

Ultra-Sensitive Immunodetection of 5′Methyl Cytosine for DNA Methylation Analysis on Oligonucleotide Microarrays

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Abstract

For the determination of methylation levels in genomic regulatory DNA sequences a high-sensitive assay for detecting 5′methyl-cytosines (5′mC) in non-bisulfite-treated DNA has been established. The system is designed for the application of immunofluorescence using a monoclonal antibody that specifically recognizes 5′mC in single-stranded DNA hybridized to oligonucleotide microarrays. For assay readout an ultra-sensitive fluorescence scanner with submicrometer resolution was used. To minimize autofluorescence 150- μm thin glass slides with an aldehyde-functionalized surface were developed. These methodological improvements allowed the detection of 5′mC in synthetic oligonucleotides hybridized to microarrays with atto molar analytical sensitivity. Using enzymatic fragmented genomic DNA from myeloid leukemia tumor cell lines differences in the methylation status of gene regulatory sequences for E-cadherin, p15/CDKN2b and p16/CDKN2a were demonstrated. Thus, this novel technique can potentially be used for DNA methylation analysis in various scientific fields.

Key words: epigenetics; DNA methylation; oligonucleotide microarray; immunofluorescence

1. Introduction

The regulation of gene expression by epigenetic mechanisms such as cytosine methylation contributes to various biological processes like parental genomic imprinting, X-chromosomal inactivation, cellular differentiation and aging as well as the development of malignant disorders.^{1–4} A variety of methods for the analysis of CpG methylation status have been developed including chromatographic separation, use of methylation-sensitive restriction enzymes and bisulfite-driven conversion of non-methylated cytosine to uracil.⁵ Despite the widespread application of the latter and the ongoing technical ameliorations regarding the bisulfite protocol and methylation-specific PCR, still several limitations exist. These concern DNA degradation during sodium-bisulfite treatment due to oxidative damage, depurination under the required acidic and thermal conditions as well as

inconsistent conversion of cytosine to uracil in a given DNA sample.^{6,7} To overcome these technological difficulties we developed a novel method for 5′mC analysis applying a microarray format with immunodetection of 5′mC directly on genomic DNA without sequence conversion or DNA amplification. This was achieved by the exploitation of recent improvements in the field of array manufacture (150- μm thin aldehyde glass slides), ultra-sensitive surface molecule detection and the utilization of a fast scanning system with sufficient geometrical resolution.^{8,9}

2. Materials and Methods

2.1. Cell lines and genomic DNA preparation

Human acute myeloid leukemia (AML) cell lines HL-60 (American Type Culture Collection) and KG1a (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany) were grown in RPMI-1640 medium (Gibco Invitrogen,

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Paisley, Scotland) supplemented with 10% FCS (Gibco), L-glutamine (300 mg/l; Gibco) and antibiotics [Streptomycin (100 μ g/ml)/Penicillin (100 U/ml); Gibco]. Both cell lines are extensively described and epigenetically characterized.^{10–13} Genomic DNA from cell lines was isolated according to Qiagen's QIAmp Blood Kit procedure manual (Qiagen, Hilden, Germany). Restriction enzyme digest was performed with *MnII* from NEB (New England Biolabs).

2.2. Methyl-specific PCR (MS-PCR)

Chemicals and oligonucleotides were purchased at Sigma-Aldrich Chemie GmbH, Steinheim, Germany and Metabion GmbH, Munich, Germany, respectively. Methyl-specific PCR (MS-PCR) was performed with primer sequences as published by Herman et al.¹⁴ Minor modifications concerned the use of HotStarTaq DNA polymerase (5 U/ μ l; Qiagen, Hilden, Germany), sodium-bisulfite treatment at 55°C for 4 h and 40 PCR cycles in a Primus 96^{plus} MWG-biotech thermal cycler starting with 400 ng bisulfite-treated DNA. All PCRs were performed with positive controls for both unmethylated and methylated alleles. PCR products were analyzed by electrophoresis on 1% agarose gels and ethidium bromide staining.

2.3. Microarray production

To minimize background fluorescence we used borosilicate glass slides (50 \times 24 \times 0.15 mm³; Stözl-Oberglas, Austria) with an aldehyde surface obtained by standard surface chemistry.¹⁵ Amino-modified capture oligonucleotides were coupled to the slide surface by reductive amination.

