MSRE-PCR for analysis of gene-specific DNA methylation

Anatoliy A. Melnikov¹, Ronald B. Gartenhaus¹,², Anait S. Levenson¹,³, Natalia A. Motchoulskaia¹ and Victor V. Levenson (Chernokhvostov)¹,*

¹Robert H. Lurie Comprehensive Cancer Center, ²Division of Hematology/Oncology, Department of Medicine and ³Department of Orthopaedic Surgery, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA

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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.

*To whom correspondence should be addressed. Tel: +1 312 503 2435; Fax: +1 312 503 3063; Email: levenson@northwestern.edu

Present address:
Ronald B. Gartenhaus, University of Maryland, Greenebaum Cancer Center, 9-011 BRB, 655 West Baltimore Street, Baltimore, Maryland 21201, USA

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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 μg/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 CGCG; Fermentas, Hanover, NY). 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) to 1.5 M final for each reaction; dNTPs without further adjustments, except the addition of betaine were used at 0.4 mM (Sigma, St Louis, MO) were added to 0.2 mM; primers and betaine were used at 1.5 M final for each reaction; dNTPs 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.

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 Fcalc (GAATTTCAGTTAGAGTTTGTGTTTT) and Rcalc (AATTCTAATTCACCTACCTAAAC), 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 CGCG 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...
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\textsuperscript{a} |                |

\textsuperscript{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

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).
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.

### 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

**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   | M²  | M²    | M²   | (36)      |
| Apaf-1    | M   | M     | UM   | NF        |
| BRCA1     | M²,c| UM²,c| UM²,c| (37),(38) |
| Calcitonin| M   | M     | UM   | NF        |
| Caspase-8 | UM  | UM    | M    | NF        |
| CycD2     | M²  | M²    | M²   | (40)      |
| DAPK      | UM²,c| M²,c | UM²,c| (41),(37),(38) |
| E-cadherin| M²,c| UM²,c| UM²,c| (42),(38) |
| EDNRB     | M   | M     | UM   | NF        |
| EP300     | UM  | UM    | UM   | NF        |
| ERα-B(dist)| M² | M²    | M²   | (43)      |
| ERα-A(prox)| M² | UM²   | UM²  | (43)      |
| Fas       | UM  | UM    | UM   | NF        |
| FHT       | UM  | UM    | UM   | NF        |
| GPC3      | M²  | M²    | UM²  | (37)      |
| GR        | UM  | UM    | M    | NF        |
| GSTP1     | M²  | M²    | M²   | (38)      |
| HIC-1     | M   | M     | M    | NF        |
| HIN-1     | M²  | M²    | M²   | (44)      |
| hMLH1     | UM² | UM²   | UM²  | (38)      |
| ICM-1     | M   | M     | M    | NF        |
| MCT1      | UM  | UM    | UM   | NF        |
| MDGI      | M²  | M²    | M²   | (45)      |
| MDR1      | M   | M     | M    | NF        |
| MGMT      | UM² | UM²   | UM²  | (38)      |
| MCI       | UM  | UM    | UM   | NF        |
| MUC2      | M   | M     | M    | NF        |
| MYF3/MYOD1| M²  | M²    | UM²  | (37)      |
| p15 INK4B | D²  | D²    | UM²  | (38)      |
| p16 INK4A | D²,c| D²,c  | M²   | (37),(38) |
| p21 WAF1  | UM  | UM    | UM   | NF        |
| p27 Kip1  | M   | M     | M    | NF        |
| p57 Kip2  | UM  | UM    | M    | NF        |
| p73       | M²  | M²    | M²   | (38)      |
| PAX5      | M²  | UM²   | M²   | (46)      |
| PR        | M   | M     | M    | NF        |
| RANKL/TRANCE | M | M     | UM   | NF        |
| Rasα/βa   | M²,c| M²,c | M²,c | (37),(38) |
| RB1       | UM  | UM    | UM   | NF        |
| RFC       | M²  | M²    | UM²  | (47)      |
| RIZ1      | M²  | UM²   | M²   | (48)      |
| S100A2    | M   | M     | M    | NF        |
| SOCS-1    | UM  | UM    | UM   | NF        |
| SSBRC     | UM² | M²    | M²   | (49)      |
| SYK       | M²  | M²    | UM²  | (50)      |
| TES       | UM² | M²    | M²   | (51)      |
| THBS1     | UM² | UM²   | UM²  | (38)      |
| TMS1      | M²  | M²    | M²   | (52)      |
| uPA       | UM² | UM²   | M²   | (37)      |
| VHL       | UM  | UM    | UM   | NF        |

*aUntested in paper(s) referenced.
*bDifferent result.
*cIdentical result.

