *Not* I, with the Amplified Fragment Length Polymorphism (AFLP) technique (Vos et al. 1995) and developed the gel electrophoresis-based DNA fingerprinting technique named Methylation Sensitive (MS)-AFLP (Yamamoto et al. 2001). Despite what its name suggests, this technique actually does not employ length polymorphism for analysis (see below). We demonstrated its utility by identifying alterations in DNA methylation, and observed hypomethylation of a satellite DNA sequence in a majority of breast tumors and breast cancer cell lines. The MS-AFLP method is especially useful for the parallel analysis of multiple samples.

Recent technological advances now allow us to prepare DNA microarrays and measure the fluorescence intensity of probes hybridized to individual DNA target spots on microarrays, using a commercially available DNA microarrayer and a fluorescence scanner. As the gel electrophoresis-based technique is not ideally suited for automatic quantification, we took advantage of the emerging DNA microarray technology and transformed the gel electrophoresis-based MS-AFLP technique into a fluorescent MS-AFLP method based on DNA microarray hybridization. There are some precedents for the application of DNA microarray hybridization methods to DNA methylation analysis (Gitan et al. 2002; Shi et al. 2002). However, our DNA Microarray MS-AFLP method is unique, because the signal is generated from unmethylated cleaved *Not* I sites rather than methylated uncleaved ones. More importantly, the technique is useful for analyzing both genetic and epigenetic changes on a genome-wide scale.

Materials and methods

Preparation of DNA microarrays

We selected 123 *Not* I-*Mse* I fragments, including five duplicates and two triplicates, and four *Mse* I-*Mse* I fragments, which were cloned into the vector pT-Adv (Clontech) and used to prepare a DNA microarray panel. This panel contained fragments cloned at random after digestion of a mixture of genomic DNAs, as well as fragments selected from bands identified in *Not* I-*Mse* I MS-AFLP fingerprinting experiments. The panel included some *Not* I-*Mse* I fragments derived from bands that exhibited alterations in intensity in tumor DNA fingerprints relative to normal DNA fingerprints of the same tissues from the same individuals.

Transformed *E. coli* cells were cultured overnight, and plasmid DNA was prepared. PCR was then performed with the plasmid DNA templates, using primers flanking the DNA inserts and Ready-to-Go Beads (Amersham-Pharmacia). Most cases used a combination of M13 Forward and Reverse Sequencing primers for PCR amplification, although in a few cases *Not* I and *Mse* I adaptor primers were used. DNA amplification was confirmed by electrophoresing small aliquots of the PCR products through a 1.5% agarose gel. The products of the remaining reactions were precipitated with ethanol, dried, and re-suspended in distilled water at a concentration of at least 0.2 μg/μl. An equal volume of DMSO was then added and mixed, and a 10-μl aliquot of DNA solution from each sample was then transferred into a 384-well microwell plate for printing. Together with the negative control, PCR-amplified DNAs were spotted onto UltraGAPS coated glass slides (Corning), using the TotalArray Microarray printer (Packard Bio-Sciences). Each DNA fragment was spotted in triplicate and each slide was printed with two DNA microarrays. DNA microarray slides were baked for 1 min in a microwave oven, and then fixed by irradiation with UV light. Prior to use, individual slides were dipped in a pre-warmed (42°C) blocking solution containing 1% BSA in 5×SSC and 0.1% SDS for 45 min. The slides were then rinsed in 2×SSC/0.1% SDS for 2 min and in 0.1×SSC for 2 min, and centrifuged for 5 min, in a 50-ml conical tube, at 1000 rpm to remove the solution. These blocked slides were used for hybridization within 45 min.

Template preparation by the MS-AFLP method

Genomic DNA from the breast carcinoma cell lines MCF7 and MDA-MB468 and the prostate cancer cell lines PC3, DU145 and LNCaP was used in the experiments. For preparation of the *Not* I-*Mse* I MS-AFLP template, a 1-μg aliquot of DNA was digested with *Not* I and *Mse* I, and ligated to adaptors as described previously (Yamamoto et al. 2001). *Not* I-*Mse* I (and short *Not* I) fragments were then selectively amplified using AccuPrime SuperMix II (Invitrogen). PCR was performed in 21-μl reactions [1.5 μl of the adaptor-ligated DNA, 1 μl each of (50 pmol/μl) adaptor primers, 10 μl of AccuPrime SuperMix II, and 7.5 μl of water]. The primers used were *Not* I+0 (5′-CCGGAATTC-GACTGCGTAGGGGCCGC-3′) and *Mse* I+0 (5′-GATGAGTCCTGAGTAA-3′). The cycle parameters were: 68°C for 10 min and 94°C for 30 s, followed by 45 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 2 min, followed by 68°C for 10 min. The reactions were then kept at 10°C until the amplified DNA fragments

were isolated using a QIA PCR Clean-up kit (Qiagen). DNA was eluted into 100 μl of elution buffer.

