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SHORT COMMUNICATION

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A microarray-based method for detecting methylated loci

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Abstract CpG island DNA methylation plays an important role in regulating gene expression in development and carcinogenesis. We developed a new microarray-based method called methylation amplification DNA chip (MAD) for detecting differences in methylation. In this method, only methylated CpG islands from the two samples that we wanted to compare were amplified and used for hybridization. The resource material for the microarray was derived from the methylated DNA library of the sample in which we wanted to detect hypermethylation. Choosing the methylated DNA library as the resource material of the microarray increased the percentage of DNA fragments derived from hypermethylated loci on the microarray.

Key words DNA methylation · DNA chip · Microarray · CpG island · Hypermethylation

Introduction

The great majority of cytosine residues of CpG dinucleotides are methylated in the human genome, but some remain unmethylated in specific GC-rich areas, called CpG islands (Antequera et al. 1990). These small stretches of DNA sequences are located in the promoter and first exon regions of 60% of human genes (Ng and Bird 1999). DNA methylation of cytosine within 5′ CpG islands is associated with loss of gene expression and plays a role in regulating gene expression during development. This epigenetic event is frequently associated with the transcriptional silencing of imprinted genes, some repetitive elements, and genes on the inactive X chromosome (Li et al. 1993; Singer-Sam and Riggs 1993). In neoplastic cells, the CpG islands of some tumor suppressor genes become aberrantly methylated (Uones 1996; Baylin et al. 1997).

Several techniques, such as restriction landmark genomic scanning (RLGS) and a representational difference analysis (RDA)-based method, have been developed to detect differences in methylation when searching for imprinted genes and aberrantly methylated genes in cancer cells (Hatada et al. 1993; Toyota et al. 1999). Although these techniques are very powerful for detecting methylation differences, the number of CpG islands cloned by using RLGS is limited, and the methylation difference of each gene is not detected by RDA. Very recently, a microarray-based technique for the detection of differences in methylation has been developed (Yan et al. 2001). In that method, both non-CpG sequences and CpG islands resistant to cleavage by the methylation-sensitive restriction enzyme *Bst*UI from two samples are amplified and cohybridized to the microarray panel. However, amplification of non-CpG islands can reduce the signal intensities derived from CpG islands. Moreover, when using that method, an enormous number of clones must be fixed in microarrays to detect the hypermethylated loci. Here, we describe a new method called methylation amplification DNA chip (MAD). In this method, only CpG islands, which are important for transcriptional regulation, are amplified. A microarray was made from the methylated clones of the sample in which we wanted to detect hypermethylation. This increased the percentage of DNA fragments derived from hypermethylated loci on the microarray (Fig. 1A).

