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MSRE-PCR for analysis of gene-specific DNA methylation

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ABSTRACT

Abnormal DNA methylation is observed in certain promoters of neoplastic cells, although the likelihood of methylation for each individual promoter varies. Simultaneous analysis of many promoters in the same sample can allow use of statistical methods for identification of neoplasia. Here we describe an assay for such analysis, based on digestion of genomic DNA with methylation-sensitive restriction enzyme and multiplexed PCR with gene-specific primers (MSRE-PCR). MSRE-PCR includes extensive digestion of genomic DNA (uncut fragments cannot be identified by PCR), can be applied to dilute samples (<1 pg/μl), requires limited amount of starting material (42 pg or genomic equivalent of seven cells) and can identify methylation in a heterogeneous mix containing <2% of cells with methylated fragments. When applied to 53 promoters of breast cancer cell lines MCF-7, MDA-MB-231 and T47D, MSRE-PCR correctly identified the methylation status of genes analyzed by other techniques. For selected genes results of MSRE-PCR were confirmed by methylation-specific PCR and bisulfite sequencing. The assay can be configured for any number of desired targets in any user-defined set of genes.

INTRODUCTION

Tumor-specific changes in DNA methylation have been observed in many different malignancies and are frequently described as global hypomethylation combined with local hypermethylation [reviewed in (1–5)]. Global hypomethylation (6) is linked to genomic instability of a tumor (7), whereas hypermethylation of specific genes correlates with their silencing (8) and can induce point mutations owing to spontaneous deamination of 5me-C (transversion C>T) (9). Silencing of a tumor suppressor gene can lead to enhanced transformation and increased tumor growth through disruption of the normal regulatory mechanisms of the affected cell (10,11). Given that DNA methylation is a specific chemical modification of one of the most stable biological macromolecules, the DNA methylation status of a selected gene is an attractive diagnostic biomarker (12), and the potential of DNA methylation for early diagnosis, outcome prediction and therapy adjustments is well recognized (13). Unfortunately, no known gene is always methylated in a given tumor: the highest frequency of methylation reported thus far is in the promoter of 14-3-3σ [stratifin; methylated in 96% of breast carcinomas and in 38% of atypical hyperplasias (14)], thus simultaneous rapid and high-throughput evaluation of methylation events in many promoters can increase the diagnostic value of promoter methylation, increasing the reliability of cancer detection (15).

In this paper we describe a procedure of methylation-sensitive restriction enzyme digestion PCR (MSRE-PCR), which can be used for rapid detection of DNA methylation in multiple fragments simultaneously. This procedure is based on extensive digestion of genomic DNA with methylation-sensitive restriction enzyme (MSRE) followed by multiplexed PCR amplification of user-defined genes using gene-specific primers. Although elimination of unmethylated fragments from the pool of potential PCR templates by MSRE digestion has been tried before (16,17), the requirements for high specificity and sensitivity of the assay present substantial problems that have been resolved in MSRE-PCR, which allows analysis of DNA methylation in a genomic equivalent of seven cells and can reliably detect methylation present in <2% of the sample.

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MATERIALS AND METHODS

Cell culture

MCF-7 and T47D cells were maintained in phenol red containing RPMI 1640 supplemented with 10% FBS, 100 µg/ml streptomycin, 100 U/ml penicillin, 6 ng/ml bovine insulin, 2 mM L-glutamine and 100 mM non-essential amino acids. Estrogen receptor-negative MDA-MB-231 cells were maintained in phenol red-containing MEM with 10% CBS and the same additives as MCF-7 and T47D. All materials were obtained from Invitrogen (Carlsbad, CA). The uPA, E-cadherin, SRBC and calcitonin cDNA containing plasmids were obtained from Invitrogen or the American Type Culture Collection (Rockville, MD).

DNA isolation

Genomic DNA was isolated from tissue culture cells using the DNeasy Tissue Kit (Qiagen, Valencia, CA), and DNA concentration was determined using DyNA Quant 2000 (Hoefer, Amersham Biosciences, Piscataway, NJ).