Fluorescent probe preparation

Fluorescently labeled probes were prepared using the Bioprime labeling system (Invitrogen). Samples (2.5 μl) of PCR-amplified DNA were each mixed with 5 μl of water and 5 μl of Random Primer Mix solution. The mixtures were boiled at 100°C for 2 min, quickly placed on ice for 1 min, and briefly centrifuged for 10 s. Then 1 μl of either CY5 Mix solution (1.56 mM each of dGTP, dATP and dTTP, 0.22 mM dCTP, and 0.11 mM Fluorolink CY5-dCTP) or CY3 Mix solution (1.56 mM each of dGTP, dATP, and dTTP, 0.22 mM dCTP, and 0.11 mM Fluorolink CY3-dCTP) was added. Fluorolink CY5-dCTP and CY3-dCTP were purchased from Amersham-Pharmacia. The Klenow fragment of *E. coli* DNA polymerase was then added to a final concentration of 0.8 U per μl. The mixtures were incubated at 37°C for 1 h before adding 2 μl of stop solution (0.5 M EDTA) to terminate the reaction. The CY5 and CY3 reactions were mixed and fluorescently labeled DNA fragments were separated from the unincorporated dNTPs by G-50 spin-column chromatography. Exposure of samples to light was minimized during these and the following experimental procedures.

DNA microarray hybridization

The probe was mixed in a siliconized Eppendorf tube with 100 μg of yeast tRNA (Sigma), and 2.5 μg of human Cot 1 DNA, which is rich in repeated sequences (Invitrogen). Then 6 μl of 3 M sodium acetate (pH 7.5) and 180 μl of absolute ethanol was added, mixed, and the samples were centrifuged at 13,000 rpm for 10 min. The supernatant was removed, and the tube was centrifuged again for 2 min, and the supernatant was removed again. The pellet was then air-dried for 1 min. The probe was re-suspended in GlassHyb hybridization solution (Clontech) by pipetting, vortexed for 30 s, and then briefly centrifuged for 10 s. The solution in the tube was heated at 100°C for 3 min and incubated for 30 min at 37°C before hybridization, to allow repetitive sequences to anneal, thus preventing labeled repeats from subsequently hybridizing to the microarray. The probe was then poured onto the DNA microarray and covered with a coverslip. The slide was then placed in a hybridization cassette (Corning) and incubated overnight in a water bath at 50°C. Two hybridization experiments were performed per slide, separately hybridizing two different probes, prepared by reciprocal labeling, to two DNA microarrays on the slide. After hybridization, the slide was removed from the cassette and transferred to a 50-ml conical tube filled with GlassHyb Washing solution. After the coverslip had detached, it was removed from the solution, and the slide was washed and rotated for 10 min at room temperature. The slide was then transferred into the 11:1 mixture of GlassHyb Washing solution and 1×SSC, and then into 0.1×SSC and washed for 10 min each. The slide was then centrifuged for 5 min at 1000 rpm.

Fluorescence of CY5 and CY3 dyes was measured using a ScanArray 4000 DNA microarray scanner (Packard BioScience), and pseudo-colored (red for CY5 and green for CY3) images were obtained in the TIFF format. The fluorescence intensity of individual spots was quantified using ImaGene microarray analysis software. The data were then imported into Excel for scatter plot and variation analyses.

Southern hybridization

Genomic DNAs from the breast cancer cell lines MCF7 and MDA-MB468, and the prostate cancer cell lines PC3, DU145 and LNCaP was digested with the methylation-insensitive enzymes *Eco* RI and *Bam* HI and the methylation-sensitive *Not* I. Approximately 1.3 μg of DNA was loaded per well and electrophoresed through 1% agarose gels. The gels were denatured and neutralized, and the DNA was transferred to Zeta-Probe blotting membrane (Bio-Rad) and fixed by irradiation with UV light. The membranes were soaked in water and then prehybridized with UltraHyb hybridization buffer (Ambion). DNA fragments prepared for microarray printing were also used to prepare radioactive probes for the Southern hybridization using the PrimeIt II kit (Stratagene) following the manufacturer's protocol. For probes that produced results that were in conflict with the data from the microarray hybridization experiments, additional Southern hybridization experiments were performed using genomic DNA digested with *Eco* RI and *Bam* HI and/or genomic DNA digested with *Not* I and *Mse* I in order to resolve the discrepancies.