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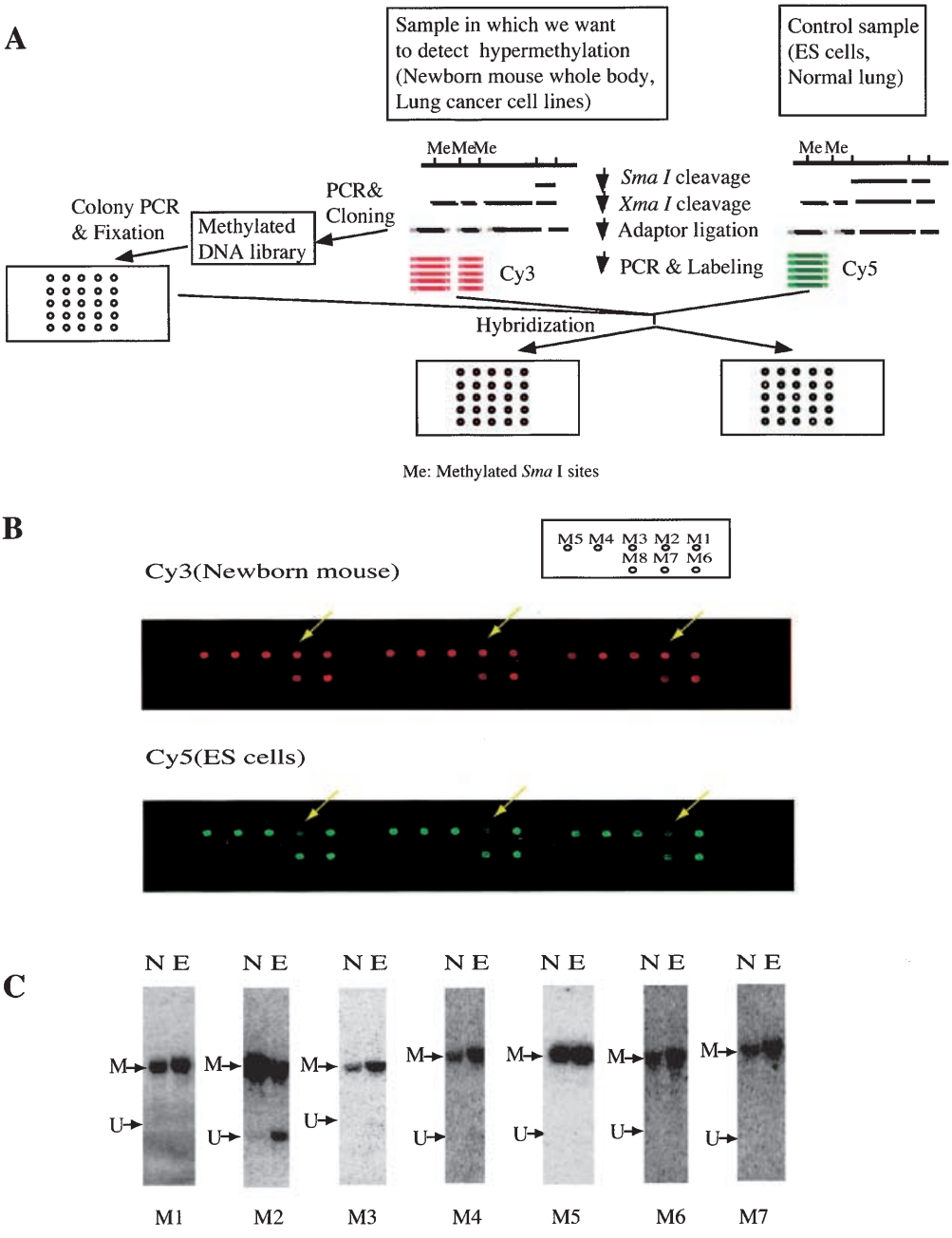

Fig. 1. A Schematic flowchart for the Methylation Amplification DNA chip (MAD) method. **B** Representative results of MAD applied to newborn mouse whole body and embryonic stem (ES) cells. Methylated CpG islands of newborn mouse whole body and ES cells were labeled with Cy3 and Cy5, respectively, and cohybridized to a microarray slide containing M1–M8 in triplicate. The hybridization output is the measured intensities of the two fluorescence reporters false-colored with *red* (newborn mouse whole body) and *green* (ES cells). M2 (*arrows*) has a stronger signal intensity (Cy3:Cy5 = 2.8) for Cy3 (newborn mouse), which indicates hypermethylation in newborn mouse compared with ES cells. **C** Southern blot analysis of newborn mouse whole body (*N*) and ES cells (*E*) using M1–M8 as probes. Digests were carried out by using the methylation-sensitive restriction enzyme *Sma*I. Methylated (*M*) and unmethylated (*U*) fragments are indicated. ME, methylated *Sma*I sites; PCR, polymerase chain reaction