NF, no references found.

Results from two or more papers are separated with a slash (/).
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 II 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 GCCG) 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 is 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 bisulfitization 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 bisulfitization 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 GC-GC 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 × 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 underdetection 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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REFERENCES

1. Feinberg, A.P. and Tycko, B. (2004) The history of cancer epigenetics. Nat. Rev. Cancer, 4, 143–153.
2. Cottrell, S.E. and Laird, P.W. (2003) Sensitive detection of DNA methylation. Ann. N. Y. Acad. Sci., 983, 120–130.
3. Geiman, T.M. and Robertson, K.D. (2000) Chromatin remodeling, histone modifications, and DNA methylation—how does it all fit together? J. Cell Biochem., 87, 117–125.
4. Jones, P.A. (2002) DNA methylation and cancer. Oncogene, 21, 5358–5360.
5. Esteller, M. and Herman, J.G. (2002) Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. J. Pathol., 196, 1–7.
6. Gaudet, F., Hodgson, J.G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J.W., Leonardt, H. and Jaenisch, R. (2003) Induction of tumors in mice by genomic hypomethylation. Science, 300, 489–492.
7. Ahuja, N., Mohan, A.L., Li, Q., Stolker, J.M., Herman, J.G., Hamilton, S.R., Baylin, S.B. and Issa, J.P. (1997) Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res., 57, 3370–3374.
8. Dao, D., Walsh, C.P., Yuan, J., Gorelov, D., Feng, L., Hensle, T., Nisen, P., Yamashiro, D.J., Bestor, T.H. and Tycko, B. (1999) Multipoint analysis of human chromosome 11p15/mouse distal chromosome 7: inclusion of H19/IGF2 in the minimal WT2 region, gene specificity of H19 silencing in Wilms’ tumorigenesis and methylation hyper-dependence of H19 imprinting. Hum. Mol. Genet., 8, 1357–1352.
9. Laird, P.W. and Jaenisch, R. (1994) DNA methylation and cancer. Hum Mol Genet., 3, 1487–1495.
10. Rountree, M.R., Bachman, K.E., Herman, J.G. and Baylin, S.B. (2001) DNA methylation, chromatin inertia and cancer. Oncogene, 20, 3156–3165.
11. Jones, P.A. (2003) Epigenetics in carcinogenesis and cancer prevention. Ann. N. Y. Acad. Sci., 983, 213–219.
12. Herman, J.G. and Baylin, S.B. (2000) Promoter-region hypermethylation and gene silencing in human cancer. Curr. Top. Microbiol. Immunol., 249, 35–54.
13. Widschwendter, M. and Jones, P.A. (2002) The potential prognostic, predictive, and therapeutic values of DNA methylation in cancer. Commentary re: J. Kwong et al., Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. Clin. Cancer Res., 8, 131–137, 2002, and H-Z. Zou et al., Detection of aberrant p16 methylation in the serum of colorectal cancer patients. Clin. Cancer Res., 8, 188–191, 2002. Clin. Cancer Res., 8, 17–21.
14. Umbricht, C.B., Evron, E., Gabrielson, E., Ferguson, A., Marks, J. and Sukumar, S. (2001) Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer. Oncogene, 20, 3348–3353.
15. Brock, M.V., Gou, M., Aliyama, Y., Muller, A., Wu, T.T., Montgomery, E., Deaseal, M., Geronmone, P., Rubinson, L., Heitmiller, R.F. et al. (2003) Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. Clin. Cancer Res., 9, 2912–2919.
16. Singer-Sam, J., Grant, M., LeBoon, J.M., Okuyama, K., Chapman, V., Monk, M. and Riggs, A.D. (1990) Use of a HpaII-polymerase chain reaction assay to study DNA methylation in the Pgk-1 CpG island of mouse embryos at the time of X-chromosome inactivation. Mol. Cell Biol., 10, 4987–4989.
17. Singer-Sam, J., LeBon, J.M., Tanguay, R.L. and Riggs, A.D. (1990) A quantitative HpaII-PCR assay to measure methylation of DNA from a small number of cells. *Nucleic Acids Res.*, 18, 687.