Results

DNA Microarray MS-AFLP: strategy

We set out to convert the gel electrophoresis-based MS-AFLP DNA fingerprinting technique into a fluorescent MS-AFLP technique based on DNA microarray hybridization. The two methods are schematically shown for comparison in Fig. 1A and B. Briefly, genomic DNA is digested with the methylation-sensitive enzyme *Not* I and the methylation-insensitive *Mse* I, and ligated with the appropriate adaptors. PCR is then performed with adaptor-ligated DNA and *Not* I and *Mse* I adaptor primers. *Not* I-*Mse* I fragments, as well as short *Not* I fragments, are amplified preferentially over the much more abundant *Mse* I fragments, by using long *Not* I and short *Mse* I primers and setting the annealing temperature of the PCR between the T_ms of those primers. In the fingerprinting method, additional

selective nucleotides are added at the 3′ ends of the primers to decrease the number of DNA fragments that are amplified. One of the primers, usually the *Not* I primer, is 5′ end labeled with ^{32}P, and discrete banding patterns rather than smears are obtained and recorded on X-ray films. The band intensity correlates with the number of unmethylated *Not* I sites that are cleaved by the enzyme. In the microarray hybridization method, additional selective nucleotides are unnecessary. Products of PCR are then fluorescently labeled with CY5 or CY3, using the Klenow enzyme and CY5-dCTP or CY3-dCTP. After the labeling reactions have been terminated, the two probes are mixed, and unincorporated dNTPs are removed. The probes are then used for hybridization to DNA microarrays on glass slides, following the comparative genomic hybridization (CGH) method (Kallioniemi et al. 1993; Pinkel et al. 1998; Albertson et al. 2000). The fluorescence signal associated with each target spot is measured using a fluorescence scanner. The fluorescence intensity is correlated with the number of unmethylated *Not* I sites cleaved by the enzyme. Because it is difficult to print exactly the same quantity of DNA per spot, wide variation exists among different spots in a DNA microarray and corresponding spots on different slides. For this reason, the CY5/CY3 intensity ratios, rather than individual intensities, are calculated.

Self-self and self-nonself hybridization experiments demonstrate the feasibility of the DNA Microarray MS-AFLP method

