RESEARCH ARTICLE

Methylation Profile of *BRCA1*, *RASSF1A* and *ER* in Vietnamese Women with Ovarian Cancer

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Abstract

DNA methylation is considered a promising biomarkers for diagnosis of cancer in general and of ovarian cancer in particular. In our study, we validated the accuracy of methylation specific polymerase chain reaction (MSP) to analyze the methylation pattern of *BRCA1*, *RASSF1A* and *ER* in 59 and 10 Vietnamese patients with epithelial ovarian cancer (EOC) and benign ovarian tumors, respectively. We found methylation of *BRCA1*, *RASSF1A* and *ER* in 11/59 (18.6%), 40/59 (67.8%) and 15/59 (25.4%) of EOC cases, while methylation of *BRCA1* was only detected in 2/10 (20%) benign ovarian patients. Forty five out of the 59 EOCs (78%) demonstrated methylation at one or more genes. The methylation frequency of *RASSF1A* was significantly associated with EOC ($p<0.0005$). No significant association was observed between methylation status of these genes and the clinical and pathological parameters of tumors collected from Vietnamese women suffering from ovarian cancer.

Keywords: Breast cancer 1 (*BRCA1*) - RAS-association domain family member 1 (RASSF1A) - estrogen receptor α (*ER*)

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Introduction

Ovarian cancer is the leading cause of death and the second most common cancer among women with gynaecological cancers (Siegel et al., 2012). The poor survival rate of ovarian cancer patients is due in part to nonspecific symptoms and the lack of sensitive and specific methods for the detection of early-stage ovarian cancer (Barnholtz et al., 2003). Thus, the identification of a sensitive and specific biomarker or a panel of biomarkers for the early detection of ovarian cancer is extensively studied to increase the survival rate of ovarian cancer patients.

Aberrant methylation of CpG islands is associated with transcriptional silencing of key tumor suppressor genes in cancer. In addition, it is a frequent epigenetic event in epithelial ovarian cancer (EOC), which is the most variant type of this cancer (Barton et al., 2008). These findings show DNA methylation as an attractive biomarker for early diagnostic cancer. Among a large number of genes that have been identified as hypermethylated in EOC, three genes *BRCA1* (breast cancer 1, early onset), *RASSF1A* (RAS-association domain family member 1) and *ER* (estrogen receptor 1) were extensively studied because (i) methylation of *BRCA1* occurs only in breast and ovarian cancers (Esteller et al., 2001), (ii) methylation of *RASSF1A* is frequently associated with the early stage of many primary tumors including ovarian one (Agathanggelou et al., 2001), and (iii) methylation of *ER* is closely involved in ER signaling that plays a key role in hormonal cancer progression (Mann et al., 2011). Thus, methylation on these genes becomes one of the most attractive biomarkers for specific and early detection of ovarian cancer (Widschwendter et al., 2013). Moreover, combination of more than one methylated DNA as a methylation panel could greatly increase the sensitivity of cancer detection without a significant reduction of specificity. Indeed, specific methylation of one panel of four genes was detected in primary ovarian tumor with 76% sensitivity while methylation of each particular gene varied from 18-41% (Rathi et al., 2001). In addition, specific methylation of one panel of six genes was detected in the serum or plasma of ovarian cancer patients with 100% specificity and 82% sensitivity (de Caceres et al., 2004).

The incidence of ovarian cancer varies among different geographic regions and racial groups, and is rapidly escalating in developing countries in which women have been frequently exposed to risk factors of the disease (Du et al., 2008; Pathy et al., 2012). The increased incidence is also associated with the methylation status of tumor suppressor genes (Mehrotra et al., 2004; Wiencke, 2004).

