Standardization of the methylation-specific PCR method for analyzing BRCA1 and ER methylation

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Abstract. The significant differences in DNA methylation that are considered to be a biomarker for the diagnosis of cancer are a barrier to the application of biomarkers in the clinical field. In the present study, new primers were designed and further standard controls were set up to validate the accuracy of the methylation-specific PCR (MSP), a method widely used to analyze DNA methylation. By analyzing the methylation pattern of breast cancer 1 (BRCA1) and estrogen receptor (ER) in 60 patients with breast cancer, the number of cases of methylated BRCA1 and ER detected by the primer was 7/60 and 21/60, respectively, whereas that detected by the previous widely used primers was 25/60 and 47/60, respectively. Sequencing of the MSP products indicated that the 18 and 26 false-positive methylations of BRCA1 and ER, respectively, were due to insufficient validation of the previously used primers. Thus, the present study proposes that all studies based on the MSP approach should incorporate more controls to validate the precision of the MSP primers.

Introduction

The methylation of deoxycytidine nucleotides distributed in CpG islands is well known as an epigenetic regulation mechanism for genomic function. Alteration of the DNA methylation pattern has been identified to be closely associated with carcinogenesis (1,2). Aberrant DNA hypermethylation at promoter sequences leads to silencing of certain critical genes, including the tumor suppressors, thus contributing to cancer development (3,4). A number of studies have focused extensively on

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the identification of DNA methylation patterns as biomarkers for diagnosing cancer (5-7).

A global change in DNA methylation on a genome-wide scale is able to be analyzed by DNA microarrays and high-throughput DNA sequencing, which may not be accessible to a number of institutions, particularly those in developing countries (8,9). Additionally, DNA methylation at local genes may be analyzed by methods based on the PCR approach, which is routinely used in every laboratory that works with DNA (10). The majority of the PCR-based methods use genomic DNA templates that have been treated with sodium bisulfite. This chemical converts unmethylated cytosine, but not methylated cytosine, to uracil residues (11). Specific primers were designed on the basis of sequences that contain an adequate number of CpG islands, thus the primers distinguish methylated from unmethylated templates (12). The methylation-specific PCR (MSP) is suitable and sensitive for the detection of the CpG methylation status at any CpG islands (10). Since the MSP primer sets are specifically designed for the DNA whose composition was changed following bisulfite conversion, a trace of unmodified DNA (native DNA), due to uncompleted conversion in principle, is not amplified during the PCR reactions (12,13). Thereby, the majority of the control tests (positive or negative controls) that are used to validate the MSP results for the DNA methylation patterns in different types of cancers have used only bisulfite-treated DNA and not untreated DNA extracted from different cell lines (cancer or non-cancer) or from patient's specimens (14).

In the present study, the false-positive effect caused by a trace of unmodified DNA on the MSP results was reported, using previously published primer sets to identify the methylation of the breast cancer 1 (*BRCA1*) and estrogen receptor (*ER*) genes in Vietnamese females with breast cancer. New primer sets and the set-up of additional standard controls for eliminating false-positive results were designed in order to improve the accurate positivity of the MSP method.

Materials and methods

Tissue samples. A total of 60 specimens of primary breast cancer were collected from patients undergoing surgical resection at the Department of Pathology, National Cancer