DNA digestion and purification

Digestions were performed with Hin6I (recognition site GCGC; Fermentas, Hanover, NJ). Typically, 500 ng of genomic DNA were mixed with 100 pg of pUC19 and digested with 40 U of the enzyme at 37°C for 72 h under a layer of mineral oil; the final volume of the reaction was 50 µL. Control samples were treated in the same way but without the addition of the enzyme. After incubation, digested samples were purified using DNA Clean Up and Concentrator-5 (Zymo Research, Orange, CA) and eluted in 100 µL of TRIS–EDTA. Control samples were ethanol precipitated and dissolved in 100 µL of TRIS–EDTA.

Primer design and PCR amplification

Genomic fragments containing at least two but no more than six Hin6I recognition sites and located within corresponding CpG islands were selected for amplification. Primer design was done using Clone Manager Suite 7, version 7.01, with Primer Designer 5, version 5.01 (Scientific and Educational Software, Durham, NC). Primers used in this study are available upon request.

KlenTaq1 was from AB Peptides (St Louis, MO) or DNA Polymerase Technology (St Louis, MO) and was used at 0.2 U per reaction. The buffer supplied with the enzyme was used without further adjustments, except the addition of betaine (Sigma, St Louis, MO) to 1.5 M final for each reaction; dNTPs (Sigma, St Louis, MO) were added to 0.2 mM; primers were used at 0.4 µM each. An ABI 9600 thermocycler was programmed for touch-down PCR (94°C, 40 s; T_a °C, 40 s; 72°C, 60 s), with T_a starting at 65°C and reducing 2°C every two cycles to 55°C followed by 35 cycles with T_a at 55°C. Final extension was for 10 min at 72°C and incubation was at 4°C. Finally, 3 µl of the product were loaded onto 2% agarose or 12% polyacrylamide and visualized after ethidium bromide staining.

Northern blot confirmation

RNA extraction and northern blot were performed as described elsewhere (18).

Methylation-specific PCR

Methylation-specific PCR (19) was performed using the CpGenome Modification kit (Intergene–Serologicals, Norcross, GA). MethPrimer (20) was used for primer design; primer sequences are available upon request. PCR conditions were as described elsewhere (21).

Bisulfite sequencing

Bisulfite sequencing was performed using bisulfite-modified DNA (see above) and sequencing primers F_{calc} (GAATTTT-AAGTTAGAGTTTTTGTTTT) and R_{calc} (AATTTCAATTC-CACTATACCTAAAC), designed using MethPrimer (20). PCR conditions were as described elsewhere (21). PCR fragments were cloned into pGEM-T-Easy (Promega, Madison, WI) as described by the manufacturer, and 10 individual clones were sequenced using M13 primer. No fewer than six clones were evaluated for CpG>TpG conversion of CpG sites.

RESULTS

The overall schema of the experiment is presented in Figure 1A. Successful extensive digestion of DNA in the sample is a critical element of the procedure, and to monitor this process we used an internal substrate (pUC19 DNA) added before digestion to control samples and samples incubated with the enzyme. After purification of digested DNA a separate PCR reaction was performed using pUC19-specific primers flanking two GCGC sites in pUC19. Results of this PCR (35 cycles) served as a quality control for the digestion procedure; a typical example is shown in Figure 1B, where control (no enzyme) incubations allow formation of PCR product (template is preserved), whereas samples incubated with the enzyme do not (template is destroyed). A separate control—incubation of *in vitro* methylated pUC19 DNA with the enzyme—invariably produced amplifiable DNA, thus confirming the purity of the restriction enzyme (data not shown).

Once the sample passed this quality control it was used for PCR with gene-specific primers designed to amplify genomic fragments located within CpG islands (Figure 1C). Multiplex PCR—4 fragments for agarose gel (Figure 1C) and up to 12 fragments for polyacrylamide gel analysis (data not shown)—was used; gel resolution and the overall requirement for size difference within amplified fragments appeared to be the limiting factors for further multiplexing. Since the absence of PCR products in digested samples can indicate either its sensitivity to the enzyme or the failure of a specific primer pair, bands in the control sample were compared with bands in the matching digested sample, and only samples producing expected bands in the undigested control were scored.