We first examined the suitability of the MS-AFLP method for preparation of fluorescent probes for DNA microarray hybridization experiments. For this purpose, we constructed a DNA microarray panel comprising of 114 different cloned *Not* I-*Mse* I fragments (a total of 123, including five duplicates and two triplicates) and four cloned *Mse* I fragments. Together with the spot numbers, those DNA fragments were numbered and are listed in Supplementary Table 1. The nucleotide sequences of all these fragments were identified in the human genome and the GenBank Accession Nos. of the sequences that contained the DNA fragments are also given in Supplementary Table 1. Most DNA fragments contained unique sequences, but three were multicopy sequences and 11 were repetitive sequences. These DNA fragments were printed on glass slides and hybridized with fluorescent probes prepared by the DNA Microarray MS-AFLP method. In order to obtain stronger signals, we employed random primer extension labeling with fluorescent nucleotides rather than labeling using fluorescent *Not* I primers, although this may have caused an increase in the background noise.
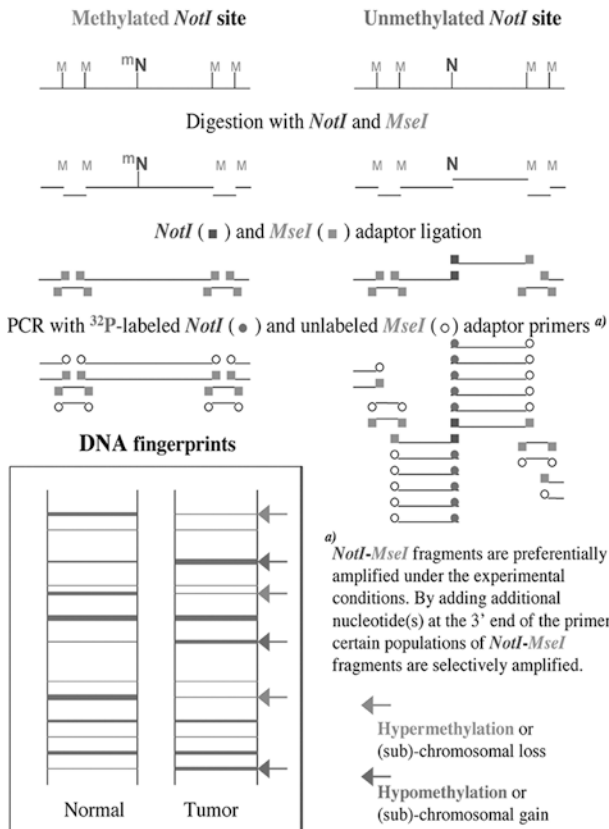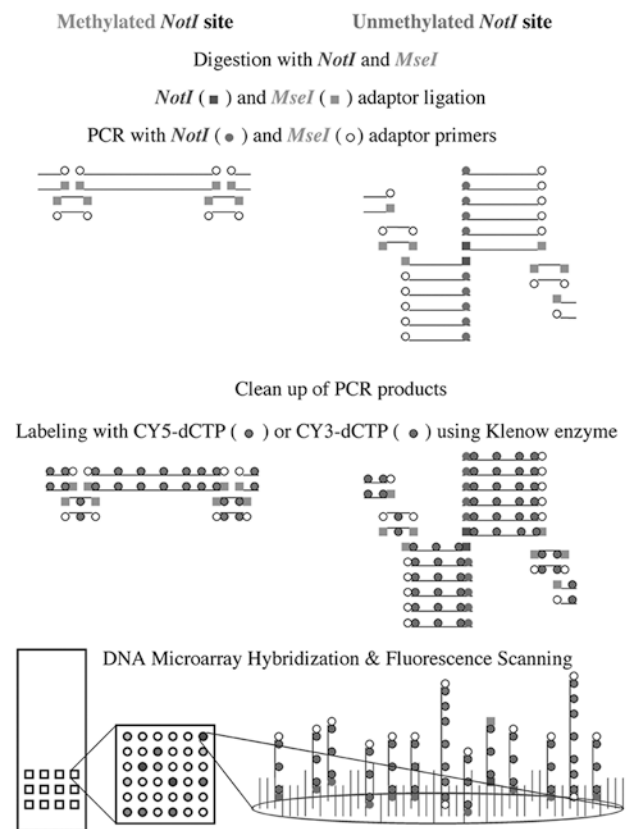
After hybridization and washing, the signal intensity of individual spots was calculated using the ImaGene software, and subjected to scatter plot analysis using the Excel program. The results of one self-self and one self-nonself hybridization experiments are shown in Fig. 1C. The two plots on the left show the results of self-self (MCF7/MCF7) hybridization (left: CY5-labeled MCF7-1 DNA probe/CY3-labeled MCF7-2 DNA probe; right: CY5-labeled MCF7-2 DNA probe/CY3-labeled MCF7-1 DNA probe). Two different preparations of MCF7 genomic DNA, MCF7-1 and -2, were separately digested, adaptor-ligated, PCR-amplified, and used for reciprocal fluorescent probe preparation by the MS-AFLP method. The two plots on the right show the results of self-nonself (MCF7/PC3) hybridization (left: CY5-labeled MCF7-1 DNA probe/CY3-labeled PC3-1 DNA probe; right: CY5-labeled PC3-2 DNA probe/CY3-labeled MCF7-2 DNA probe). Again, PC3-1 and -2 represent two different preparations of PC3 genomic DNA. The MCF7/MCF7 hybridization experiments resulted in significantly lower variation than the MCF7/PC3 experiments.
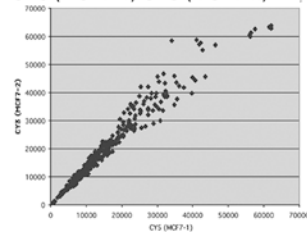
Differences in the susceptibility of genomic DNA to digestion by *Not* I among two breast and three prostate cancer cell lines

We next performed the DNA Microarray MS-AFLP hybridization experiments for every pairwise combination of genomic DNAs from MCF7, MDA-MB468, PC3, DU145, and LNCaP cancer cell lines. The same DNA Microarray MS-AFLP protocols were employed, except that different DNA was used to prepare fluorescent probes. Fluorescence intensity was measured and the data were analyzed. The $\log_{10}$ values of normalized CY5/CY3 signal intensity ratios were calculated and plotted, and a representative sample of the data is shown in Fig. 2, where the $\log_{10}$ values are plotted on the Y-axis against the spot number on the X-axis. The complete data set is available in Electronic Supplementary Material (Supplementary Fig. 2). Two line graphs in blue and pink represent the results of reciprocal labeling experiments. All the line graphs are on the same scale. The self-nonself hybridization experiments resulted in more differences than the self-self hybridization experiments. We anticipated that there would be more differences in the breast-prostate hybridization experiments than in the breast-breast and prostate-prostate hybridization experiments. However, this was not always the case.
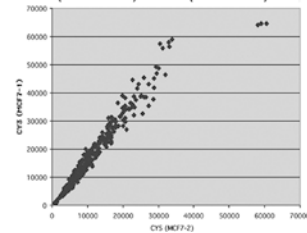
We calculated the subtracted values of the $\log_{10}$ of normalized CY5/CY3 signal intensity ratios between reciprocal labeling experiments. The average values of the triplicate spots were then calculated and are shown in the MA (microarray) columns in Supplementary Table 1, and we summarize these data here. We color-coded the subtracted $\log_{10}$ values of normalized CY5/CY3 signal intensity ratios to facilitate the identification of differences (dark red for values ≤ -1, red for > -1 and ≤ -0.5, orange for > -0.5 and ≤ -0.3, light orange for > -0.3 and ≤ -0.25, yellow for > -0.25 and < 0.25, bright green for ≥ 0.25 and < 0.3, sea green for ≥ 0.3 and < 0.5, green for ≥ 0.5 and < 1.0, and dark green for values ≥ 1.0). When reciprocal labeling resulted in the reversal of fluorescent

A.