Materials and methods

MAD

The procedure is illustrated in Fig. 1A. One microgram of genomic DNA was digested with 40 units of *Sma*I overnight. The DNA was further digested with 50 units of *Xma*I for 8 h. The digests were extracted with phenol:chloroform and precipitated with ethanol. The adaptor were prepared by annealing the two oligonucleotides AGCACTCTCCAG CCTCTCACCGAC and CCGGGTCGGTGA. Approximately 0.5 μg of DNA was ligated to 0.8 pmol of the adaptor by using T4 DNA ligase. Polymerase chain reaction (PCR) was performed by using 0.1 μg of each ligation mix as a template in a 50-μl volume containing 50 pmol of the primer AGCACTCTCCAGCCTCTCACCGAC, 1.25 units of *Taq* DNA polymerase, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 μM dNTP, and 15% dimethylsulfoxide (DMSO). The reaction mixture was incubated for 5 min at 72°C and for 3 min at 94°C and subjected to 25 cycles of amplification consisting of 10 s denaturation at 94°C, 30 s annealing at 70°C, and 2.5 min extension at 72°C. The final extension was lengthened to 9.5 min. PCR products were purified by using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA). One hundred monograms of PCR product was labeled as described (Pollack et al. 1999) by using Cy3-dCTP or Cy5-dCTP.

To make resource material for the microarrays, the PCR products were digested with *Xma*I and ligated to *Xma*I-

digested pBluescript. After transformation, each colony was amplified by PCR by using primers GATATCGAA TTCCTGCAGCC and CGCTCTAGAACTAGTGGATC in the presence of 1.25 units of *Taq* DNA polymerase, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 μM dNTP, and 15% DMSO. The reaction mixture was incubated for 5 min at 94°C and subjected to 40 cycles of amplification consisting of 10 s denaturation at 94°C, 30 s annealing at 60°C, and 1 min extension at 72°C. Amplified DNA fragments were fixed on poly-L-lysine-coated microscope slides as described by Schena et al. (1995) by using the SPBIO-2000 (Hitachi Software Engineering, Yokohama, Japan) arrayer. Labeled DNA was cohybridzed to the microarray and scanned by using the Scan Array4000 (GSI Lumonics, Tokyo, Japan) arrayer. Global mean normalization was used to normalize Cy3 and Cy5 signals (Roberts et al. 2000).

Methylation-specific PCR

Genomic DNA was treated with sodium bisulfite by using a CpGenome DNA Modification Kit (Intergen, Norcross, GA, USA) and subjected to methylation-specific PCR (MSP). The MSP primer sequences that specifically recognized the methylated MIN26 sequence were TTTTAGAT TAACGAGTTGGGCGAC and CGACTACATCAAAA ACACGCCGA and those that recognized the unmethylated MIN26 sequence were TTGTGGAGTGATATA TTGGAAGTG and CATCAAAAACACACCAACCAA TATCA.

Results and discussion

Toyota et al. (1999) described a method called methylated CpG island amplification (MCA) for amplifying only methylated CpG islands. We utilized this method to prepare both probes and targets for hybridization. About 70%–80% of CpG islands contain at least two closely spaced (<1 kb) *Sma*I sites (CCCGGG) (Toyota et al. 1999). Only those *Sma*I sites within these short distances can be amplified by using the MCA method, ensuring representation of the most CpG-rich sequences. The DNA is cleaved with a methylation-sensitive restriction enzyme *Sma*I, which makes blunt ends, followed by cleavage with the *Xma*I, which is a methylation-insensitive isoschizomer of *Sma*I and makes 5′ protruding ends. Adaptors specific for *Xma*I-cleavage ends are ligated, and PCR amplification in the presence of 15% DMSO is performed by using primers complementary to these adaptors. As a result, only DNA fragments between two methylated and closely spaced *Sma*I/*Xma*I sites can be amplified. Methylated DNAs from the two samples that we wanted to compare were amplified by MCA and labeled as described by Pollack et al. (1999) by using Cy3-dCTP and Cy5-dCTP, respectively, and used for cohybridization. For resource material for making the microarrays, we constructed a methylated DNA library from the sample in which we wanted to detect hypermethylation. DNA from the samples in which we wanted to detect hypermethylation was amplified by MCA. Amplified DNA fragments were cleaved with *Xma*I and cloned into *Xma*I sites of pBluescript. After transformation, each colony was amplified by PCR by using the primers GATATCGAATTCCTGCAGCC and CGCTCTAGA ACTAGTGGATC in the presence of 15% DMSO. Amplified DNA fragments were fixed on poly-L-lysine-coated microscope slides as described by Schena et al. (1995) by using the SPBIO-2000 (Hitachi Software Engineering) arrayer. Labeled DNA was cohybridzed to the microarray and scanned by using the Scan Array4000 (GSI Lumonics) arrayer. Global mean normalization was used to normalize Cy3 and Cy5 signals (Roberts et al. 2000).

