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Original Article

Peripheral blood *BRCA1* methylation profiling to predict familial ovarian cancer

Yuyeon Jung [1,2] **Sooyoung Hur** [1,2] **JingJing Liu** [1,3] **Sanha Lee** [1] **Byung Soo Kang** [1] **Myungshin Kim** [4,5] **Youn Jin Choi** [1,2]

[1]Department of Obstetrics and Gynecology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea
[2]Cancer Research Institute, College of Medicine, The Catholic University of Korea, Seoul, Korea
[3]Department of Obstetrics and Gynecology, Yantai Affiliated Hospital of Binzhou Medical University, Yantai, China
[4]Department of Laboratory Medicine, College of Medicine, The Catholic University of Korea, Seoul, Korea
[5]Catholic Genetic Laboratory Center, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea

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Correspondence to
Myungshin Kim
Department of Laboratory Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 222, Banpo-daero, Seocho-gu, Seoul 06591, Korea.
E-mail: microkim@catholic.ac.kr

Youn Jin Choi
Department of Obstetrics and Gynecology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 06591, Korea.
E-mail: yunno@catholic.ac.kr

ORCID iDs
Yuyeon Jung
https://orcid.org/0000-0001-8862-3971
Sooyoung Hur
https://orcid.org/0000-0001-6563-2293

ABSTRACT

Objective: Familial cancer appears at a young age and its incidence is increasing. About 12% of familial ovarian cancer cases are associated with *BRCA1/2* mutations (BRCAm). In this study, we investigated *BRCA1* methylation may predict ovarian cancer in those with a family history of cancer (FHC) but without *BRCA1/2* mutations (BRCAwt).
Methods: Using peripheral blood DNA from 55 subjects without a history of cancer [cancer(−)] and 52 ovarian cancer patients, we examined *BRCA1* promoter methylation through bisulfite sequencing of the promoter and expressed the results as the cumulative methylation index. Then, we evaluated the *BRCA1* promoter methylation according to *BRCA1/2* germline mutations.
Results: *BRCA1* methylation was more prevalent in the BRCAm cancer(−) group than in the BRCAwt cancer(−) group and ovarian cancer patients (p=0.031 and p=0.019, respectively). In the BRCAwt cancer(−) group, *BRCA1* methylation was more prevalent in those with an FHC than in those without one and in the BRCAm cancer(−) group with an FHC (p=0.001 and p<0.001, respectively).
Conclusion: Our data suggest a predictive role of *BRCA1* methylation profile for ovarian cancer in those without a history of cancer but with an FHC. *BRCA1* methylation has important implications for diagnostic and predictive testing of those with BRCAwt cancer(−) status with FHC.

Keywords: Genes *BRCA1*; Genes *BRCA2*; DNA Methylation; Hereditary Breast and Ovarian Cancer Syndrome; Ovarian Cancer

INTRODUCTION

Ovarian cancer, the second most common gynecologic cancer, has a high fatality rate [1]. Notably, about 12% of ovarian cancer cases are hereditary and those affected have at least one first-degree female relative with the disease, mostly due to germline mutations in the *BRCA1* (17q21.31) or *BRCA2* (13q13.1) genes. Heterozygous carriers of *BRCA1/2* mutations (BRCAm) have an increased lifetime risk of ovarian cancer development (*BRCA1*, 40%–60%; *BRCA2*, 11%–30%) [2,3].

JingJing Liu
https://orcid.org/0000-0002-3828-1608
Sanha Lee
https://orcid.org/0000-0003-0269-0087
Byung Soo Kang
https://orcid.org/0000-0003-2515-8536
Myungshin Kim
https://orcid.org/0000-0001-8632-0168
Youn Jin Choi
https://orcid.org/0000-0003-4699-2116

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Author Contributions

Conceptualization: J.Y., K.M., C.Y.J.; Data curation: J.Y.; Formal analysis: L.J., K.M.; Project administration: H.S., K.M., C.Y.J.; Supervision: J.Y., K.M., C.Y.J.; Writing - original draft: J.Y.; Writing - review & editing: L.S., K.B.S., C.Y.J.

BRCA1/2 carriers are advised to undergo evaluation and management to reduce the risk of ovarian cancer development. In a study of 283 ovarian cancer families, only 27% of families had ovarian cancer due to *BRCA1* or *BRCA2* mutations, which strongly suggests the existence of other genetic and epidemiologic risk factors [4,5]. Recent work found that two genes—*AKRD11*, a putative tumor suppressor, and *POLE*, a DNA repair and replication enzyme—predispose the development of ovarian cancer. Despite ongoing research to identify predisposing genes, a proportion of ovarian cancer risk cannot yet be fully explained [6].