The assay can be used with small amounts of DNA: 20 pg (the genomic equivalent of three cells) were sufficient for PCR analysis (Figure 1D, lane 3). Digestion parameters were optimized for small amounts of DNA; in selected conditions we always observed successful digestion even when DNA concentration was extremely low (Figure 1D; lanes 4–6). Significantly, a 100-fold increase in the amount of digested genomic DNA did not result in formation of PCR product for estrogen receptor promoter α (Figure 1D; lanes 4–6), much less in formation of the product to the extent seen in undigested

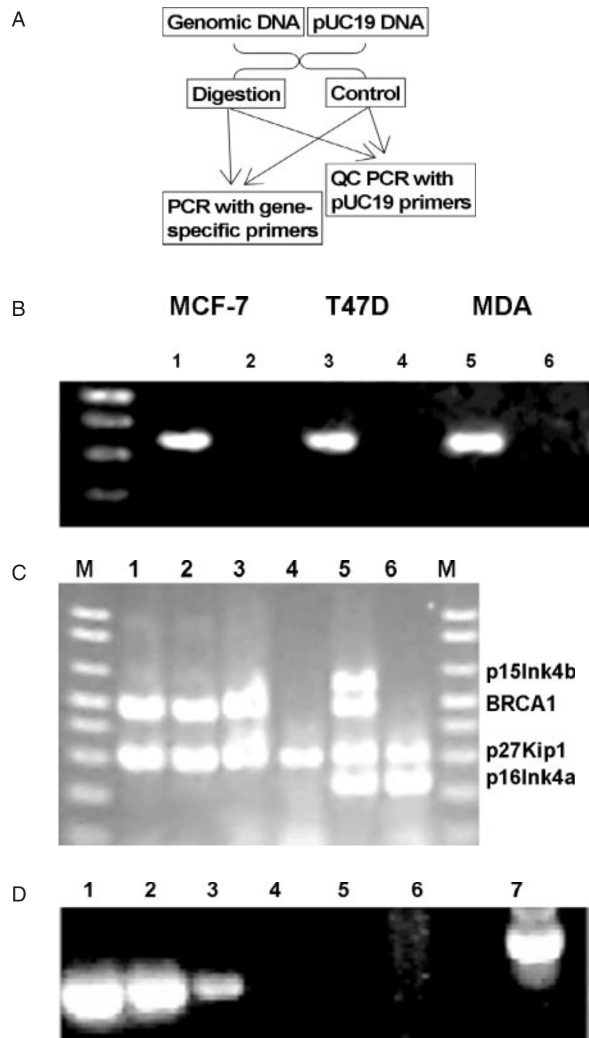

Figure 1. Methylation-sensitive restriction enzyme digestion and PCR with gene-specific primers (MSRE-PCR). (**A**) Schema of the experiment. Genomic DNA is mixed with pUC19 DNA (internal control) and separated into two aliquots. One of them is treated with Hin6I, and the other is incubated in identical conditions but without the enzyme. Quality control PCR is then performed with pUC19-specific primers, and successfully digested samples are used for PCR with gene-specific primers. (**B**) An example of quality control PCR using primers for pUC19. Lanes 1, 3 and 5 contain PCR products obtained with control (undigested) DNA. Lanes 2, 4 and 6 were loaded with a PCR reaction mixture performed with experimental (digested) DNA. All three samples (MCF-7, T47D and MDA-MB-231) successfully passed quality control. (**C**) An example of MSRE-PCR. Gene-specific primers for p15Ink4b, p16Ink4a, p27Kip1 and BRCA1 were used in a tetraplexed format. Lanes 1, 3 and 5 contain PCR products from control (undigested) DNA; lanes 2, 4 and 6 contain PCR products from experimental (digested) DNA from MDA-MB-231 (lanes 1 and 2), MCF-7 (lanes 3 and 4) and T47D (lanes 5 and 6). The absence of p15Ink4b-specific and p16Ink4a-specific fragments in both undigested (control) and digested (experimental) samples for MDA-MB-231 and MCF-7 (lanes 1, 3 and 2, 4) suggests that both genes located in 9p21 are deleted. (**D**) MSRE-PCR can be performed with 200 pg of genomic DNA. Minimal amounts of DNA are required for digestion and PCR amplification was established using DNA from T47D cells. Digestion with Hin6I was done using 20 ng (lanes 1 and 4), 2 ng (lanes 2 and 5) and 0.2 ng (lanes 3 and 6). One-tenth of the digestion mixture was used for PCR, so the amount of the template is 2 ng (lanes 1 and 4), 0.2 ng (lanes 2 and 5) and 0.02 ng (lanes 3 and 6). Primers for TMS1 (lanes 1–3) and estrogen receptor α promoter A (lanes 4–7) were used. Lane 7, undigested T47D DNA was used as a control. TMS1 promoter is methylated (bands in digested samples), while estrogen receptor α promoter A is unmethylated (no bands in digested samples and a band in undigested sample).