For a model experiment, DNAs from newborn mouse whole body (Cy3) and embryonic stem (ES) cells (Cy5) were analyzed by using MAD. The resource material for preparing the microarray was derived from a methylated DNA library of newborn mouse (M1–M7: Accession numbers AB083485-AB083491) and human control DNA (M8). Each DNA was spotted in triplicate. The spot intensity derived from each DNA was reproducible (Fig. 1B). The signal intensity of the M1–M7 spots is strong, whereas the human-derived M8 spot gives no signal. Among seven spots, the signal intensity of M2 was stronger (Cy3:Cy5 = 2.8) for Cy3 (newborn mouse), which indicates hypermethylation in newborn mouse compared with ES cells. These results were confirmed by Southern blot analysis with *Sma*I (Fig. 1C).

We applied MAD to three lung cancer cell lines (EBC-1, 1-87, and LK79). The resource material for preparing the microarray was derived from the methylated DNA library (H1–H192) of four cancer cell lines (EBC-1, 1-87, PCI19, and PK1; the latter two are pancreatic cancer cell lines) and hypermethylated DNA fragments (MINT1–MINT32) in a colon cancer cell line Caco2 (Toyota et al. 1999). The methylated DNAs from the cancer cell lines were labeled with Cy3, and DNA from normal lung was labeled with Cy5. Each Cy3-labeled methylated DNA from a cancer cell line was cohybridized with Cy5-labeled methylated DNA from normal lung. The percentages of hypermethylated loci in the cancer-cell DNA (Cy3:Cy5 > 10) were 3.2% (H130, MINT23, H180, H156, MINT26, MINT19, H23), 2.3% (H130, H23, MINT6, H139, MINT5), and 6.3% (H130, MINT23, MINT26, H23, MINT6, H139, MINT5, MIN31, MINT12, MINT32, MINT24, MINT20, H152, H9) for EBC-1, 1-87, and LK79, respectively. These percentages are much higher than those described by a previous report (Yan et al. 2001). There are two reasons for this. The first is that our microarray included 29 DNA fragments with hypermethylated loci (MINT1–MINT32) in a colon cancer cell line. This indicates that common loci were hypermethylated in lung and colon cancer cells. The second reason is that only DNA fragments with methylated loci in cancer cells were fixed on our microarray. In the previous study (Yan et al. 2001), the origins of the fixed DNAs were not restricted to methylated loci. By choosing the methylated DNA library for the resource material of the microarray, we increased the percentage of DNA fragments derived from hypermethylated loci on the microarray. The loci that were hypermethylated in at least two cancer cell lines are listed in Fig. 2A. We picked up MINT26 for further