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Table 1. MSP Primers for Analysis of *β-globin, BRCA1, RASSF1A* and *ER* Methylation

Gene name	Primers	Sequence (5'–3')	References	Amplicon size (bp)	MSP conditions
β-globin	β-globin F	CAACTTCATCCACGTTCACC	Wilcox et al. (2005)	268	94°C 5 min, 40 cycles of (94°C 30 sec, 62°C 10 sec, 72°C 10 sec), 72°C 5 min
U01317.1	β-globin R	GAAGAGCCAAGGACAGGTAC			
	β-globin UN F	AGAAGAGTTAAGGATAGGTATGGTTGT	Present study	Round 1: 250	94°C 5 min, 40 cycles of (94°C 30 sec, 62°C 10 sec, 72°C 10 sec), 72°C 5 min
	β-globin UN R	CTTACCCCACAAAACAATAACAACAAA			
	β-globin UN F	AGAAGAGTTAAGGATAGGTATGGTTGT	Present study	Round 2: 224	94°C 5 min, 40 cycles of (94°C 30 sec, 65°C 10 sec, 72°C 10 sec), 72°C 5 min
	β-globin UN R1	ACTTCTCCTCAAAAATCAAATACACCA			
BRCA1	BRCA-R	AAATCTCAACGAACTCACGCCG	Esteller et al. (2000)	Round 1: 200	94°C 1 min, 40 cycles of (94°C 30 sec, 65°C 10 sec, 72°C 10 sec), 72°C 5 min
NG_005905.2	BM-F	GGGTAGATTGGGTGGTTAATT	Present study		
	BM-R	TACACGAACTCACGCCGCGCAA	Present study	Round 2: 195	94°C 1 min, 40 cycles of (94°C 30 sec, 68°C 10 sec, 72°C 10 sec), 72°C 5 min
	BM-F	GGGTAGATTGGGTGGTTAATT			
	BU-F	TTAATTTAGAGTTTTGAGAGAT	Present study	Round 1: 191	94°C 5 min, 40 cycles of (94°C 30 sec, 57°C 10 sec, 72°C 10 sec), 72°C 5 min
	BRCA-UN R	CAAAAAATCTCAACAAACTCACACCA	Esteller et al. (2000)		
	BRCA-UN F	TTGGTTTTTGTGGTAATGGAAAAGTG	Esteller et al. (2000)	Round 2: 76	94°C 5 min, 40 cycles of (94°C 30 sec, 60°C 10 sec, 72°C 10 sec), 72°C 5 min
	BU-R	CAACAAACTCACACCACACAA	Present study		
RASSF1A	RM F	GGTTTTGCGAGAGCGCGTTTA	Present study	170	94°C 1 min, 40 cycles of (94°C 30 sec, 68°C 10 sec, 72°C 10 sec), 72°C 5 min
NG_023270.1	RM R	CTACGCGTTAACGACGACCG			
	UM240 F	GGGGTTTTGTGAGAGTGTGTTTAG	Liu et al. (2002)	Round 1: 175	94°C 5 min, 40 cycles of (94°C 30 sec, 60°C 10 sec, 72°C 10 sec), 72°C 5 min
	UM241 R	TAAACACTAACAAACACAAACCAAAC			
	Un F	GAGAGTGTGTTTAGTTTTGTTT	Present study	Round 2: 135	94°C 5 min, 40 cycles of (94°C 30 sec, 65°C 10 sec, 72°C 10 sec), 72°C 5 min
	Un R	CCACAAAACAAACCCCAACTT			
ER	EM-F	GATACGGTTTGTATTTTGTTCG	Present study	247	94°C 1 min, 40 cycles of (94°C 30 sec, 65°C 10 sec, 72°C 10 sec), 72°C 5 min
NC_000006.11	ER4-R	CTACGCGTTAACGACGACCG			
	EU4-F	GTGGGGATATGGTTTGTATTTTGTTTG	Present study	Round 1: 258	94°C 1 min, 40 cycles of (94°C 30 sec, 60°C 10 sec, 72°C 10 sec), 72°C 5 min
	ER4-Un R	ATAAACCTACACATTAACAACAACCA	Lapidus et al. (1998)		
	ER4-Un F	ATGAGTTGGAGTTTTTGAATTGTTT	Lapidus et al. (1998)	Round 2: 154	94°C 1 min, 40 cycles of (94°C 30 sec, 65°C 10 sec, 72°C 10 sec), 72°C 5 min
	EU4-R	ACCTACACATTAACAACCACAACA	Present study		

So far DNA methylation in ovarian cancer has not been reported in Vietnamese women yet; therefore, we primarily investigated the methylation status of *BRCA1*, *RASSF1A* and *ER* in ovarian cancer patients in Vietnam by using the methylation specific polymerase chain reaction (MSP). This highly sensitive method is widely used for the detection of CpG methylation status on any CpG island (Kristensen and Hansen, 2009).