2.4. Capture oligonucleotide dilution and spotting process

Sense capture oligonucleotides were diluted to 5 μ M in 1 \times SSC [15 mM sodium citrate, 150 mM sodium chloride, pH 7.0 with 2.5% glycerol (Sigma-Aldrich)]. The contact spotting robot (SpotBot), from ArrayIt, CA, was used with Tungsten Split Pins (PT3000; Point Technologies, Boulder, CO) for the microarray printing process, resulting in spots of \sim 50- μ m diameter with a volume of 100 pl. Oligonucleotide immobilization was performed at room temperature by incubation in a humid environment for 12 h. For stabilization of the intermediary carbon–nitrogen double bond between aldehyde surface and amino-modified capture oligonucleotides to form a secondary amine, sodium cyanoborohydride [0.02 M in 0.1 M carbonate buffer; pH 8.2 (Sigma-Aldrich)] was applied by additional 2 h incubation. After a 15 min washing step in carbonate buffer (0.1 M; pH 8.2) microarrays were dried and immediately used for hybridization experiments.

Table 1. Oligonucleotide sequences

Oligonucleotide name	Sequence 5'–3'
Target oligonucleotide: (52mer)	atxgatxgatxgatxgatxgcgctttttccagagcaatccaggcgcgccc
Positive control (33mer)	atxgatxgatxgatxgatxgtccaggcgcgccc
Nonsense: (24mer)	cccagaagcaatccaggcgcgccc
Sense p15/CDKN2b: (59mer; NT_008413.16)	ggcgcgctggtattgcttctggaaaagcgcc-tagcgcgagcagccgagctcaaa
Sense p16/CDKN2a: (58mer; NT_008413.16)	ccagcagtcagcgaaggtccatgctgctccccgcggcgctccatgctgctccc
Sense E-cadherin: (40mer; NT_010498.15)	cgccacggaggccccgagtgaccgacgtcggt-gcgtggg

Oligonucleotide description with length, sequence and NT number when indicated: All oligonucleotides, except target oligonucleotide, carried a 3'-terminal amino group with (CH)₇-linker for surface immobilization. Positive control, sense p15/CDKN2b, sense p16/CDKN2a and sense E-cadherin carried an additional 5'-terminal fluorescence label (cyanine 5) used for spot quality control; x in sequence marks 5' methyl-cytosine; positive control oligonucleotide was spotted on each microarray and was used as internal standard; nonsense, sense p15/CDKN2b and target oligonucleotide were used to test antibody specificity and sensitivity; all sense oligonucleotides were used as capture oligonucleotides for hybridization with genomic DNA.

2.5. Capture oligonucleotide design

Capture oligonucleotide design was based upon analyses of predicted promoter sequences for possible methylation sites (CpG islands).^{14,16} Capture oligonucleotides suitable to hybridize to DNA-targets generated by restriction enzyme digestion of genomic DNA were designed using OligoWiz software (Center for Biological Sequence Analysis BioCentrum-DTU Technical University of Denmark). Sequence information concerning capture and hybridization oligonucleotides (VBC-genomics, Vienna, Austria) including modifications and fluorescence labels are summarized in Table 1.

2.6. Hybridization experiments

Hybridizations were performed at 55°C (oligonucleotides) or 65°C (genomic DNA), respectively, in a hybridization buffer mix containing 4 \times SSC and 0.1% SDS (Sigma-Aldrich, Chemie). The used surface inactivation (sodiumcyanoborohydride buffer) and blocking conditions [0.2% BSA (Sigma-Aldrich) in Dulbecco's phosphate-buffered saline (PBS; Gibco)] allowed to omit prehybridization or the use of competitive DNA. For single subgrid hybridization (2.5- μ l hybridization mixtures) 5 mm diameter cover slips, (Menzel Gläser, Braunschweig, Germany) and for multiple subgrid hybridization (10 μ l hybridization mix) 22 \times 22 mm² cover slips (Assistent, Sondheim, Germany) were used in Corning hybridization chambers (Fischer Scientific, Loughborough, UK) submerged in a water bath. After

overnight hybridization washes were performed with $2\times$ SSC, 0.01% SDS for 15 min, $2\times$ SSC for 15 min and $0.2\times$ SSC for 15 min with all washing solutions preheated to 42°C .