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Gene name	Primers	Sequence (5'-3')	Size, bp	First author (ref.)
BRCA1	BRCA-F	TCGTGGTAACGGAAAAGCGC	75	Esteller et al (15)
	BRCA-Un F BRCA-Un R		86	Esteller et al (15)
	BM-F BRCA-R	GGGTAGATTGGGTGGTTAATT	Round 1: 200	Present study
	BM-F BM-R	GGGTAGATTGGGTGGTTAATT TACACGAACTCACGCCGCGCAA	Round 2: 195	Present study
	BU-F BRCA-Un R	TTAATTTAGAGTTTTGAGAGAT CAAAAAATCTCAACAAACTCACACCA	Round 1: 191	Present study
	BRCA-Un F BU-R	TTGGTTTTTGTGGTAATGGAAAAGTG CAACAAACTCACACCACAAA	Round 2: 76	Present study
ER	ER4-F ER4-R	CGAGTTGGAGTTTTTGAATCGTTC CTACGCGTTAACGACGACCG	151	Lapidus <i>et al</i> (16)
	ER4-Un F ER4-Un R	ATGAGTTGGAGTTTTTGAATTGTTT ATAAACCTACACATTAACAACAACCA	158	Lapidus et al (16)
	EM-F ER4-R	GATACGGTTTGTATTTTGTTCG CTACGCGTTAACGACGACCG	247	Present study
	EU4-F ER4-Un R	GTGGGGATATGGTTTGTATTTTGTTTG ATAAACCTACACATTAACAACAACCA	Round 1: 258	Present study
	ER4-Un F EU4-R	ATGAGTTGGAGTTTTTGAATTGTTT ACCTACACATTAACAACAACCACAACA	Round 2: 154	Present study

Table I. MSP primers for analysis of BRCA1 and ER gene methylation.

BU and EU indicated the primers specific to unmethylated targets. BM and EM indicated the primers specific to methylated targets. F, forward; R, reverse; MSP, methylation-specific PCR; *BRCA1*, breast cancer 1; *ER*, estrogen receptor.

Hospital K, Hanoi, the largest cancer hospital in Vietnam. Informed consent was obtained from patients in written form (ICF-ATF-FP-005-VN), and the study was approved by the guidelines of the local ethical committee in Vietnam (2205/QĐ-KHCN; Vietnam National University, Hanoi, Vietnam).

Genomic DNA extraction and bisulfite modification. Genomic DNA was extracted using a QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA) and treated with sodium bisulfite using an EpiTect Bisulfite kit (Qiagen). During the modification, the unmethylated cytosines of the genomic DNA were converted to uracils, but the methylated cytosines remained unchanged (11). PCR that used β -globin-F/R primer for the native DNA and Un-globin-F, -R and -R1 for treated DNA (Fig. 1) was performed to determine the efficiency of bisulfite conversion.

MSP. The methylation status of BRCA1 and ER was evaluated using two primer sets for the MSP. The first set included BRCA1 and ER primers that were originally designed and reported by Esteller *et al* (15) and Lapidus *et al* (16), respectively. The second set included new primers that were designed using the free online tool from MethPrimer (http://www.urogene.org/methprimer/index1.html). The primer sequences and amplicon lengths are shown in Table I. PCR amplification with the first primer set was performed as described previously (15,16). Bisulfite-treated DNA was subjected to a single round of PCR with the new EM-F and ER4-R ER primers. Two rounds of PCR, the first round with the BM-F/BRCA-R and the second round with BM-F/BM-R primers, were performed to detect BRCA1 methylation. The 25 μ l of the PCR reaction contained 0.3 μ mol/l primers, 100 μ mol/l dNTPs, 2.0 U JumpStart Taq polymerase (Sigma-Aldrich, St. Louis, MO, USA) and 1-2 µl of bisulfitetreated DNA. The PCR conditions were follows: 94°C for 1 min, 40 cycles of (94°C for 30 sec, 65°C for 10 sec and 72°C for 10 sec), and 72°C for 5 min. The second 25 µl nested PCR reaction contained 1 μ l of the first PCR product and was performed with the conditions as follows: 94°C for 1 min, 40 cycles of (94°C for 30 sec, 68°C for 10 sec and 72°C for 10 sec) and 72°C for 5 min. Two rounds of PCR were performed with the new primer sets specific to unmethylated BRCA1 and ER. The PCR products were subjected to electrophoresis on a 12% polyacrylamide gel. All the PCR reactions were replicated at least three times.