The silencing of tumor suppressor genes (e.g., *BRCA1/2*) by methylation of CpG islands is key to the tumorigenic process, contributing to all of the typical hallmarks of cancer that result from transcriptional inactivation of tumor suppressor genes and the loss of their normal cellular functions [7]. Methylated DNA is an extremely sensitive and specific tumor marker and its detection has recently been exploited for the early diagnosis of primary or recurrent cancer. Methylated *GSTP1* aids in the early diagnosis of prostate cancer, methylated *PITX2* predicts outcomes in lymph node-negative breast cancer patients, and methylated *MGMT* predicts the benefit from alkylating agents in patients with glioblastomas [8-10]. In ovarian cancer, silencing of DNA repair, cell cycle, and growth regulation genes such as *p16**INK4a*, *PTEN*, *CDKN2A*, *MLH1*, *RASSF1A*, and *CHD1* have established promoter methylation as a common mechanism for tumor suppressor inactivation [11].

Epigenetic modifications of *BRCA1/2* genes promote early-onset hereditary breast/ovarian cancer [2]. A recent study suggested the existence of a strong link between breast cancer and *BRCA1* methylation in white blood cells, indicating the potential cause of breast cancer [12]. Because another recent study suggested that *BRCA1* promoter methylation is positively associated with risk of ovarian cancer development [13], the association between carriers and *BRCA1* methylation status requires further study.

In this study, we used pyrosequencing to analyze *BRCA1* methylation status at known promoter regions and CpG islands in peripheral blood from subjects without a history of cancer [cancer(−)] and ovarian cancer patients. We investigated the extent of *BRCA1* methylation according to *BRCA1/2* germline mutations.

MATERIALS AND METHODS

1. Patient samples

Peripheral blood from 55 cancer(−) individuals (44 BRCAwt, 11 BRCAm) and 52 ovarian cancer patients (33 BRCAwt; 19 BRCAm) was provided by the Korea Gynecologic Cancer Bank, Seoul St. Mary's Hospital Biobank, and the Department of Obstetrics and Gynecology at the Catholic University of Korea. Inclusion criteria were consented to participate in a BRCA genetic study, presence of breast, ovarian, or pancreatic cancer, a type of family history of cancer (FHC; breast, ovarian, or pancreatic cancer), known *BRCA* mutation carriers, or those who donated human material for study. The ones who did not agree to pursue the study were excluded. In addition, the patients/ participants with FHC are defined as the ones who have at least one of breast cancer, ovarian cancer or pancreatic cancer in first- or second-degree relatives. The blood of ovarian cancer patients was taken before surgery and that of the cancer(−) group was provided as donations of human material for a Cancer Bank or Biobank. We used this to analyze BRCA mutation and methylation. All samples were obtained with appropriate consent. This study was approved by the Institutional Review Board of Seoul

St. Mary's Hospital, the Catholic University of Korea, College of Medicine (IRB approval, KC17TESI0690). Informed consent was obtained from the patients included in the study.

2. *BRCA1/2* mutation analysis

We performed targeted NGS using the BRCAaccuTest PLUS panel (NGeneBio Co. Ltd., Seoul, Korea) and MiSeq instrument (Illumina Inc., San Diego, CA, USA) or Axen BRCA panel (Macrogen, Seoul, Korea) and Nextseq500 instrument (Illumina Inc.), in accordance with the manufacturers' protocols. After FASTQ files were generated, the Bam file obtained from sequencing was processed with NGeneAnalySys software (NGeneBio Co. Ltd.). Variant calling was performed on the variant call format (VCF) output files by evaluating the coverage (the number of times that targeted, during the sequencing) and the quality score. Then, we filtered only non-synonymous exonic single-nucleotide variants (SNVs) according to the quality criteria: 1) coverage of at least 20×; 2) a Q-score ≥30 (an error rate in base calling of 1 in 1,000); and 3) at least 30% of the reads indicating the variant (variant frequency). Copy number variations were also detected by calculating the ratio of each of the total amplicons in each sample based on the normalized amplicon coverage derived from other samples within the same run [14,15]. This analysis included read alignment to the human reference genome (Genome Reference Consortium, GRCh37). Genetic variants were classified using a five-tier system according to the guidelines of the American College of Medical Genetics and Genomics (ACMG): pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign, or benign. Pathogenic and likely pathogenic variants were considered significant. Multiplex ligation-dependent probe amplification (MLPA) analyses were performed for large genomic rearrangement (LGR) of *BRCA1/2* in our previous study. Probe mixes P002 and P045 were used for screening of LGR, and P087 and P077 were used for confirmation (MRC-Holland, Amsterdam, Netherlands). MLPA data were analyzed using Genemarker v1.91 (Softgenetics, State College, PA, USA) [14,16].