2.7. Microarray immunolabeling

Slides were blocked for 30 min at room temperature with 0.2% BSA in Dulbecco's PBS and incubated with a monoclonal anti-5'mC antibody (clone: 33D3; mouse IgG1; Serotec, Düsseldorf, Germany) or isotype-matched control antibody (anti-collagen clone: COL-1; mouse IgG1) diluted in PBS-BSA-Tween [BSA 0.1%; Tween-20 0.05% (Sigma-Aldrich Chemie)] for 1 h at room temperature at a concentration of $1\ \mu\text{g}/\text{ml}$ each. Primary antibody incubation was followed by a washing step in PBS for 5 min and secondary antibody incubation (cyanine 3 dye conjugated secondary goat anti-mouse antibody; 1 : 2000 diluted in PBS-BSA-Tween, Amersham) for 1 h at room temperature. After three 5 min washing steps in PBS, microarrays were dried and scanned immediately.

2.8. Ultra-sensitive microarray readout

The device applies a novel readout technology that combines wide-field illumination as well as time delay and integration (TDI) mode for readout.⁹ In brief, the apparatus used in our measurements comprises an inverted microscope (Axiovert 200, Zeiss, Germany), a high-precision scanning stage (Märzhäuser Wetzlar, Germany) and a charge-coupled device camera (CCD camera) with high quantum efficiency and low readout noise running in TDI mode (CoolSNAP HQ, Photometrics, Roper Scientific, USA). For illumination of cyanine 3 and cyanine 5 we used an Nd : Yag laser (Neodym-Ion doted Yttrium-Aluminium-Granat) with $\lambda = 532\ \text{nm}$ (Millennia II, Spectra-Physics, USA) and a Krypton-Ion laser with $\lambda = 647\ \text{nm}$ (43 Series Ion Laser, Melles Griot, USA), respectively. High laser intensities, $\times 100$ optical magnification (0.516 nm pixel resolution) and short exposure times (250 ms) allowed ultra-sensitive microarray readout within minutes.

2.9. Image analysis and statistics

For image and data analysis we used ImaGene® 5.6 (BioDiscovery, CA, USA) together with ArrayNorm® 1.7 (Graz University of Technology Bioinformatics Group, Austria),¹⁷ a software for array normalization and data visualization control. After background correction, statistical analysis of fluorescence signal intensities comparing the cell lines was done with two-tailed Student's *t*-test.

3. Results and discussion

Designed amino-modified and fluorescence-labeled capture oligonucleotides were spotted on to the aldehyde

surface-coated glass cover slips, hybridized with selected target oligonucleotides or enzyme digested genomic DNA. The consecutive indirect anti-5mC antibody staining was followed by readout on the fluorescence scanning platform (schematically outlined in Fig. 1). Cell line and target gene selection were based solely on well-established promoter/gene methylation models: E-cadherin, p15/CDKN2b and p16/CDKN2a from two AML-derived tumor cell lines (HL-60, KG1a). To validate the results obtained with the anti-5'mC microarray we analyzed representative CpG-island containing DNA fragments by MS-PCR.

3.1. Antibody specificity

To test the anti-5mC antibody specificity (Clone 33D3¹⁸), target oligonucleotides designed with five consecutive 5'methyl CpG dinucleotides (100 aM, 600 molecules per μl), spaced by 2 nt, and nonsense oligonucleotides without 5'mC were hybridized to the immobilized capture oligonucleotide ($1\ \mu\text{M}$) (Fig. 2). The anti-5mC antibody signal-to-noise ratio (SNR) of spot mean fluorescence intensity (mfi in counts) for the 5'mC positive target oligonucleotide was $\text{SNR} = 7$, in contrast to 5'mC negative nonsense control with an average SNR of 1. The minimum relevant signal is defined as the signal for which the SNR is ≥ 3 .¹⁹ Control experiments on sense and nonsense capture