DNA that was extracted from the lymphocytes of the healthy volunteers and then treated with bisulfite was used as a positive control for *BRCA1* and *ER* unmethylation. A mixture of plasmid DNA containing methylated *BRCA1* or *ER* sequences and DNA extracted from normal lymphocytes was used as a positive control for *BRCA1* and *ER* methylation. Water without a DNA template was included in each PCR reaction as a control for any contamination. The meth-



Figure 1. Representative result for efficiency of bisulfite conversion. (A) Detection of a band of 268 bp amplified by the β -globin primer set. (B) Detection of a band of 244 bp amplified by the nested Un globin primer set. (C) Nucleotide sequence of the 5' region of β -globin gene (accession no. U01317.1) and primer location. F, forward; R, reverse; UT, untreated DNA; BT, bisulfite-treated DNA; L, lymphocytes of the healthy volunteer; 1-6, breast cancer specimens; M, 100-bp DNA ladder; (--), negative control without DNA template.



Figure 2. Representative analysis of MSP products amplified by (A and B) the first primer sets of *BRCA1* and (C and D) *ER*. UnFT, incompletely converted DNA; FT, completely converted DNA; mx, mixture of untreated and completely converted DNA; UT, untreated DNA; BT, bisulfite-treated DNA without verifying the efficiency of full conversion; S1, S3, S11 and S47, different samples of breast cancer tissue; M, DNA ladder; (--), negative control without DNA templates; MSP, methylation-specific PCR; *BRCA1*, breast cancer 1; *ER*, estrogen receptor.

ylation status was confirmed by sequencing the cloned MSP products for a subset of samples from each assay.

Results

The full conversion of genomic DNA that was extracted from the primary breast cancer specimens was verified by PCR with a β -globin primer set (Fig. 1). Using primers designed from native DNA sequences, the majority of the PCR products were revealed to be amplified from untreated and not bisulfite-converted DNA (Fig. 1A). By contrast, the PCR products amplified by primers designed for unmethylated globin sequences were detected from the bisulfite-treated DNA, but not the native DNA (Fig. 1B). Negligible PCR products were amplified from several treated DNA samples possibly due to an incomplete conversion. Incompletely and completely



Figure 3. Representative analysis of MSP products amplified by the new primer sets of (A) *BRCA1* and (B) *ER*. BT, bisulfite-treated DNA without verifying the efficiency of full conversion; FT, completely converted DNA; UT, untreated DNA; UnFT, incompletely converted DNA; S2 and S11, breast cancer tissue samples; M, DNA ladder; (--), negative control without DNA templates; MSP, methylation-specific PCR; *BRCA1*, breast cancer 1; *ER*, estrogen receptor.



Figure 4. Representative analysis of *BRCA1*-MSP products amplified by (A) the first primer set and (B) the second primer set without verifying the efficiency of full conversion of the DNA templates. 1-7, breast cancer samples; Me, the presence of *BRCA1* methylation; Un, the presence of BRCA1 unmethylation; L, lymphocytes of the healthy volunteer; P, plasmid DNA, including *BRCA1* methylated sequence mixed with DNA extracted from lymphocytes of the healthy volunteer; M, DNA ladder; (--), negative control without DNA template. (C) The nucleotide sequence of the 5' region of *BRCA1* (accession no. NG-005905.2) and the location of the BRCA1-MSP primers listed in Table I. BM indicated the primers specific to methylated *BRCA1. BRCA1*, breast cancer ; MSP, methyl-ation-specific PCR; F, forward; R, reverse.

converted DNA were applied separately to MSP with the first *BRCA1* and *ER* primer sets. Unexpectedly, in several samples, methylation of *BRCA1* and *ER* was detected from the incompletely modified DNA and not from the fully modified DNA (Fig. 2A and C). It was likely that the primer sets specifically designed for methylated *BRCA1* and *ER* wrongly amplified the native DNA template that was not modified, and this template remained through the bisulfite reaction.

To confirm this hypothesis, untreated genomic (native) DNA was subjected to MSP with the first BRCA1 and ER primer sets, which were appropriate for detecting

methylation (Fig. 2B and D). PCR products were amplified from untreated genomic DNA and from a mixture of untreated genomic DNA and completely modified DNA. In addition, the PCR products were also amplified from untreated DNA by using the primer sets specifically designed for unmethylated *ER* and *BRCA1* (data not shown). The analysis indicated a false-positive result that was due to a trace of native DNA not being converted, but being retained through bisulfite treatment.