Materials and Methods

Tissue samples: Fifty nine specimens of epithelial ovarian carcinomas and 10 specimens from benign ovarian tissues were collected when the patients were undergoing surgical resection at the Department of Pathology, National Cancer Hospital K in Hanoi, the largest cancer hospital in Vietnam. Informed consent was obtained, and the study was approved by the guidelines of the local ethical committee in Vietnam.

Genomic DNAs extraction and bisulfite modification: Genomic DNAs were extracted by using QIAamp DNA Mini Kit (Qiagen, Valencia, CA), and treated with sodium bisulfite by using EpiTect Bisulfite Kit (Qiagen). During the treatment, the unmethylated cytosines of the genomic DNAs were converted to uracils, but the methylated cytosines remained unchanged (Clark et al., 1994). Polymerase chain reaction which used native DNA and bisulfite-treated DNA with β-globin primer sets was performed to determine the efficiency of bisulfite conversion.

Methylation specific PCR (MSP): Methylation status of *BRCA1*, *RASSF1A* and *ER* was evaluated by using methylation specific polymerase chain reaction (MSP-PCR) to amplify bisulfite treated DNA with primers that distinguish methylated (M) and unmethylated (U) DNA. Based on the primer designing tool for MSP method (http://www.urogene.org/methprimer/index1.html), we designed the primers for *BRCA1*, *RASSF1A* and ER, and some of these primers were used in combination with the published ones. Primer sequences used in this study and amplicon lengths are shown in Table 1. Bisulfite treated DNAs were subjected to single or nested PCR dependent on particular targeted genes. Then the PCR products were subjected to electrophoresis on 12% acrylamide gel. All PCR reactions were replicated.

DNA that was extracted from

lymphocytes of the healthy volunteers and then treated with bisulfite was used as a positive control for *BRCA1*, *RASSF1A* and *ER* unmethylation. A mixture of plasmid DNA containing methylated *BRCA1*, *RASSF1A* or *ER* sequences and DNA extracted from normal lymphocytes was used as a positive control for methylation. Water with no DNA template was included in each PCR reaction as a control for contamination. Methylation status was confirmed by sequencing the cloned MSP products for a subset of samples from each assay.

Statistical analysis

Associations between clinicopathological characteristics and individual promoter methylation status were examined by using Fisher exact test. For all statistical analyses, a p-value of ≤ 0.05 was considered significant. All analyses were done by using the Simple Interactive Statistical Analysis Web-based program.

Results

Verification of the specificity of MSP primers

A false positive result for DNA methylation analysis could be due to MSP primers that amplified nonspecifically untreated genomic DNAs. Thus, we verified the efficiency of bisulfite treatment of genomic DNAs, and the specificity of designed MSP primer sets by using bisulfite treated and untreated DNAs as templates for PCR. Full conversion of genomic DNAs was verified by PCR with β globin primer sets that discriminated treated DNAs from untreated ones. By using β globin primers designed from native DNA sequences, we found that most PCR products were amplified from only untreated DNAs. In contrast to that, PCR products amplified by β globin primers designed for unmethylated sequences were detected from bisulfite-treated DNAs instead from the native DNAs (data not shown). Bisulfite treated DNAs and native DNAs were separately applied to MSP with the *BRCA1*, *RASSF1A* or *ER* primer sets. The results indicated that no MSP products were amplified from untreated DNAs extracted either from lymphocytes or from ovarian cancer tissues (Figure 1). The respective MSP products were amplified only from bisulfite treated DNAs extracted from lymphocytes by using MSP primer sets specifically designed for detection of unmethylated targets (Figure 1A). These results indicated that the specificity of designed MSP primers for unmethylated targets was reliable. On the other hand, no MSP products were amplified from treated DNAs extracted from lymphocytes by using MSP primer sets that were specifically designed for detection of methylated targets (Figure 1B). MSP products were amplified from a mixture of plasmid DNAs containing methylated *BRCA1*, *RASSF1A* or *ER* sequences and DNAs extracted from normal lymphocytes (Figure 1). These results ensured that the accuracy of MSP primers designed for only methylated targets was guaranteed.