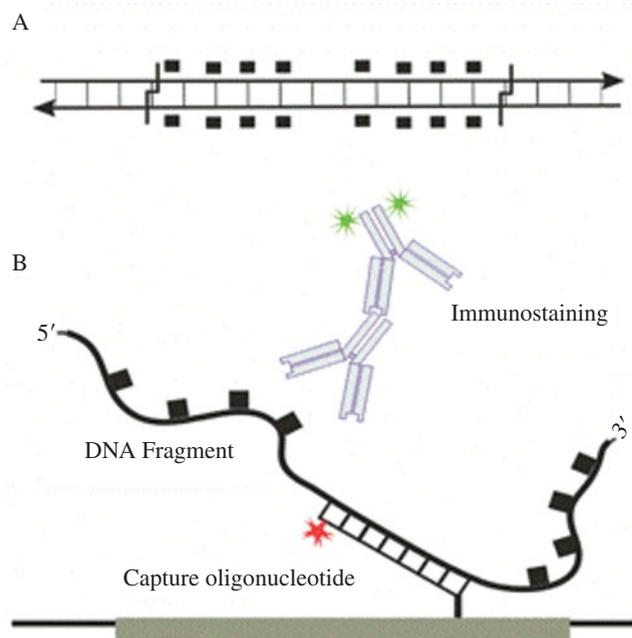


Figure 1. Schematic assay system. (A) Specific promoter or CpG island region with 5'mC and restriction enzyme digest. (B) 5'Methyl-cytosine detection via indirect immunofluorescence on DNA fragments hybridized to capture oligonucleotides. Black squares, 5'methyl-cytosine; empty black squares, normal nucleotides; black line, single strand DNA fragment; antibody symbols, primary and secondary antibodies; capture oligonucleotide, cyanine 3 labeled; detection antibody, cyanine 5 labeled; gray, aldehyde-modified microarray glass surface.

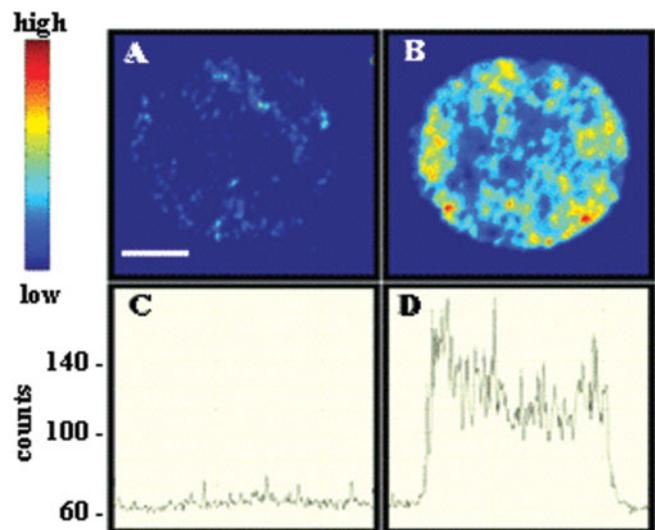


Figure 2. Antibody specificity and target oligonucleotide hybridization. 5-Methyl-cytosine detection via immunofluorescence on target oligonucleotide (100 aM) after hybridization to oligonucleotide microarray. Ultra-sensitive readout of cyanine 3 labeled secondary antibody with (A) negative control spot with nonsense capture oligonucleotide (1 μ M), (B) positive control spot with sense capture oligonucleotide (1 μ M), (C) fluorescence intensity profile of negative control spot and (D) fluorescence intensity profile of positive control spot (SNR = 7). A and B original scan images (magnification $\times 100$, spot diameter = 30 μ m; bar 10 μ m). C and D fluorescence intensity in counts/pixel, pixel resolution 64 nm.

oligonucleotides after target hybridization with unspecific primary monoclonal antibody (anti-collagen) and labeled anti-IgG1 secondary antibody gave no signals (data not shown).

3.2. Assay sensitivity

Hybridization experiments were performed with sense and nonsense capture oligonucleotides hybridized with a series of increasing target oligonucleotide concentrations (600 aM to 38.4 fM; Fig. 3). Signals of 10 repeats from 2 hybridization experiments plotted against the target oligonucleotide concentration are linear ($R^2 = 0.96$) from 600 aM to 38.4 fM. On a minimal slide background the 5'-mC-specific signals resulted in an SNR of 8 (mfi/SD 18/8) for the 600 aM spot and up to an SNR of 83 (mfi/SD 249/125) for the 38.4 fM spot. Signal intensities for target oligonucleotide hybridized to nonsense capture oligonucleotide are similar to slide background. A detection limit at 600 aM target oligonucleotide represents 1.5 fg DNA per hybridization volume of 3 μ l, corresponding to the DNA content of nuclei from 540 cells. The detection limit of methylation-specific PCR is in the range of 50–500 pg DNA representing 10–100 cells.²⁰

3.3. MS-PCR

MS-PCR was done using DNA from the cell lines HL-60 and KG1a which are well characterized with regard to the methylation status of p15/CDKN2b, p16/CDKN2a and