3. *BRCA1* methylation analysis

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Bisulfate conversion was performed with 2 mg DNA using the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions. Denaturation was performed at 95°C for 5 minutes. PCR and pyrosequencing primers used for *BRCA1* methylation analysis were as follows: forward, GGTTAATTTA GAGTTTCGAG AGACG; and reverse, TCAACGAACT CACGCCGCGC AATCG. The polymerase chain reaction (PCR) mixture contained 100 ng bisulfite-converted DNA, 10× PCR buffer, 10 mM dNTP, DNA Taq polymerase, and 10 pmol of the forward and 5′ biotinylated reverse primers in a 25 mL reaction volume. Pyrosequencing was performed with the PyroGold Q96 Reagent kit (Qiagen AG, Basel, Switzerland) and PyroMark ID system™ (Qiagen AG) according to the manufacturer's recommendations. CpG site quantification was performed with PyroQ-CpG™ methylation software (version 1.0.11.14; Qiagen AG). Typical pyrograms for quantitative PCR samples are illustrated in **Supplementary Fig. 1**. The intensity was measured at each dispensation sequence and presented graphically in a pyrogram. The dispensation order was generated automatically by Pyromark. The y-axis represents the signal intensity in arbitrary units, while the x-axis shows the dispensation sequence. Percentages indicated above the pyrograms represent the proportion of C at each CpG site after bisulfite conversion, and the methylation level of each CpG site is estimated by the proportion of C (%). The overall BRCA1 promoter methylation level is calculated by the sum of the methylation percentage as the cumulative methylation index (CMI) [17-19].

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4. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 16.0 software (IBM, Armonk, NY, USA). Associations between dichotomized clinicopathological characteristics were assessed with the Fisher's exact test and an independent t-test. *BRCA1* methylation status was compared by one-way analysis of variance with Tukey honestly significant difference post hoc testing. Differences were considered statistically significant at $p<0.05$.

RESULTS

1. Patient characteristics

A total of 107 subjects (55 in the cancer[−] group and 52 ovarian cancer patients) were enrolled in this study (**Supplementary Table 1**). The clinical demographics of the cancer(−) group and ovarian cancer patients are shown in **Table 1**. Of the 55 individuals in the cancer(−) group, 32 had a FHC (e.g., breast, ovarian, or pancreatic cancer in first- or second-degree relatives), 34.4% of whom (11 of 32) showed BRCAm. Of the 52 ovarian cancer patients, 7 had an FHC, 57% of whom (4 of 7) showed BRCAm. Notably, all individuals in the cancer(−) BRCAm group had an FHC (n=11), whereas 4 of 19 (21.1%) BRCAm ovarian cancer patients had one. The pathological characteristics of the 52 ovarian cancer patients are summarized in **Supplementary Table 2**.

2. BRCAm

Of the 107 subjects, BRCAm were identified in 30:11 in the cancer(−) group and 19 ovarian cancer patients (**Supplementary Table 1**). Of those in the cancer(−) group, 9 had pathogenic/likely pathogenic variants and 2 had deletions. There was no difference in the proportion of *BRCA1/2* status between the cancer(−) group and ovarian cancer patients (**Supplementary Table 3**). Among the cases with an FHC, there were 13 pathogenic variants/likely pathogenic variants (33.3%), 9 VUSs (23.1%), and 2 deletions (5.1%). Among all of the cases without an FHC, there were 12 pathogenic/likely pathogenic variants (17.7%), 27 VUSs (39.7%), and 3 deletions (4.4%).

3. *BRCA1* methylation

The CMI of *BRCA1* did not differ between the cancer(−) group and ovarian cancer patients ($p > 0.05$, data not shown). The *BRCA1* CMI was significantly higher in the BRCAm cancer(−) group than in the BRCAwt cancer(−) group (mean CMI, 47.0 vs. 84.4; p=0.031). The *BRCA1* CMI in ovarian cancer patients did not differ between BRCAwt and BRCAm (mean CMI, 63.2 vs. 79.5; p=0.484) (**Fig. 1**). Next, we subclassified those in the cancer(−) group according

Table 1. Patients' clinical characteristic (n=107)