Methylated *NotI* site   Unmethylated *NotI* site

Digestion with *NotI* and *MseI*

NotI (■) and *MseI* (■) adaptor ligation

PCR with ^{32}P-labeled *NotI* (●) and unlabeled *MseI* (○) adaptor primers [a)]

DNA fingerprints

[a)] *NotI-MseI* fragments are preferentially amplified under the experimental conditions. By adding additional nucleotide(s) at the 3' end of the primers, certain populations of *NotI-MseI* fragments are selectively amplified.

← Hypermethylation or (sub)-chromosomal loss

← Hypomethylation or (sub)-chromosomal gain

Normal   Tumor

B.

Methylated *NotI* site   Unmethylated *NotI* site

Digestion with *NotI* and *MseI*

NotI (■) and *MseI* (■) adaptor ligation

PCR with *NotI* (●) and *MseI* (○) adaptor primers

Clean up of PCR products

Labeling with CY5-dCTP (●) or CY3-dCTP (●) using Klenow enzyme

DNA Microarray Hybridization & Fluorescence Scanning
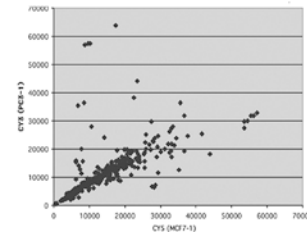
C.

MCF7/MCF7
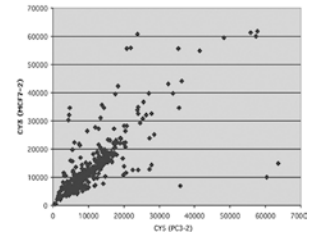
CY5 (MCF7-1)/CY3 (MCF7-2)   CY5 (MCF7-2)/CY3 (MCF7-1)

MCF7/PC3

CY5 (MCF7-1)/CY3 (PC3-1)   CY5 (PC3-2)/CY3 (MCF7-2)

signal colors, the values were printed in bold face. Inspection of the data (Supplementary Table 1) revealed that, with the exception of one green cell (the *Mse* I DNA fragment #117), all of the differences were observed in the *Not* I-*Mse* I DNA fragments. Similarly, only one green and one red cell were observed in the self-self hybridization experiments; all the other differences were observed in the self-nonself hybridization experiments. Judging from the line graphs of the reciprocal labeling experiments, these unexpected differences all seem to be technical artifacts. Out of the 1920 (128×15) cells, 208 (10.8%) were either < −0.25 or > 0.25 and were not colored yellow. However, this percentage may be an overestimate of the differences among those cell lines, because some of the

DNA targets were pre-selected from bands that were frequently altered in cancer cell lines in the *Not* I-*Mse* I MS-AFLP DNA fingerprinting experiments. Although the matches were not perfect, the results of the duplicate and triplicate experiments using different preparations of DNA fragments from different plasmid clones showed a relatively good correlation.
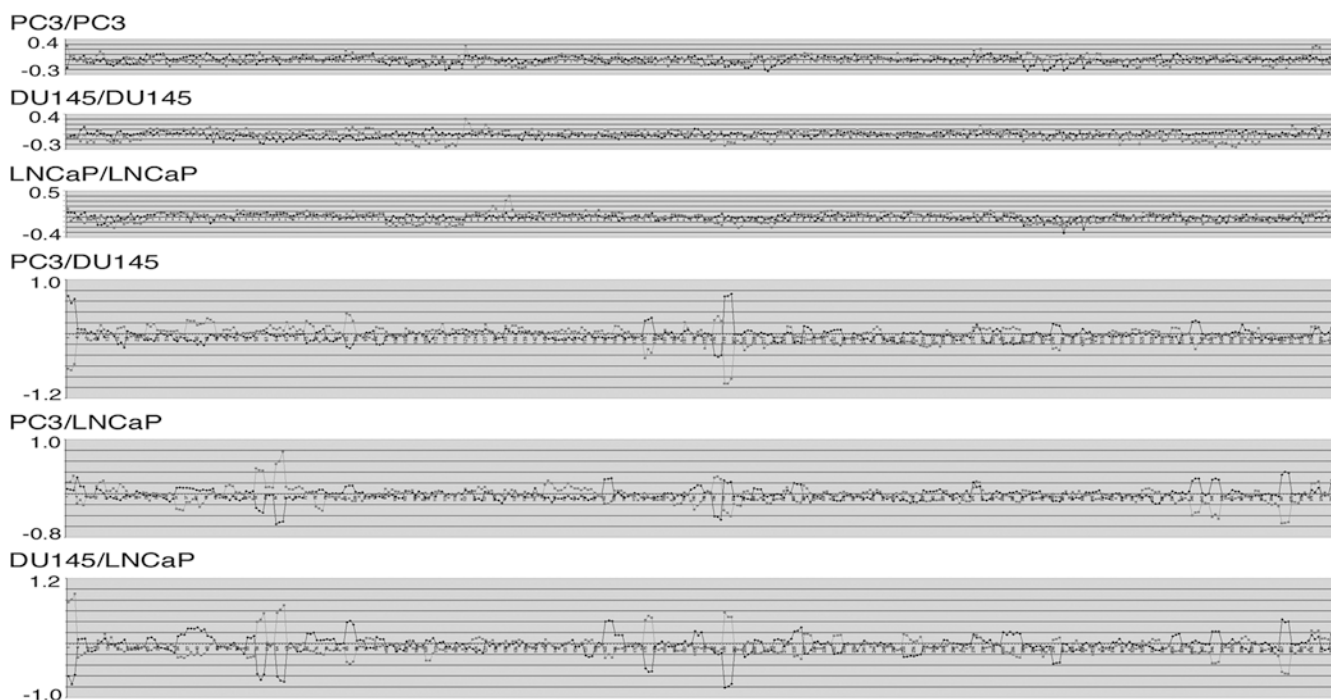
The DNA microarray MS-AFLP method provides results comparable to Southern hybridization