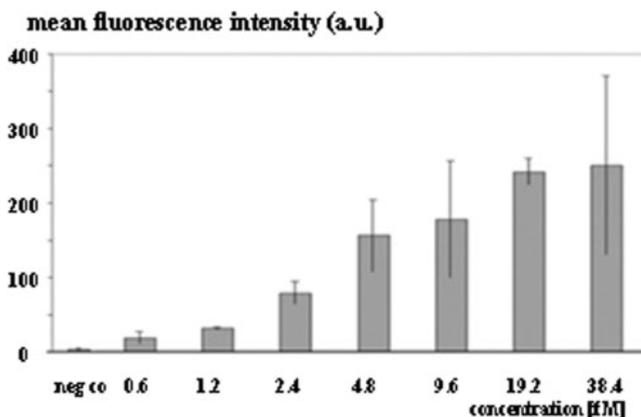


Figure 3. Detection limit and dynamic range of the 5-methyl-cytosine microarray immunostaining. Microarrays were hybridized with various concentrations from 600 aM to 38.4 fM of target oligonucleotide carrying five 5-methyl-CpG nucleotides. Capture oligonucleotide probes were 10-fold printed at a concentration of 5 μ M; mfi/SD (y -axis); fM concentrations of target oligonucleotide and no DNA (x -axis).

E-cadherin promoter regions.^{14,12} Observed results were concordant with reported data: promoters for p15/CDKN2b and p16/CDKN2a are unmethylated in HL-60 cells and methylated in KG1a cells (Fig. 4).

3.4. Genomic DNA hybridization

Microarray capture oligonucleotides and restriction enzymes were designed to bind and cut exactly the same CpG islands detected by MS-PCR. *MnII* digest result in a 147 bp fragment for p15/CDKN2b and a 111 bp fragment for p16/CDKN2a including the region amplified by MS-PCR. *MnII* digest concerning E-cadherin results in a 105 bp fragment in the preproprotein region for E-cadherin, a hypermethylated CpG region in certain tumors.²¹ Genomic DNA was digested with *MnII* over night at 37°C, checked on agarose gels, quantified, split and used for MS-PCR analysis as well as for hybridization experiments in parallel. Experiments consisted of the hybridization with 1.5 μ g DNA, representing 3×10^5 cells, on individual microarray slides with p15/CDKN2b, p16/CDKN2a and E-cadherin capture oligonucleotides. Each microarray subgrid contained 15 identical spots per gene, and negative (nonsense oligonucleotide) and positive control spots (33mer, see Table 1 and Fig. 5 first two lines left). Data analysis relying on 45 single spots from three successive hybridization experiments represented a reliable number of data for adequate statistical analysis. Original microarray scans of genomic DNA hybridization after immunoassaying for p15/CDKN2b, p16/CDKN2a and E-cadherin promoter methylation are shown in Fig. 5. Spot signal quantification and data analysis revealed a clear discrimination between methylated and unmethylated promoter DNA for all three genes selected. Mean fluorescence intensities of promoter regions from p15/CDKN2b (mfi/SD 222/33)

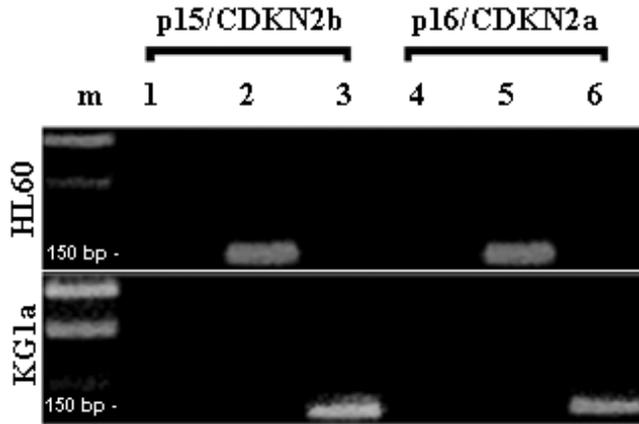


Figure 4. Methylation-specific PCR results. MS-PCR results for p15/CDKN2b and p16/CDKN2a after bisulfite treatment with wild-type primer (lanes 1 and 4), methylated (lanes 2 and 5) and unmethylated (lanes 3 and 6) primer sets on genomic DNA extracted from cell lines HL-60 and KG1a. m = marker; amplified fragment length = ~150 bp.