Clinical characteristics	Cancer(−)			Cancer(+)		
	BRCAwt	BRCAm	p-value	BRCAwt	BRCAm	p-value
Number of cases	44 (41.1)	11 (10.3)		33 (30.8)	19 (17.8)	
Age (yr)	43 (43.1, 23–72)	35 (39.3, 22–69)	0.172	59 (58.2, 39–78)	54.5 (56.7, 44–80)	0.638
Personal history of other cancer			NA			0.011
Negative	44 (41.1)	11 (10.3)		30 (28.0)	11 (10.3)	
Positive	0	0		3 (2.8)	8 (7.5)	
Family history of cancer			0.001			0.400
Negative	23 (21.5)	0		30 (28.0)	15 (14.0)	
Positive	21 (19.6)	11 (10.3)		3 (2.8)	4 (3.7)	

Values are presented as number of patients (%) or median (mean with range).
BRCAm, *BRCA1/2* mutation; BRCAwt, *BRCA1/2* wild type; NA, not available.

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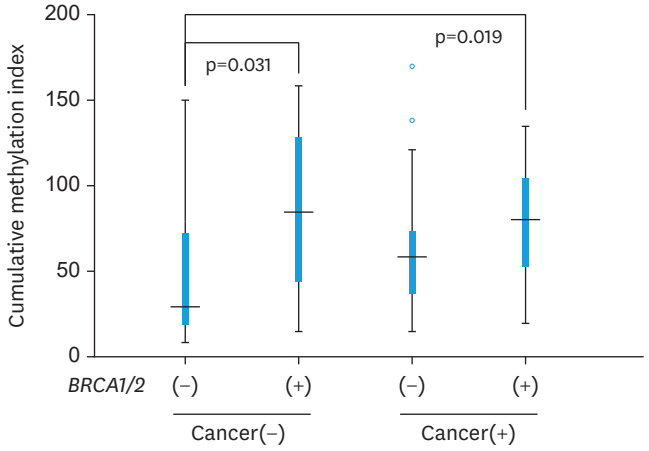

Fig. 1. Methylation status of the *BRCA1* promoter.

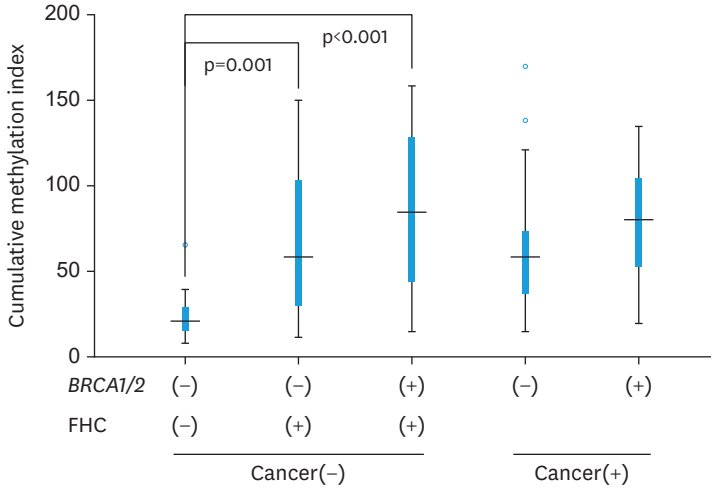

Fig. 2. *BRCA1* promoter methylation according to FHC.
FHC, family history of cancer.

to the presence of an FHC. All of those in the BRCAm cancer(−) group had an FHC (n=11). Of those in the BRCAwt cancer(−) group, 21 had an FHC and 23 did not (**Supplementary Table 1**). In the cancer(−) group, *BRCA1* CMI was higher in those with an FHC than in those without one in both BRCAwt and BRCAm (p=0.001 and p<0.001, respectively). Among those in the cancer(−) group with an FHC, the *BRCA1* CMI was not significantly different between BRCAwt and BRCAm (p=0.861) (**Fig. 2**). In ovarian cancer patients, the *BRCA1* CMI was not significantly different according to the presence of an FHC.

DISCUSSION

We investigated *BRCA1* methylation status in the peripheral blood of cancer(−) subjects and ovarian cancer patients with or without BRCAm. First, we found that *BRCA1* methylation was more common in BRCAm cancer(−) than in BRCAwt individuals. Second, those in the BRCAm cancer(−) group with an FHC had a significantly higher frequency of methylation than those who were BRCAwt and lacked an FHC.