For comparison, we also performed Southern hybridization experiments. Genomic DNA from the five cancer

◄

Fig. 1A–C Schematic presentation of the two MS-AFLP methods (**A, B**) and scatter-plot analysis of CY5/CY3 signal intensity values (**C**). **A** The gel electrophoresis-based MS-AFLP DNA fingerprinting method. **B** The newly developed DNA microarray-based MS-AFLP method of fluorescent probe hybridization. Alterations are identified by the increase/decrease in band intensity in the fingerprinting method, and by the altered ratios of two fluorochrome signals in the hybridization method. Because individual spots in DNA microarray are represented by single species of DNA fragments, the results are more quantitative with the hybridization method. **C** Scatter plots of fluorescence intensity. The CY5 and CY3 fluorescence intensities were measured and plotted for individual spots in the MCF7/MCF7 self-self and MCF7/PC3 self-nonself hybridization experiments. The X-axis and Y-axis indicate the CY5 and CY3 signal intensities, respectively. The two panels on the *left* show the results of MCF7/MCF7 hybridization, those on the *right* the data for MCF7/PC3 hybridization. The results of two reciprocal labeling experiments using different preparations of genomic DNA are shown

cell lines used for the DNA Microarray MS-AFLP studies was digested with *Eco* RI, *Bam* HI and *Not* I. We chose this triple digestion, instead of digestion with *Not* I alone, because *Not* I sites are rare and it was anticipated that size discrimination of DNA fragments larger than

10 kb would be difficult. The *Not* I-*Mse* I and *Mse* I fragments used for microarray printing were also used as templates for radioactive probe preparation. Some representative results of the Southern hybridization experiments that exhibited a difference in DNA methylation and in copy number are shown in Fig. 3A and B, respectively. Based on the results of the Southern hybridization experiments, the anticipated results of microarray experiments were color-coded as red (R), green (G), or yellow (Y), and are shown in the S-columns of Supplementary Table 1. When the Southern hybridization experiments did not produce useful information, no color is indicated. All of the cells for the self-self hybridization experiments were automatically given the color yellow in the S-columns. Assuming that the results of the Southern hybridization experiments are always correct, the total numbers of matches, true positives, true negatives, false positives, and false negatives were then counted. We calculated the rates of false positives and negatives and obtained values of 2.6% (42/1605) and 3.6% (58/1605), respectively. We also determined the sensitivity and specificity of the DNA Microarray MS-AFLP method. Sensitivity is defined by the number of true positive results divided by the sum of the true positive results and the false negative results, while specificity is the number of true negative results divided by the sum of the true negative results and the false positive results. We obtained values of 78.5% [212/(212 + 58)] for sensitivity and 97.1% [1393/(1393 + 42)] for specificity.