Based on the primer design strategies for the MSP method, new primers for *BRCA1* and *ER* were designed. A number of



Figure 5. Representative analysis of *ER*-MSP products amplified by (A) the first primer set and (B) the second primer set without verifying the efficiency of the full conversion of the templates. 1-7, breast cancer samples; Me, the presence of *ER* methylation; Un, the presence of *ER* demethylation; L, lymphocytes of the healthy volunteer; P, plasmid DNA, including *ER* methylated sequence mixed with DNA extracted from lymphocytes of the healthy volunteer; M, DNA ladder 100 bp; (--), negative control without DNA template. (C) The nucleotide sequence of the 5' region of *ER* (accession no. AL356311.6) and the location of the ER-MSP primers listed in Table I. EM indicated the primers specific to methylated *ER*. *ER*, estrogen receptor; MSP, methylation-specific polymerase chain reaction.

these primers were used in combination with the published primers (Table I). PCR was performed in which either untreated or bisulfite-treated genomic DNA was used as a template. The methylation of *BRCA1* and *ER* was detected from the treated DNA, but not from the untreated DNA (Fig. 3), and unmethylation of *BRCA1* and *ER* was also detected from the treated DNA, but not from the untreated DNA (data not shown). This indicates the precision and specificity of the new primer sets in distinguishing methylated from unmethylated and untreated sequences.

Genomic DNA extracted from 60 breast cancer specimens was treated with bisulfite and subjected directly to MSP without verifying the full conversion following treatment. The number of cases of methylated *BRCA1* and *ER* detected by the first primer set was 25/60 and 47/60, respectively and that detected by the second primer set was 7/60 and 21/60, respectively (Figs. 4 and 5). When treated DNA whose full conversion was examined through PCR with the β -globin primers and with the new primers were used as templates for the two primer sets, the same result (7/60 and 21/60 methylated DNA, respectively) was obtained. Therefore, incompletely converted DNA resulted in 18 and 26 cases of false-positive methylation of *BRCA1* and *ER*, respectively. Unmethylation of *BRCA1* and *ER*, was detected in the DNA of all 60 breast cancer patients.

False priming events of the first primer set were confirmed by cloning and sequencing the MSP products that were amplified from untreated DNA templates (data not shown). The nucleotide sequences amplified by the first primer set specific to *BRCA1* and *ER* methylation were revealed to be identical to native sequences. In addition, three representatives of the MSP products amplified from either incompletely converted or fully converted DNA by the second *BRCA1* and *ER* primer set were also cloned and subsequently sequenced. The nucleotide sequences revealed that all cytosine residues were converted to thymidines in *BRCA1* and *ER* unmethylated products, and that all cytosines in the CpG sites remained as cytosines. The cytosines that were not in the CpG sites were converted to thymidines in the *BRCA1* and *ER* methylated products.

Discussion

Among the different types of markers that are capable of distinguishing tumors from normal tissue, the DNA methylation marker has become the most attractive due to its sensitivity, specificity and applicability to a variety of clinical specimens (12,17). MSP is the most widely used method for the sensitive detection of DNA methylation (10). As this method requires common equipment only, MSP may allow every laboratory to approach and develop the DNA methylation marker for the purpose of diagnosis and prognosis of cancers (5-7).

Using the MSP method, aberrant methylation at the 5' region has been reported on a number of genes in different types of cancer (18-20). The MSP result for one gene is dependent on the analyzed sequence of the 5' region and the type of cancer. Thus, for a specific type of cancer, utilization of

the same panel of targeted genes and of the same region of the gene for analysis of DNA methylation should be validated and reproduced to increase the accuracy of DNA methylation markers in clinical applications (14).