Methylation status of BRCA1, RASSF1A and ER in EOC and benign ovarian tissues

Genomic DNAs extracted from 59 EOC and 10 control specimens taken from benign ovarian tissues were treated with bisulfite and then subjected to MSP assays. No biallelic methylation (M/M) signals were detected whereas biallelic unmethylation (U/U) and monoallelic methylation (M/U) signals were detected for *BRCA1*. In contrast, three patterns of M/M, M/U and U/U signals were observed for *RASSF1A* and *ER* (Table 2). Biallelic unmethylation (U/U) and monoallelic methylation (M/U) signals of *BRCA1* were detected whereas only biallelic unmethylation (U/U) signal of *RASSF1A* and *ER* was detected from 10 benign ovarian tissues. The number of methylated *BRCA1*, *RASSF1A* and *ER* detected from 59 EOC was 11/59 (18.6%), 40/59 (67.8%) and 15/59

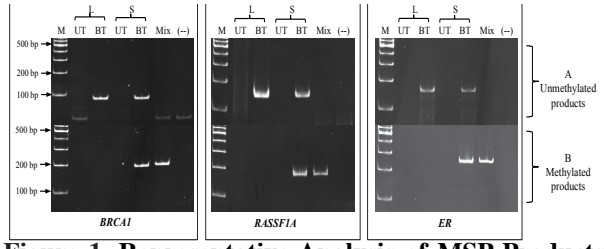

Figure 1. Representative Analysis of MSP Products Amplified from Untreated DNAs (UT) and Bisulfite Treated DNAs (BT) with the Primer Sets of BRCA1, RASSF1A and ER for the Detection of Unmethylated Sequences (A) and Methylated ones (B). L: lymphocytes of the healthy volunteers. S: ovarian cancer samples. Mix: a mixture of plasmid DNAs containing methylated *BRCA1*, *RASSF1A* or *ER* sequences and DNA extracted from normal lymphocytes. (--): Negative control without DNA templates. M: 100 bp DNA ladder

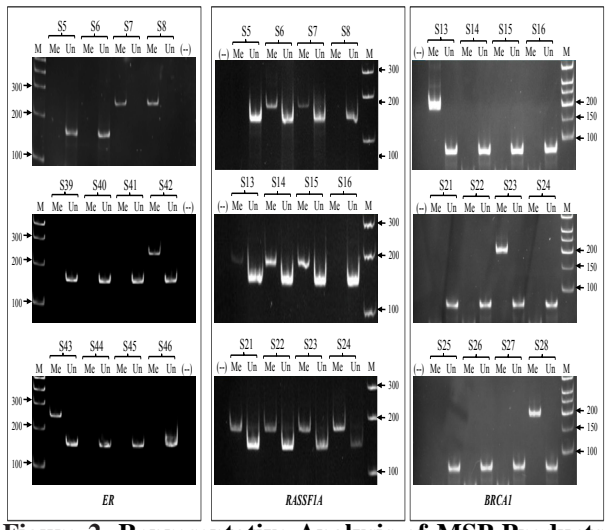

Figure 2. Representative Analysis of MSP Products Amplified by the Primer Sets of BRCA1, RASSF1A and ER for the Detection of Unmethylated Sequences (Un) and Methylated Ones (Me). S: ovarian cancer samples. (--): Negative control without DNA templates. M: DNA ladders

Table 2. Status and Frequency of Methylation of *BRCA1*, *RASSF1A* and *ER* in EOC

Genes	*BRCA1*	*RASSF1A*	*ER*
M/M	0	5	5
M/U	11	35	10
U/U	48	19	44
Methylation ratio	11/59 (18.6%)	40/59 (67.8%)	15/59 (24.5%)

Table 3. Association of Pathologic Characteristics of the 59 Ovarian Cancer Patients and 10 Benign Ovarian Tissues with Methylation Status of *BRCA1*, *RASSF1A* and *ER*