Discussion

By performing a total of 30 (15×2 reciprocal labeling) DNA Microarray MS-AFLP hybridization experiments,

Fig. 2 Line graphs of $\log_{10}$ values of normalized CY5/CY3 signal intensity ratios. The $\log_{10}$ values of normalized CY5/CY3 signal intensity ratios are shown in a line graph. The six panels show the results for PC3/PC3, DU145/DU145, LNCaP/LNCaP, PC3/DU145, PC3/LNCaP, and DU145/LNCaP hybridization experiments. The results of two reciprocal labeling experiments are shown in *blue* and *pink*. The X-axis indicates the spot number, and the Y-axis indicates the $\log_{10}$ value of normalized CY5/CY3 signal intensity ratio. The Y-axes of the graphs are all on the same scale. The data for all comparisons performed in the course of this study are included in Fig. 2 of Electronic Supplementary Material
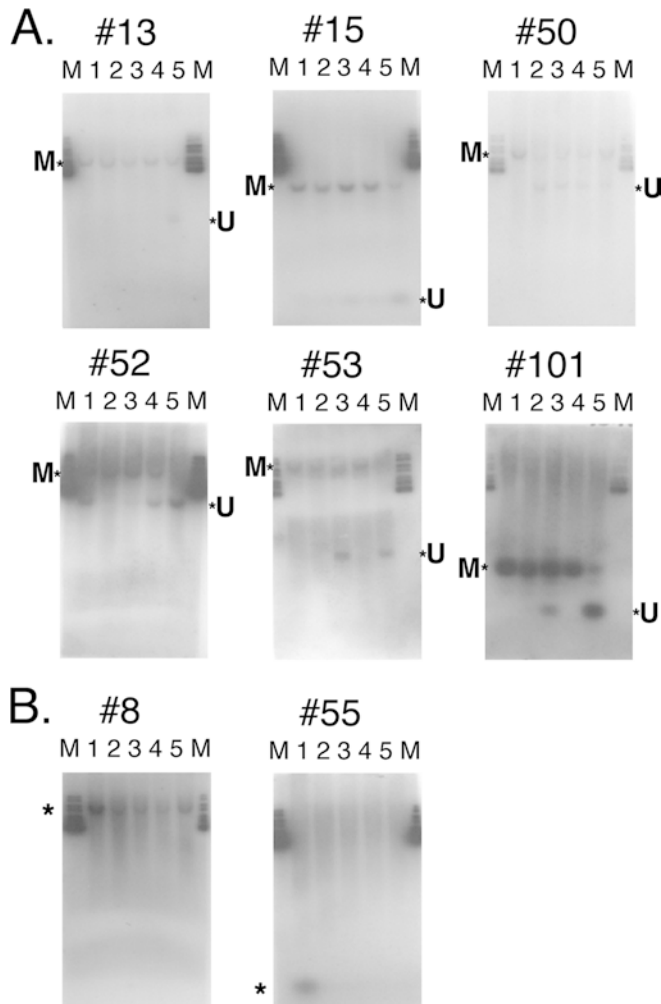
Fig. 3A, B Confirmation by Southern hybridization analysis of changes in DNA methylation and copy number. Southern hybridization experiments were performed using genomic DNA from five cancer cell lines: MCF7, MDA-MB468, PC3, DU145, and LNCaP (lanes 1–5, respectively). DNA was digested with a mixture of the methylation sensitive *Not* I and methylation-insensitive *Eco* RI and *Bam* HI, electrophoresed through a 1% agarose gel, and transferred onto nylon membranes. ^{32}P-Labeled probes were prepared from isolated DNA fragments by the random-primer method and used for hybridization. Some of the results are shown. **A** Difference in DNA methylation. **B** Difference in copy number. The locations of the bands resulting from cleavage at unmethylated (U*) and methylated (M*) *Not* I sites, as well as the locations of bands arising from copy number changes (*asterisks*) are shown. The M lanes were loaded with 1-kb ladder markers

as well as Southern hybridizations with more than 100 different probes, we have successfully demonstrated that DNA Microarray MS-AFLP is a reliable method for genetic and epigenetic analyses. Using the gel electrophoresis-based method, it is often difficult to quantify the signal due to noise from the bands in the background. However, using the more quantitative DNA Microarray MS-AFLP method, differences were observed in copy number in addition to DNA methylation. True differences should be consistently observed in triplicate target DNA spots printed with a single species