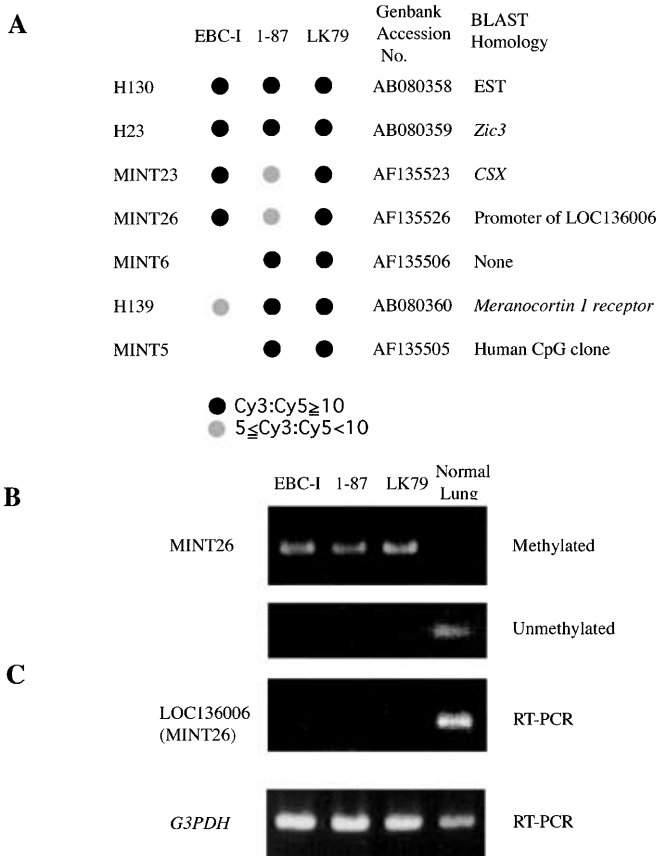

Fig. 2. A Summary of the seven differentially methylated loci. **B** Methylation analysis of MINT26 (LOC136006). Methylation-specific PCR primer sequences that specifically recognized the methylated MIN26 sequence were TTTTAGATTAACGAGTTGGGCGAC and CGACTACATCAAAAACACGCCGA and those that recognized the unmethylated MIN26 sequence were TTGTGGAGTGATATAT TGGAAGTG and CATCAAAAACACACCAACCAATATCA. **C** Reverse transcriptase (RT)-PCR analysis of LOC136006 (MINT26). The primers used for LOC136006 were GGAGGAGTTCGTGGT AAGATG and GTGCTCTGGGAAGGATTACC. The primers used for *G3PDH* were ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA

analysis because this locus is in the promoter of *LOC136006* (GenBank accession number: XM_069647), which is similar to *Neuralin 1*. DNAs from each cancer cell line and from normal lung were treated with sodium bisulfite as described previously (Herman et al. 1996) and amplified by PCR. In three cancer cell lines, 150-bp fragments were amplified by primers that specifically recognized the methylated MIN26 sequence, thus indicating the methylation of this gene (Fig. 2B). In contrast, 61-bp fragments were amplified by primers that specifically recognized the unmethylated MINT26 sequence in normal lung. Therefore, the hypermethylation of this gene was confirmed. To determine whether this hypermethylation was related to the expression of this gene, we performed reverse transcriptase (RT)-PCR analysis on these samples (Fig. 2C). No fragment was amplified from any of the three cancer cell lines, whereas a 198-bp fragment was amplified from normal lung, indicating a correlation between methylation and expression.

Very recently, a microarray-based technique for the detection of differences in methylation was developed (Yan et al. 2001). In that method, genomic DNA is cleaved with *Mse*I followed by linker ligation. PCR amplification after cleavage with the methylation-sensitive restriction enzyme *Bst* UI results in the amplification of methylated *Mse*I fragments. PCR products are labeled and hybridized to a microarray containing clones from a CpG island library. *Mse*I fragments are not abundant in CpG islands, indicating that both non-CpG and CpG islands are used for hybridization. In our method, only CpG islands were amplified and used for hybridization, resulting in high sensitivity. In addition to this merit, our method has the great advantage of reducing the number of fixed clones on the microarray by restricting the resource to a methylated DNA library of the sample in which we want to detect hypermethylation. Moreover, our method would be useful for finding imprinted genes or tumor suppressor genes whose expression levels are too low to be found by an expression-based method.

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