18. Levenson, A.S., Gehm, B.D., Pearce, S.T., Horiguchi, J., Simons, L.A., Ward, J.E., III, Jameson, J.L. and Jordan, V.C. (2003) Resveratrol acts as an estrogen receptor (ER) agonist in breast cancer cells stably transfected with ER alpha. *Int. J. Cancer*, 104, 587–596.

19. Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D. and Baylin, S.B. (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA*, 93, 9821–9826.

20. Murata, H., Khattra, N.K., Yang, Y., Gu, L. and Li, G.M. (2002) Genetic and epigenetic modification of mismatch repair genes hMSH2 and hMLH1 in sporadic breast cancer with microsatellite instability. *Oncogene*, 21, 5696–5703.

21. Soria, J.C., Rodriguez, M., Liu, D.D., Lee, J.J., Hong, W.K. and Mao, L. (2002) Aberrant promoter methylation of multiple genes in bronchial brush samples from former cigarette smokers. *Cancer Res.*, 62, 351–355.

22. Clark, S.J., Harrison, J., Paul, C.L. and Frommer, M. (1994) High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.*, 22, 2990–2997.

23. Xiong, Z. and Laird, P.W. (1997) COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.*, 25, 2532–2534.

24. Eads, C.A., Danenberg, K.D., Kawakami, K., Saltz, L.B., Blake, C., Shibata, D., Danenberg, P.V. and Laird, P.W. (2000) MethLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.*, 28, e32.

25. Zeschnigk, M., Bohringer, S., Price, E.A., Onadim, Z., Masshofer, L. and Lohmann, D.R. (2004) A novel real-time PCR assay for quantitative analysis of methylated alleles (QAMA): analysis of the retinoblastoma locus. *Nucleic Acids Res.*, 32, e125.

26. Thomasin, H., Kress, C. and Grange, T. (2004) MethylQuant: a sensitive protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Mol. Cell Biol.*, 24, 416–426.

27. Harrison, J., Stirzaker, C. and Clark, S.J. (1998) Cytosines adjacent to methylated CpG sites can be partially resistant to conversion in inherently methotrexate-resistant human breast cancer cells. *Cancer Res.*, 58, 1972–1977.

28. Shelton, A.S., Eguchi, H., Nakachi, K., Tanimoto, K., Higashi, Y., Suenasu, K., Iino, Y., Morishita, Y. and Hayashi, S. (2000) Distinct mechanisms of loss of estrogen receptor alpha gene expression in human breast cancer: methylation of the gene and alteration of trans-acting factors. *Carcinogenesis*, 21, 2193–2201.

29. Reeben, M., Celiikkaya, G., Hasemeier, B., Langer, F. and Kreipe, H. (2002) Promoter hypermethylation of the death-associated protein kinase gene in breast cancer is associated with the invasive lobular subtype. *Cancer Res.*, 62, 6634–6638.

30. Krop, I.E., Sgroi, D., Porter, D.A., Lunetta, K.L., LeVangie, R., Seth, P., Kaelin, C.M., Rhee, E., Bosenberg, M., Schnitt, S. et al. (2001) HIN-1, a putative cytokine highly expressed in normal but not cancerous mammary epithelial cells. *Proc. Natl. Acad. Sci. USA*, 98, 9796–9801.

31. Huyhn, H., Alpert, L. and Pollak, M. (1996) Silencing of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasms is associated with epigenetic changes. *Cancer Res.*, 56, 4865–4870.