Table 1. Results of MSRE-PCR and the template status

PCR product in Undigested control	MSRE digested sample	Template status
Present	Present	Methylated
Present	Absent	Unmethylated
Absent	Absent	Deleted[a]

[a]Deletion of the corresponding fragment has to be verified by an alternative approach.

DNA (Figure 1D; lane 7) or in methylated DNA (Figure 1D; lanes 1–3). Apparently, digestion eliminated at least 99% of unmethylated template (20 pg versus 2 ng). Quantitative PCR results confirmed this result (data not shown).

MSRE-PCR can produce three types of outcomes (Table 1): methylation or its absence in a given region is clearly defined by the readout, whereas the absence of a band in the control (undigested) sample can indicate a deletion of a corresponding region or a single nucleotide polymorphism within the primer-binding sequence. Such regions can then be tested by independent methods.

Validation of MSRE-PCR has been carried out by comparison of methylation data for breast cancer cell lines with previously published results obtained with alternative techniques (Table 2). In the vast majority of cases MSRE-PCR and other techniques gave identical results, confirming MSRE-PCR performance. In several cases the differences can be attributed to variations in sequences examined (first exon versus promoter) or to clonal variability (see Discussion).

Besides comparison of our results with previously published data (Table 2) we validated MSRE-PCR results with three independent assays: methylation-specific PCR (MSP) (19), bisulfite sequencing (22) and northern blot analysis of gene expression (Figure 2). Primers for MSP and bisulfite sequencing were designed to analyze a fragment within the MSRE-PCR-amplified region.

MSP analysis confirmed MSRE-PCR data for all fragments tested (compare Table 2 and Figure 2A). Another confirmation was obtained with direct bisulfite sequencing (22) of calcitonin promoter in T47D and MCF-7 cells (compare Table 2 and Figure 2B). For MCF-7 cells the probability of methylated cytosine in the region analyzed is very high (90–100% for six CpG dinucleotides located within Hin6I sites), suggesting that this template would be resistant to Hin6I treatment. At the same time, in good agreement with MSRE-PCR data in T47D cells, this region is significantly less methylated (the probability of cytosine methylation within Hin6I site can reach 0%, indicating that all fragments will be cut at least once).

Northern blot analysis provided another confirmation of MSRE-PCR data, indicating a reverse correlation between mRNA expression and promoter methylation (Table 2 and Figure 2C). Importantly, although methylation of the promoter strongly correlates with the absence of expression, the reverse is not necessarily true, since negative transcriptional control can be linked to mechanisms other than methylation. This is the case with calcitonin: methylation of its promoter explains the absence of the message in MCF-7 and MDA-MB-231, whereas silencing in T47D is probably achieved through different mechanisms.