Pathologic characteristics	Overall (N=69)	Proportion of methylated targets (%)							
		BRCA1	p	*RASSF1A*	p	*ER*	p		
Ovarian tumors									
Age	59	11		40		15			
≥ 50	31	5	0.125	20	0.387	6	0.180		
≤ 50	28	6		20		9			
FIGO stage									
I	44	8 (72.8%)	0.573	30 (75.0%)	0.576	11 (73.4%)	0.573		
II	13	2 (18.2%)		9 (22.5%)		2 (13.3%)			
III	2	1 (9%)		1 (2.5%)		2 (13.3%)			
Benign ovarian tissues									
	10	2 (20%)	0.605	0 (0%)	**0.0005**	0 (0%)	0.07		

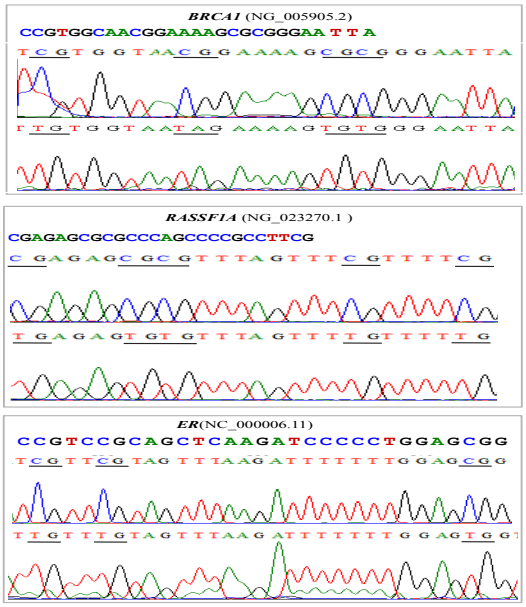

Figure 3. The Native Sequences of *BRCA1* (NG_005905.2), *RASSF1A* (NG_023270.1) and *ER* (NC_000006.11) in Comparison with their Methylated (upper) and Unmethylated (under) Sequences. Cytosines in the CpG sites (underlined) remained to be cytosines in the methylated *BRCA1*, *RASSF1A* and *ER* sequences but they converted to thymidines in the unmethylated ones. All cytosines alone were converted to thymidines in both methylated and unmethylated sequences

(25.4%), respectively. Forty five out of 59 EOC (78%) showed methylated alleles of one or more of the three genes. Representative results of the MSP reactions of the three genes were illustrated in Figure 2.

Finally, the methylation frequency of *RASSF1A* was significantly associated with EOC (p<0.0005). However, no significant differences in the methylation frequencies of *BRCA1*, *RASSF1A* and *ER* were observed between stages and ages of ovarian cancer patients (Table 3).

Sequencing MSP products representative to BRCA1, RASSF1A and ER in EOC

MSP products amplified by using the primer sets specific to methylated and unmethylated sequences of three genes were cloned into pGEM-T Easy vector (Promega). Three representatives of each of the MSP products were sequenced (Figure 3). The nucleotide sequences showed that all cytosines were converted to thymidines in unmethylated *BRCA1*, *RASSF1A* and *ER* products, and that all cytosines in the CpG sites remained to be cytosines and the cytosines alone were converted to thymidines in the methylated *BRCA1*, *RASSF1A* and *ER* products. The sequencing analysis confirmed the accuracy of specific MSP primers designed in this study.

Discussion

Aberrant methylation on CpG islands is one of the earliest molecular alterations occurring during carcinogenesis (Heyn and Esteller, 2012); thus, it has emerged as promising biomarkers for the early detection of cancers including ovarian cancer (Heichman and Warren, 2012; Montavon et al., 2012). DNA methylation profile of thousand genes can be quantitatively assessed at once by technological advances such as DNA microarrays or high-throughput DNA sequencing, which may not be accessible to many institutions in developing countries (Houshdaran et al., 2010; Feng et al., 2011). Besides, DNA methylation has been usually analyzed for particular genes by the methylation specific PCR (MSP) approach that is simple, highly sensitive, very cost-effective, and does not require special equipments; thus, the approach is routinely used in every DNA dealing laboratory (Kristensen and Hansen, 2009). The weakness of MSP method is the co-amplification of methylated and incompletely converted sequences; thus, the full conversion of each bisulfite-treated DNA template should be verified through PCR by using housekeeping gene primers (Wilcox et al., 2005). However, it is laborious and inconvenient to analyze a large number of samples. To overcome this barrier, we tested the accuracy of MSP primers specific to methylated sequences only through PCR, in which no MSP products were amplified from native or bisulfite treated DNAs extracted from lymphocytes of the healthy volunteers (Figure 1). This finding confirmed that unmethylated targets as well as a trace of uncompleted treated DNA were not inferred from the MSP results; thus, false positive results were avoided. This finding also guarantees that we could apply the designed MSP primers in order to analyse the methylation status of *BRCA1*, *RASSF1A* and *ER* in EOC samples.