of DNA fragments. In the Southern hybridization experiments, the detection of different degrees of susceptibility to *Not* I, due to DNA methylation, is relatively easy because of the shift in the position of the DNA fragments. However, differences in copy number are difficult to observe, due to many technical pitfalls that may change the band intensity. For example, uneven transfer of DNA onto the membrane and uneven background hybridization are such pitfalls. Therefore, the assumption that the results of the Southern hybridization experiments are always correct may be wrong. Copy number changes shown with DNA fragments (#8 and 55) in Fig. 3B were recognized only because the DNA Microarray MS-AFLP hybridization experiments provided conclusive quantitative evidence that could only be explained by a change in the copy number or a change in DNA methylation. Pollack and colleagues (1999) utilized genomic DNA from cell lines containing varying numbers of X chromosomes to demonstrate the quantitative nature of copy number analysis by the cDNA microarray hybridization method they developed. However, we decided not to perform similar experiments because X chromosome inactivation by DNA methylation might complicate the analysis. Therefore, it remains to be determined whether the DNA Microarray MS-AFLP technique can detect small changes in copy number—single-copy deletions, for example. Nonetheless, the acquisition of more quantitative data is a great advantage of the DNA Microarray MS-AFLP hybridization method over the original gel electrophoresis-based DNA fingerprinting method. Once the differences are identified, it should not be too difficult to distinguish changes in DNA methylation from alterations in copy number. Other benefits of this gel-to-microarray transformation are laboratory safety (since radioisotopes are not used) and freedom from the need to perform tedious gel electrophoresis. One drawback is that it is not possible to analyze many samples simultaneously in parallel, since one hybridization experiment only allows the comparison of two samples at a time. Another drawback is its limited use as a gene discovery tool because only the sequences on the microarrays can be analyzed. On the other hand, gene identification is straightforward as each dot is represented by a single sequence. We therefore conclude that the two methods are complementary to each other. Depending on the purpose, one may choose either the gel electrophoresis method or the microarray method. For example, alterations associated with a certain biological phenomenon may be initially identified by the DNA Microarray MS-AFLP method, using a small number of samples. A larger number of samples may then be analyzed by the gel electrophoresis-based MS-AFLP DNA fingerprinting method using a pair of primers selected on the basis of the sequence and size of the DNA fragments of interest.

MS-AFLP does not analyze changes in specific gene sequences, but is a tool for the detection of genome-wide alterations (Yamamoto et al. 2001). Signal is derived

from cleaved unmethylated *Not* I sites, and an increase in signal is caused either by hypomethylation or an increase in copy number, or both. Conversely, a decrease in signal is caused either by hypermethylation or a decrease in copy number, or both. The DNA Microarray MS-AFLP method may thus offer the advantage that the difference in signal intensity is not diminished but rather enhanced when one allele is deleted and the other allele is methylated, as is often the case with tumor suppressor genes during tumorigenesis.

An interesting observation was made in our experiments with the DNA Microarray MS-AFLP hybridization method. Assuming that the DNA methylation pattern would be tissue-specific, we anticipated that more differences would be found between breast and prostate cancer cell lines than between different breast or prostate cancer cell lines. The results in Supplementary Fig. 2 and Table 1 show that this is not always the case. The numbers of clear differences observed in the self-nonself breast-breast and prostate-prostate hybridization experiments were 14, 14, 13 and 22 for MCF7/MDA-MB468, PC3/DU145, PC3/LNCaP and DU145/LNCaP, respectively. Similarly, the numbers of clear differences in the self-nonself breast-prostate hybridization experiments were 17, 19, 24, 11, 17 and 17 for MCF7/PC3, MCF7/DU145, MCF7/LNCaP, MDA-MB468/PC3, MDA-MB468/DU145, and MDA-MB468/LNCaP, respectively. No statistically significant differences were observed between the breast-prostate hybridization experiments and either the breast-breast or prostate-prostate comparisons. Because the cell lines used in this study were established from different individuals, it should be noted that several, if not many, of the differences observed might have been due to single nucleotide polymorphisms.

In an effort to increase representation, we compared the results of DNA Microarray MS-AFLP using *Eag* I (whose recognition site is CGGCCG) with those obtained using *Not* I (GCGGCCGC). Regrettably, many more false negatives were observed with *Eag* I (data not shown). The probe labeling method employed in the *Eag* I experiments failed to achieve a specific activity high enough to detect some of the changes. Nonetheless, this goal may be achieved using another labeling method, as technological progress is very rapid. BAC DNA microarrays (Pinkel et al. 1998; Albertson et al. 2000) are not suitable for DNA Microarray MS-AFLP at the present time. The average BAC insert size is 140 kb, and the estimated numbers of *Not* I and *Eag* I sites per insert are calculated to be only 0.8 and 6, respectively. However, the use of methylation-sensitive restriction enzymes that cleave more frequently may alleviate the sensitivity problem, because the large BAC inserts facilitate the detection of signals that are summed from many different fragments in the inserts.

We used the *Not* I-*Mse* I fragment microarray in our study. If long oligonucleotides can act as a substitute for the *Not* I-*Mse* I DNA fragments, preparation of DNA microarrays for high-throughput analysis will be facilitated. More than 3000 *Not* I targets can easily be se-lected that will cover the entire genome at approximately 1-Mb intervals, based on the human genome sequence. We have recently constructed an oligo array consisting of 32 oligos (40–60mers) that represents the 114 *Not* I-*Mse* I fragments in the panel used for the present experiments, and started to evaluate the utility of this oligo array for MS-AFLP experiments. Preliminary results show that comparable specificity is obtained, although sensitivity is inferior to that with DNA fragments. We hope that the sensitivity can be increased by optimizing the experimental conditions, including primer design.

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