Sensitivity of the assay was tested with a mixture of DNA from T47D and MCF-7 cells (Figure 3), starting with digestion

Table 2. DNA methylation in promoters of MDA-MB-231, MCF-7 and T47D: comparison of MSRE-PCR with available data

Gene	MDA	MCF-7	T47D	Reference
14-3-3σ	M[b]	M[b]	M[a]	(36)
Apaf-1	M	M	UM	NF
BRCA1	M[b,c]	UM[a,c]	UM[b,c]	(37),(38)
Calcitonin	M	M	UM	NF
Caspase-8	UM	UM	M	NF
CycD2	M[c]	M[c]	M[a]	(40)
DAPK	UM[a,c,b]	M[c,c,c]	UM[a,c,c]	(41),(37),(38)
E-cadherin	M[a,c,c]	UM[c,c,c]	UM[c,c,c]	(42),(38),(39)
EDNRB	M	M	UM	NF
EP300	UM	UM	UM	NF
ERα-B(dist)	M[c]	M[c]	M[c]	(43)
ERα-A(prox)	M[c]	UM[c]	UM[c]	(43)
Fas	UM	UM	UM	NF
FHIT	UM	UM	UM	NF
GPC3	M[c]	M[a]	UM[b]	(37)
GR	UM	UM	M	NF
GSTP1	M[c]	M[c]	M[b]	(38)
HIC-1	M	M	M	NF
HIN-1	M[b]	M[c]	M[a]	(44)
hMLH1	UM[c]	UM[c]	UM[c]	(38)
ICAM-1	M	M	M	NF
MCT1	UM	UM	UM	NF
MDGI	M[c]	M[c]	M[a]	(45)
MDR1	M	M	M	NF
MGMT	UM[b]	UM[c]	UM[c]	(38)
MCJ	UM	UM	UM	NF
MUC2	M	M	M	NF
MYF3/MYOD1	M	M	UM	NF
p15 INK4B	D[c]	D[c]	UM[b]	(38)
p16INK4A	D[b,c]	D[a,b]	M[c,c]	(37),(38)
p21WAF1	UM	UM	UM	NF
p27Kip1	M	M	M	NF
p57Kip2	UM	UM	M	NF
p73	M[c]	M[b]	M[b]	(38)
PAX5	M[c]	UM[c]	M[c]	(46)
PR	M	M	UM	NF
RANKL/TRANCE	M	M	UM	NF
Rassf1a	M[c,c]	M[a,c]	M[c,c]	(37),(38)
RB1	UM	UM	UM	NF
RFC	M[c]	UM[c]	UM[a]	(47)
RIZ1	M[c]	UM[c]	M[b]	(48)
S100A2	M	M	M	NF
SOCS-1	UM	UM	UM	NF
SRBC	UM[a]	M[c]	M[a]	(49)
SYK	M[a]	M[b]	UM[c]	(50)
TES	UM[a]	M[c]	M[a]	(51)
THBS1	UM[c]	UM[c]	UM[c]	(38)
TMS1	M[c]	M[b]	M[c]	(52)
uPA	UM[b]	UM[a]	M[c]	(37)
VHL	UM	UM	UM	NF

[a]Untested in paper(s) referenced.
[b]Different result.
[c]Identical result.
NF, no references found.
Results from two or more papers are separated with a slash (/).

to account for potential losses throughout the procedure. DNA of MCF-7 was added as a genomic equivalent of seven cells (42 pg), and one-third of the material was used for PCR after digestion and purification. No product was observed when MCF-7 DNA was omitted from the reaction (lanes 2 and 4), whereas addition of this DNA before the digestion allowed formation of a band (lanes 1 and 3); thus DNA from seven cells (42 pg) is sufficient for MSRE-PCR, while one-third of that amount (14 pg) is sufficient for PCR-based detection.