In our study, methylation of *BRCA1*, *RASSF1A* and *ER* was found in 11/59 (18.6%), 40/59 (67.8%) and 15/59 (25.4%) in EOC, respectively. Forty five out of the 59 EOC (78%) were methylated at one or more genes. In comparision with the previous reports in which MSP method was performed to the *BRCA1* and *RASSF1A* sequences, we found that the methylation frequencies of *BRCA1* and *RASSF1A* in Vietnamese ovarian cancer patients were comparable to those detected in different racial groups (Barton et al., 2008). For instance, methylation frequency of *BRCA1* was 16% (8/50) in American, and that of *RASSF1A* was 66% (33/50) in Pakistan patients (Wilcox et al., 2005; Matoo et al., 2013). Methylation frequencies of both genes were 17.1% (6/35) and 42.9% (15/35) in Chinese women with EOC (Pan et al., 2010). The methylation status on the entire CpG island of *ER* was usually analyzed by six MSP primer sets in previous reports (Lapidus et al., 1998). We selected the

primer sets that covered the most significantly methylated loci and verified their accuracy by using non-treated DNAs as templates. Unexpectedly, these primers co-amplified nonspecifically, which resulted in false positive amplification from incompletely treated DNA templates. By designing the new primers for the same loci, some of these primers were used in combination with the published ones (Table 1), we found that *ER* methylation was 25.4% in ovarian tumors from Vietnamese patients. The methylation frequency of this locus was 56.5% in ovarian tumors from Italian patients (Wiley et al., 2006). This frequency was relatively higher than that of our result, and it might be due to MSP primer's specificity as well as the full conversion of treated DNAs that both have not been minded yet.

In this study, the methylation frequency of *RASSF1A* was significantly correlated with ovarian tumors, almost all of these tumors were in the early stage (FIGO I/II) (Table 3). This result was consistent with previous reports in which *RASSF1A* methylation was frequently associated with the early stage of many primary tumors including ovarian one (Agathanggelou et al., 2001). However, no significant differences in the methylation frequencies of *BRCA1*, *RASSF1A* and *ER* were observed between stages and ages of ovarian cancer patients. Our results were similar to those reported by Rathi et al. (2002) and Pan et al. (2010), who performed MSP method to analyze the methylation status of *BRCA1* and *RASSF1A* in 49 and 35 ovarian cancer patients, respectively. In addition, no correlations between the methylation levels of *BRCA1* and *ER* that were determined by MSP method and the clinical and pathological parameters of tumors were observed in 215 ovarian cancer patients (Wiley et al., 2006). Moreover, methylation status of *BRCA1* and *ER* determined by MS-MLPA method and that of *BRCA1* and *RASSF1A* determined by head loop PCR showed no association with tumor stages in 75 and 80 ovarian tumors, respectively (Montavon et al., 2012; Ozdemir et al., 2012). It is notable that the correlation between methylation of these genes and clinic-pathological tumor has been reported at the late stage (FIGO III/IV) of ovarian tumors whereas almost all of the examined specimens in our study were in the early stage (FIGO I/II) (Teodoridis et al., 2005; BonDurant et al., 2011). Additionally, methylation occurred at some other genes which have a role in different cellular pathways was observed in stage I EOC with high sensitivity (82-85%) and specificity (90-100%) (de Caceres et al., 2004; Zhang et al., 2013). Thus, identifying methylation status of these genes that analyzed in this study and other genes could provide the promising potential of methylation biomarker for early detection and prognosis/drug resistance as well as targeted treatment of ovarian cancer (Barton et al., 2008; Asadollahi et al., 2010). The MSP method, which was standardized in this study, is advantageous for us in screening and identifying the key gene(s) whose methylation status is clinical biomarkers in diagnosis and prognosis of ovarian cancer in particular and of different types of cancers in general.

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