32. Palmisano, W.A., Crume, K.P., Grimes, J.M., Winters, S.A., Toyama, Y., Esteller, M., Joste, N., Baylin, S.B. and Belinsky, S.A. (2003) Aberrant promoter methylation of the transcription factor genes PAX5 alpha and beta in human cancers. *Cancer Res.*, 63, 4620–4625.

33. Hira, T., Tomlinson, I.G., Graff, J.J., Birkeland, S.K., Iino, Y., Morishita, Y. and Hayashi, S. (2000) Distinct mechanisms of loss of estrogen receptor alpha gene expression in human breast cancer: methylation of the gene and alteration of trans-acting factors. *Carcinogenesis*, 21, 2193–2201.

34. Du, Y., Carling, T., Fang, W., Pao, Z., Sheu, J.C. and Huang, S. (2001) Hypermethylation in human cancers of the RIZ1 tumor suppressor gene, a member of a histone/protein methyltransferase superfamily. *Cancer Res.*, 61, 8094–8099.

35. Xu, X.L., Wu, L.C., Du, F., Davis, A., Peyton, M., Tomizawa, Y., Maitra, A., Tomlinson, G., Gazdar, A.F., Weissman, B.E. et al. (2001) Inactivation of human SRBC, located within the 11p15.5-p15.4 tumor suppressor region, in breast and lung cancers. *Cancer Res.*, 61, 7943–7949.

36. Yuan, Y., Mendez, R., Sahin, A. and Dai, J.L. (2001) Hypermethylation leads to silencing of the SYK gene in human breast cancer. *Cancer Res.*, 61, 5558–5561.

37. Tobias, E.S., Hurlstone, A.F., MacKenzie, E., McFarlane, R. and Black, D.M. (2001) The TES gene at 7q31.1 is methylated in tumours of the large intestine. *J. Pathol.*, 197, 131–138.

38. Ferguson, A.T., Evron, E., Umbricht, C.B., Korz, D., Raman, V., Loeb, D.M., Niranjan, B., Godfrey, T., Nakamura, H., Graff, J.J., Collins, C., Shayesteh, L., Doggett, N., Johnson, K., Wheelock, M., Herman, J. et al. (1998) Mechanisms of inactivation of E-cadherin in breast cancer cell lines. *Cancer Res.*, 58, 1972–1977.

39. Yoshida, T., Eguchi, H., Nakachi, K., Tanimoto, K., Higashi, Y., Suenasu, K., Iino, Y., Morishita, Y. and Hayashi, S. (2000) Distinct mechanisms of loss of estrogen receptor alpha gene expression in human breast cancer: methylation of the gene and alteration of trans-acting factors. *Carcinogenesis*, 21, 2193–2201.

40. Hiraguri, S., Nehmey, T., Nakamura, H., Graff, J.J., Collins, C., Shaye, W. et al. (1998) Mechanisms of inactivation of E-cadherin in breast cancer cell lines. *Cancer Res.*, 58, 1972–1977.

41. Borgen, P.I. and Van Zee, K.J. (2001) Molecular analysis of the INK4A cyclin-dependent kinase inhibitors p16INK4, p15INK4B and p18INK4A in human breast cancer. *Cancer Res.*, 61, 8094–8099.

42. Borgen, P.I. and Van Zee, K.J. (2001) Molecular analysis of the INK4A cyclin-dependent kinase inhibitors p16INK4, p15INK4B and p18INK4A in human breast cancer. *Cancer Res.*, 61, 8094–8099.

43. Borgen, P.I. and Van Zee, K.J. (2001) Molecular analysis of the INK4A cyclin-dependent kinase inhibitors p16INK4, p15INK4B and p18INK4A in human breast cancer. *Cancer Res.*, 61, 8094–8099.

44. Borgen, P.I. and Van Zee, K.J. (2001) Molecular analysis of the INK4A cyclin-dependent kinase inhibitors p16INK4, p15INK4B and p18INK4A in human breast cancer. *Cancer Res.*, 61, 8094–8099.

45. Borgen, P.I. and Van Zee, K.J. (2001) Molecular analysis of the INK4A cyclin-dependent kinase inhibitors p16INK4, p15INK4B and p18INK4A in human breast cancer. *Cancer Res.*, 61, 8094–8099.