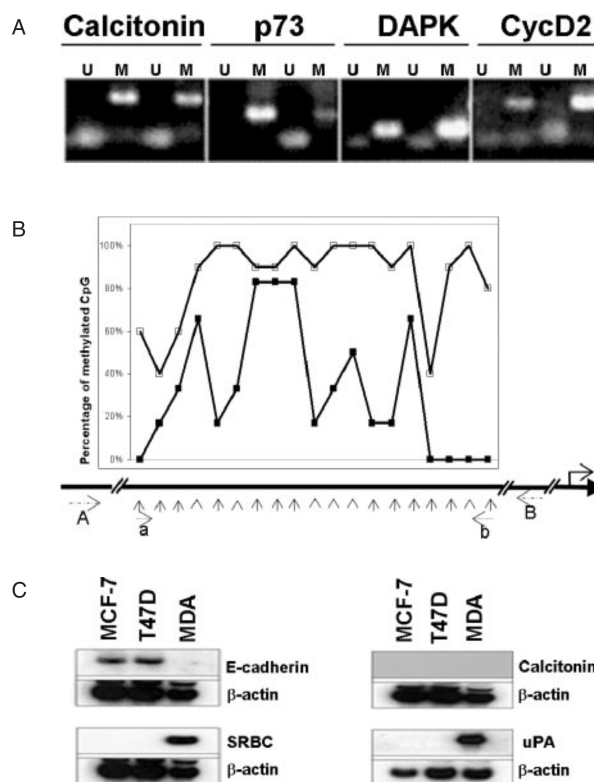

Figure 2. Validation of MSRE-PCR results with selected genes. (**A**) Methylation-specific PCR (MSP) assay of four genes in MCF-7 cells (duplicate reactions for each gene). Primers for unmethylated (U) and methylated (M) DNA of corresponding CpG islands were used. Fragments amplified for MSP are located within regions analyzed by MSRE-PCR. (**B**) Bisulfite sequencing of a calcitonin promoter fragment located within a larger fragment used for MSRE-PCR. Probability of methylation for each of 19 CpG dinucleotides for MCF-7 (open squares) and T47D (filled squares) is plotted. Vertical arrows mark the position of CpG dinucleotides; arrowheads indicate CpG dinucleotides within the Hin6I recognition site. Horizontal arrows mark the position of PCR primers for MSRE-PCR (dashed arrows, primers A and B) and primers for amplification of bisulfite-modified DNA (dotted arrows, primers a and b). (**C**) Northern blot analysis of gene expression in MCF-7, T47D and MDA-MB-231 cells. β-Actin was used as a loading control.

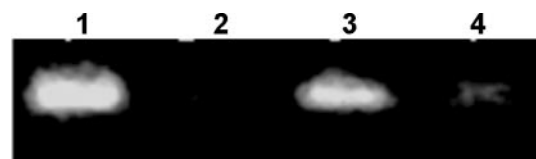

Figure 3. Sensitivity of MSRE-PCR assay for heterogeneous genomic DNA. MSRE-PCR can successfully identify methylated fragments in a mixed sample. Genomic DNA of T47D (2.6 ng, lanes 1, 2; or 1.3 ng, lanes 3,4) was mixed with 42 pg of MCF-7 genomic DNA (lanes 1 and 3) before digestion with Hin6I. Control samples (lanes 2 and 4) contained only T47D DNA. After digestion, DNA was purified and one-third of it was used for PCR with RANKL/TRANCE primers; RANKL/TRANCE is unmethylated in T47D and methylated in MCF-7. Digestion, purification and PCR were performed as described in Materials and Methods.

DISCUSSION

In this report we describe the development and validation of a technique for rapid DNA methylation analysis in a user-defined set of genes, which is based on extensive digestion

of genomic DNA with a methylation-sensitive restriction enzyme and PCR amplification of surviving fragments. A fragment will be designated 'unmethylated' if no PCR product is observed after digestion; alternatively, the fragment will be called 'methylated' if it can be amplified after digestion. In MSRE-PCR, methylation of restriction sites determines the result of the assay: if all sites are methylated the fragment will be scored as methylated even if all the CpGs outside of Hin6I sites are unmethylated. Conversely, a single unmethylated recognition site within the fragment can cause elimination of the template, and the whole fragment will be scored as unmethylated. Thus, MSRE-PCR extrapolates results of a limited number of sites for the whole fragment.