and p16/CDKN2a (mfi/SD 159/34) from HL-60 cells showed significantly lower signals than from KG1a with p15/CDKN2b (mfi/SD 672/130) and p16/CDKN2a (mfi/SD 568/138) ($P < 0.0001$ in both cases), whereas E-cadherin promoter region displayed inverted measures with significantly higher methylation signals in HL-60 (mfi/SD 183/23) than in KG1a (mfi/SD 63/4) ($P < 0.0001$) and reduced CpG methylation density compared to p15/CDKN2b and p16/CDK2a. The intra-assay analytical correlation of variance for p15/CDKN2b was 8.5%, for p16/CDKN2a 10.0% and for E-cadherin 7.6%. The overall consistency expressed as inter-assay variability between hybridization experiments was 15% on average, showing that the presented microarray experiments met general MIAME quality criteria.²²

Unlike other currently used methods, such as bisulfite conversion dependent MS-PCR or microarray techniques which focus on sequence details within individual primer pairs or capture oligonucleotide sequences, the presented method is capable of detecting all methylated cytosines per hybridized DNA fragment at once. Moreover our approach can be used for any region of the genome whether or not methylation-sensitive restriction enzyme sites are present. Practically, as a consequence of target DNA length (less than few hundred nucleotides), hybridization specificity can be optimized with the possibility to reduce the capture oligonucleotide numbers significantly compared with other microarray techniques for DNA methylation analysis.

4. Conclusion

We have examined the feasibility of a novel microarray for 5'mC detection using immunofluorescence measurement. This method combines the advantages of

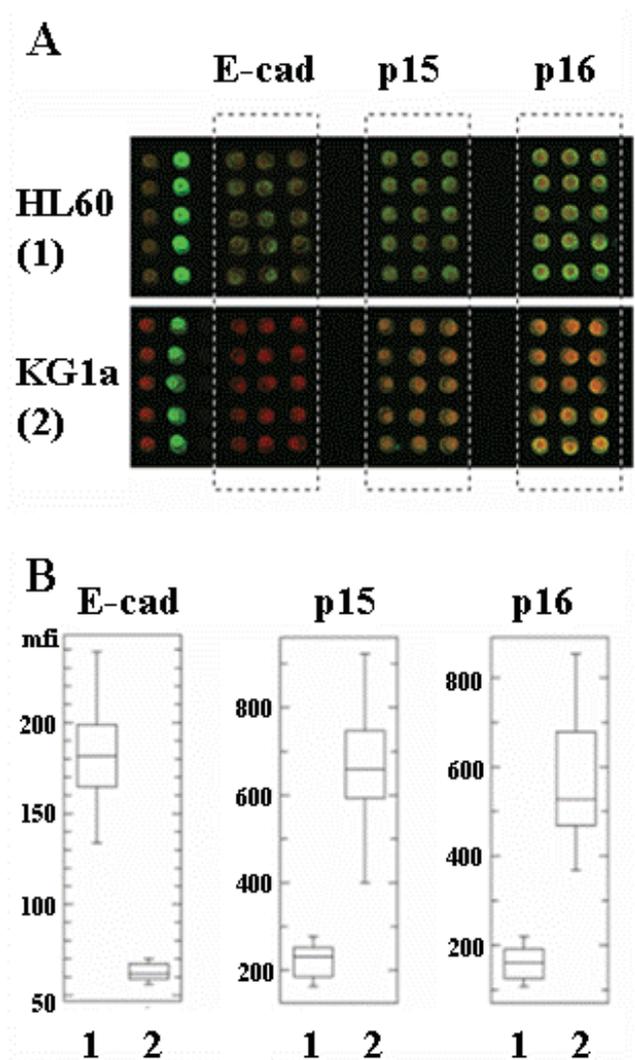


Figure 5. Microarray methyl-cytosine immunodetection on genomic DNA. Hybridization of restriction enzyme digested genomic DNA of two AML tumor cell lines. **A.** Top scan of HL-60 (1) and bottom scan of KG1a (2) microarray. Overlaid scans display methylation signal (cyanine 3: green signal) of KG1a DNA compared to HL-60 and capture oligonucleotide scan (cyanine 5: red signal). For negative control (-) spotted nonsense oligonucleotide without 5'mC were used; positive control (+) with 5'mC modified control oligonucleotide. Original magnification: $\times 100$; spot distance: 150 μm ; pixel resolution: 0.516 μm . **B.** Box plot analysis of A reveal that HL-60 is methylation negative for p15/CDKN2b and p16/CDKN2a but methylation positive for E-cadherin. Results for KG1a are inverted. Box plots of 45 individual spots per gene show median, 75% percentile and outliers. $P < 0.01$ for all three genes.

microarray technology (high-throughput), fluorescence detection (high sensitivity) and immunolabeling (high specificity) and might become an adequate tool for investigating DNA methylation in various scientific fields.

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