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The methylation status of the CpG dinucleotides in the promoter and/or transcriptional regulatory region of certain cancer susceptibility genes has been studied in various types of cancers, such as breast, ovary, and leukocyte-derived cells. The mechanisms underlying cancer development [15] are as follows: 1) germline mutation, 2) somatic mutation, 3) loss of heterozygosity or an allelic deletion, and 4) epigenetic modifications of the promoter region of tumor suppressor and DNA repair genes. Germline mutations disrupt the function of the encoded proteins and affect first-degree relatives with the disease. Heterozygous *BRCA1* and *BRCA2* carriers respectively have 40%–60% and 11%–30% increased lifetime risks of ovarian cancer. *BRCA1* carriers are recommended to undergo risk-reducing salpingo-oophorectomy at 35–40 years old [20], whereas *BRCA1/2* carriers have been recommended to undergo surveillance and management to prevent ovarian cancer. Somatic mutations in *BRCA1/2* are rare causes of ovarian cancer. Allelic instability in the *BRCA* genes might also affect their expression. Aberrant cytosine methylation of the CpG dinucleotides in the promoter region alters the transcriptional level of the *BRCA* genes and methylation of the *BRCA1* promoter has been shown to reduce mRNA expression [15].

Numerous studies have been conducted on germline *BRCA* mutations. However, *BRCA* methylation is considered a sporadic occurrence and was not deemed as important as germline *BRCA* mutations [18]. In recent work, the incidence of *BRCA1/2* methylation in ovarian cancer was more frequent than germline mutations. *BRCA1* methylation occurred in 11%–89% and *BRCA2* methylation in 0%–98.7% of ovarian cancer cases [21]. *BRCA* methylation can account for a large proportion of ovarian cancer occurrence.

This study examined *BRCA1* methylation in BRCAwt and BRCAm individuals with an FHC. In the cancer(−) participants of **Fig. 2**, BRCAwt and BRCAm participants with a FHC exhibited significant methylation compared with BRCAwt participants without a FHC ($p=0.001$ and $p<0.001$, respectively). On the other hand, BRCAm participants with a FHC showed no significant differences versus BRCAwt participants with a FHC ($p=0.861$). This finding suggested that *BRCA1* methylation is a key predictive factor in BRCAwt with an FHC. Previous work also showed that ovarian cancer patients with methylated *BRCA1* (n=11, 35.6 months) had significantly shorter survival than BRCA1m (n=22, 78.6 months) [22]. We also confirmed that *BRCA1* methylation affected the overall survival of ovarian cancer patients (data not shown). Our study showed that *BRCA1* methylation may promote ovarian cancer development and reduce survival. We propose that a *BRCA1* methylation study be performed in potential BRCAwt individuals with a FHC, although further work is needed. In previous breast cancer studies, *BRCA* methylation was measured as a pathologic marker of *BRCA1* variant pathogenicity [23] and as a prescreening testing tool for germline genetic testing [18]. In addition, Anjum et al. [24] suggested that *BRCA* methylation predisposed women to breast cancer. BRCAwt ovarian cancer patients with a FHC do not undergo any specific management. We suggest that BRCAwt individuals with a FHC undergo an evaluation of *BRCA1* methylation to predict ovarian cancer at early stages. But this study has a limitation in its small number of patient samples. Whereas the present study is limited by small sample size, our data suggests that in FHC with BRCAwt, *BRCA1* methylation represents a significant source of ovarian cancer. It is suggested that the future study be designed with a large number of samples in prospective manner.

It is believed that our study may become a foundation to establish a genetic/familial high-risk assessment guideline for the population with BRCAwt with FHC in the future. Although BRCAm carriers have guidelines of genetic/familial high-risk assessment [20], the ones

with *BRCA1* methylation do not. As described in the National Comprehensive Cancer Network guideline, germline *KILLIN* methylation is suggested to be associated with Cowden syndrome, a hereditary disease [25]. In addition, another study showed the transmission of *BRCA1* and *MGMT* epimutations from mother to daughter [26]. It is believed that the roles of epigenetic regulation of cancer development have not yet been fully elucidated and further studies are needed.

In conclusion, *BRCA1* methylation may contribute to familial ovarian cancer development as *BRCA1/2* germline mutation in those with a cancer(−) status but with an FHC. This study found that evaluation of *BRCA1* methylation might be indispensable for proper surveillance and management in BRCAwt individuals with an FHC.

SUPPLEMENTARY MATERIALS

Supplementary Table 1
Description of the samples obtained from healthy participants and ovarian cancer patients

Click here to view

Supplementary Table 2
Pathological characteristics of ovarian cancer patients

Click here to view

Supplementary Table 3
BRCA1/2 genomic variations

Click here to view

Supplementary Fig. 1
Methylation analysis by pyrosequencing. (A) FHC(−) with BRCAwt in cancer(−) on quantitative PCR. (B) FHC(+) with BRCAwt in cancer(−) on quantitative PCR. The methylation percentage at each position is noted above the pyrogram.

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