This feature of MSRE-PCR is similar to almost every method of methylation analysis: MSP (19) evaluates methylation status of primer-binding sites to make a conclusion regarding the complete amplified region; COBRA (23) tests restriction sites left unmodified by bisulfite treatment; MethyLight (24) detects methylation in 1–5 CpG sites covered by primers and probe; QAMA (25) measures methylation in a 14 bp fragment covered by a TaqMan probe; MethylQuant (26) assays methylation of a single cytosine in bisulfite-modified DNA. It appears that bisulfite sequencing (27) is the only technique that provides the methylation profile of each and every cytosine within the fragment.

Although similar to bisulfite treatment-based methods of methylation analysis in evaluating methylation in a limited number of sites, MSRE-PCR allows simple multiplexing and avoids some of the problems inherent in bisulfite conversion, in particular the poorly controlled efficiency of modification, which can be incomplete owing to incomplete denaturation or partial renaturation of DNA during treatment (28). Comprehensive modification of unmethylated cytosines is required for correct readout, which can be influenced by various factors (28,29), including DNA apurinization during bisulfite treatment (30). Downstream differentiation of the methylated versus unmethylated sequence in many bisulfite-based methods requires two pairs of primers and two PCR reactions for each potentially methylated fragment (19), which reduces the throughput of MSP and similar techniques, making screening of clinical samples more labor-intensive. Finally, the yield of each product depends on the quality of the corresponding primers and can result in biased PCR if the amplification efficiency is different (31).

The rationale behind the use of MSRE is elimination of non-methylated templates, which allows a single-step detection of templates that survived MSRE digestion. This rationale was used by Singer-Sam and colleagues (16,17), who treated genomic DNA with HpaII and were able to use HpaII digestion with subsequent PCR amplification to analyze DNA methylation in the mouse H-7 locus (16). In our case, however, HpaII repeatedly produced incomplete digestion in an overwhelming majority of samples (data not shown), probably as a result of increased sensitivity of the assay procedure. This may be due to specific features of HpaII, a type IIe restriction enzyme, which contains two recognition site-binding pockets, only one of which is catalytically active (32).

Among several methylation-sensitive enzymes that we tested, Hin6I (recognition sequence GCGC) proved to be the most robust, with good survival in the reaction at 37°C (data not shown) and ample activity on diluted samples (digestion of 100 pg of pUC19 in 50 μL reaction, Figure 1B). In our hands <5% of treated samples fail the pUC19 quality control step (data not shown), making it sufficient for practical applications.

Once the MSRE treatment step has been successfully performed, amplification of remaining templates can be done in a multiplexed format using gene-specific primers. Primers were designed to amplify fragments of corresponding CpG islands of 120–560 bp, with the vast majority of them containing no more than six and no fewer than two sites for Hin6I. Within this range and using the digestion conditions described in Materials and Methods, the chance of scoring any given fragment as methylated did not depend on the number of Hin6I sites (46% of all fragments with two and 49% of fragments with six sites were scored as methylated; data not shown).

We have observed three types of results using this assay: presence or absence of a band in the sample with MSRE-treated template denotes either resistance or sensitivity of the corresponding template fragment to MSRE, and therefore either presence or absence of methylation (Table 1). The third outcome, absence of PCR products in both control and MSRE-treated samples, can be interpreted as a homozygous deletion of a corresponding promoter region. This outcome was observed in the case of p16Ink4a (Figure 1C and Table 2), which was consistent with the deletion of this gene demonstrated for MCF-7 and MDA-MB-231 by several investigators (33,34). On the other hand, Musgrove and colleagues (33) did not find the deletion of p15Ink4b in MCF-7, and results obtained by MSRE-PCR (Figure 1C and Table 2) might reflect genomic variations in different MCF-7 cell lines (35). Another explanation—single nucleotide polymorphism in a primer-binding site—has to be considered as well.

Validation of the assay with breast cancer cell lines demonstrated a very good match between our data and results from different previously published studies (see Table 2). For the vast majority of the promoters examined, MSRE-PCR results were exactly identical to those obtained with other methods. In some cases, observed differences depend upon the choice of the DNA region analyzed [e.g. first exon of 14-3-3σ in (36) versus promoter region in our work] or by variability of cancer cell lines [e.g. methylation of uPA promoter in MDA-MB-231 described in (37) versus unmethylated status and expression of this gene in our work; see Table 2 and Figure 2C].