The BRCA1 and ER genes are the targets of aberrant DNA methylation in breast tumors; thus, they are a subject being studied extensively (21-24). The BRCA1 gene encodes a multifunctional protein that is involved in DNA repair, cell cycle control and chromatin remodeling (25). The ER has a central role in an important signaling pathway of mammary cells (26). The primers that were first designed for analysis of BRCA1 (15) and ER methylation (16) by the MSP method have been subsequently applied to numerous studies to detect the BRCA1 and ER methylation status in different types of cancer, including breast cancer (27-30). In the present study, these primers were also employed for the analysis of the BRCA1 and ER methylation status in females with breast cancer, using untreated and treated DNA as templates. The results shown in Fig. 2 revealed that methylation of BRCA1 and ER was detected in both types of DNA, and this indicates that these primers did not discriminate between methylated and unconverted sequences. The sequencing data confirmed that the first set of BRCA1 and ER primers amplified the unconverted sequences whose cytosine residues were retained. In replicated experiments, the co-amplification of untreated sequences by only the first primer set was confirmed by MSP and sequencing (data not shown). The number of cases of methylated BRCA1 and ER detected by the first primer set was 25/60 (41.7%) and 47/60 (78.3%), respectively, and that detected by the second primer set was 7/60 (11.7%) and 21/60 (35.0%), respectively. A big difference in the methylation levels (4-fold in BRCA1 methylation and 2-fold in ER methylation) was revealed between the two primer sets. A significant difference in the DNA methylation of the same gene(s) in one cancer type, for example, eight-fold difference (5-40%) in the BRCA1 methylation in breast cancer was reviewed by a number of different laboratories, thus barriers in the performance of DNA methylation as cancer biomarkers have been observed (14,31). The results of the present study indicate that in numerous previous studies, the significant difference in gene methylation analyzed by the MSP in general, and in particular for BRCA1 and ER methylation in breast cancer, was an overestimation that resulted from the shortcomings of control tests for the accuracy of MSP primers specific to the treated sequences only. An overestimation may be prevented by the full conversion of the DNA template, which may be verified through PCR with housekeeping gene primers (Fig. 1) (32). However, such test controls are required for each bisulfite-treated DNA template; thus, they are laborious. The present study provided a simple control test that eliminated the overestimation without verifying the full conversion. Since the precision of the MSP primers was affirmed through PCR with untreated DNA, a trace of uncompleted treated DNA was not inferred from the MSP results (Fig. 3). Indeed, in the present study, the BRCA1 and ER methylation levels detected by the new primers, BM-F/BRCA-R and BM-F/BM-R, and EM-F and ER4-R (Table I) were four- and two-fold less than that detected by the set of primers reported by Esteller et al (15) and Lapidus et al (16), respectively, and much less than that detected by the first set of primers from previous studies (26-56%), in which no control tests for the full conversion through PCR were reported (22,33). Thus, an accurate evaluation of the MSP primer specificity to treated sequences only must avoid false-positive results.

MSP is a highly sensitive method; thus, different approaches developed from or in combination with MSP, including BS-MSP (Bisulfite conversion-Specific and Methylation-Specific PCR), MEP (Methylation Enrichment Pyrosequencing) and ConLight MSP (MSP, Conversionspecific hybridization and MethyLight), for analysis of DNA methylation have been reported (34-36). However, the precision of MSP primers specific to methylated sequences only has not been verified in these methods to date. Previous results have demonstrated that incomplete conversion may typically be in the order of 2%, even when a commercial kit is used (37). Considering the data of the present study, it is proposed that all studies based on the MSP approach should incorporate more steps in the control of the specificity and precision of primers. By using untreated sequences as the template for amplification with MSP primer sets, overestimation of DNA methylation may be avoided. MSP is simple, highly sensitive, extremely cost-effective and does not require any special equipment; thus, MSP is the most widely used method for the analysis of DNA methylation in the majority of laboratories, particularly in those that are moderately equipped in developing countries. The present study contributed to the standardization of the MSP method and the validation of its precision. The study may also promote the fast progression of the DNA methylation marker towards its clinical application.

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