One of the advantages of MSRE-PCR is a side-by-side comparison between control (undigested) and experimental (digested) samples even for very low amounts of starting DNA (Figure 1D). Direct comparison increases the reliability of data by reducing false-negative readings, which are sometimes observed with methylation target arrays (MTAs) and MSP assays [e.g. p16Ink4a in MDA-MB-231 scored as unmethylated in (37) and (38), whereas it is deleted in at least some variants of this cell line (33,34)].

In most cases the MSRE-PCR data (Table 2) and MSP results for the same set of genes (37–39) are identical, despite the fact that MSP is designed to detect methylation in relatively short fragments corresponding to primer-binding sequences (19), whereas MSRE-PCR detects methylation in a region flanked by selected primers (see Materials and Methods). When tried side by side, MSP and MSRE-PCR produce exactly the same results for the genes tested (Figure 2A).

Similar to MSP and many other bisulfite modification-based methods, MSRE-PCR evaluates DNA methylation in a relatively few sites located within the MSRE recognition sequence. This feature of MSRE-PCR is illustrated by bisulfite sequencing of calcitonin promoter in T47D and MCF-7 (Figure 2B): although the overall probability of at least one cut is very high for T47D (e.g. the last CpG site within the GCGC sequence is unmethylated in all sequenced clones, Figure 2B), the same is not true for MCF-7 (relevant CpG sites have at least 90% methylation probability, Figure 2B). Consequently, calcitonin promoter is scored as methylated in MCF-7 and as unmethylated in T47D by MSRE-PCR (Table 2), although methylation of non-Hin6I CpG is relatively high in T47D cells.

Despite this limitation a good correlation was observed between MSRE-PCR data and mRNA expression examined by northern blot hybridization (Figure 2C), when promoter methylation always predicted the absence of corresponding mRNA. Importantly, the opposite is not true, because methylation is but one mechanism of negative promoter regulation, e.g. lack of calcitonin expression in T47D (Figure 2C) and unmethylated status of its promoter (Figure 2B and Table 2).

Probably the most important feature of the MSRE-PCR assay is its ability to detect promoter methylation in heterogeneous samples, even when methylated sequences represent a small fraction of the overall specimen. In our hands the assay could detect the presence of 42 pg of MCF-7 genomic DNA, which contains methylated promoter of RANKL/TRANCE, against the background of 2.6 ng of T47D genomic DNA, where the same promoter is unmethylated (Figure 3). Simple calculations indicate that the assay has sufficient power to detect $42{:}2600 \times 100\% = 1.6\%$ of methylated templates against the background of 98.4%, or, taking into account that a genomic equivalent of 7 cells (42 pg) is sufficient to detect methylation in a heterogeneous mixture, 350 cells (7×50) from a clinical sample will be sufficient to detect methylation. Since only one-third of the sample was used for PCR, the demonstrated sensitivity of the assay is at least 2 cells per 100 cells in the sample. We believe that fine needle biopsy or a similar method will provide sufficient material for the assay at this level of sensitivity.

For clinical applications where the primary goal is differentiation between normal and cancerous tissue this level of sensitivity can be unsafe. For example, heterogeneity of the sample (e.g. presence of stromal cells), insufficiently clean genomic DNA that cannot be digested, PCR contamination or incomplete digestion will cause overdetection of methylated sequences. We have already encountered this situation with formalin-fixed paraffin-embedded samples from breast cancer patients, when the vast majority of analyzed genes was scored as methylated and could not be used for differentiation of cancerous samples. Apparently, this can be carried out by scoring unmethylated genes, where there is no danger of overdetection by MSRE-PCR (manuscript in preparation); further studies with a large cohort of patients will test this conclusion.

Overall, MSRE-PCR data for cultured breast cancer cells are virtually identical to data obtained with other methods. MSRE-PCR requires a very small amount of starting material and can be used with heterogeneous samples, suggesting that it can be further developed for high-throughput analysis of clinical material.

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