MicroRNA-126 expression in the peripheral white blood cells of patients with breast and ovarian cancer is a potential biomarker for the early prediction of cancer risk in the carriers of methylated *BRCA1*

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Abstract. Constitutive breast cancer type 1 gene (*BRCA1*) promoter methylation is associated with increased cancer risk, but its role in cancer-free (CF) female carriers is incompletely understood. MicroRNA (miR) is modulated during early tumorigenesis. The present study assessed the modulation of miR-126 expression in the peripheral white blood cells (WBC) of patients with breast cancer (BC) and ovarian cancer (OC) as a biomarker of cancer risk in *BRCA1* methylation carriers. A total of 1,114 female subjects [502 patients with BC, 187 patients with OC and 425 CF volunteers] were involved. Screening for *BRCA1* promoter methylation in WBC was performed using the methylation-specific polymerase chain reaction (PCR) assay, *BRCA1* mRNA was analyzed using a reverse transcription-quantitative PCR assay and miR-126 expression was analyzed using a stem-loop RT-qPCR assay. WBC *BRCA1* promoter methylation status was significantly associated with OC (P=0.0266), early-onset BC (P=0.0003) and triple-negative BC (P=0.0066). Notably, 9.4% of the CF group exhibited WBC *BRCA1* promoter methylation. In addition, high levels of miR-126 in WBCs were detected in all three groups. The increased level of miR-126 was significantly associated with a lower risk of distant metastasis (P=0.045) in BC, but a higher risk of disease progression and death (P=0.0029) in OC. There was a positive correlation between *BRCA1* mRNA and miR-126 levels in the WBCs of all three groups, regardless of *BRCA1* promoter methylation status. Notably, circulating miR-126 level was decreased in the BC and OC groups, but not in the CF group. Together, these results suggest the likely involvement of miR-126 in the constitutional methylation of *BRCA1* promoter-related malignancies. Therefore, miR-126 may be a candidate biomarker for the early prediction of BC and OC risk in CF *BRCA1* methylation carriers.

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Abbreviations: BC, breast cancer; *BRCA1*, breast cancer type 1 gene; CF, cancer-free; miR-126, microRNA-126; OC, ovarian cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WBC, white blood cell

Key words: BRCA1, methylation, WBC, miR-126, breast cancer, ovarian cancer

Introduction

Distant metastases of cancer are responsible for most cancer-associated deaths. Breast cancer (BC), which is the most common cancer type in women worldwide, has enormous socio-economic and public health impacts. The progression of BC occurs through a series of gradually abnormal stages, beginning with ductal carcinoma *in situ* (DCIS), which, if untreated, might progress to invasive ductal carcinoma (IDC) (1). IDC, the most common form of BC (2), is a histologically heterogeneous group of breast lesions with the potential for progression to metastatic BC (3).

Ovarian cancer (OC) is less common than BC but, due to the absence of signs or symptoms associated with early-stage disease, it is responsible for more deaths than other cancer types in women. High-grade serous OC (HGSOCs), which account for 68% of OC cases, are aggressive neoplasms commonly diagnosed at an advanced stage and have the worst prognosis (4). Complications associated with OC progression that have an ultimate fatal outcome occur in ~75% of patients despite good initial responses to chemotherapy (5).

BC type 1 gene (*BRCA1*) is a DNA repair and cancer suppressor gene that plays an essential role in maintaining genome integrity (6). Cells lacking BRCA1 protein are inclined to repair DNA damage by an error-prone mechanism resulting in gross chromosomal rearrangements and the generation of mutations that lead to carcinogenesis (7).

Thus, female carriers of germline *BRCA1* pathogenic mutations are at an increased risk of developing aggressive BC and OC at an early age. The inactivation of *BRCA1* by epigenetic alteration is an alternative mechanism during sporadic BC and OC carcinogenesis (8,9). BC tumors, harboring hypermethylated *BRCA1* promoter, display pathological features similar to *BRCA1*-mutated hereditary BC (8,10). Both types of tumors occur at an early age and are associated with the triple-negative (TNG) BC subtype (11,12). Moreover, the methylated *BRCA1* promoter occurs in all histological types of epithelial OC, including serous, endometrioid and clear cell carcinomas (8,13,14).

In 2008, Snell *et al* (15) made a breakthrough, finding that methylated *BRCA1* promoter is observed in peripheral white blood cells (WBC) of patients with mutation-negative familial BC and cancer-free controls. Since then, several studies have shown the association of WBC *BRCA1* promoter methylation with the risk of developing early onset BC and high-grade serous OC, with pathological features similar to those of patients with germline mutated *BRCA1* (15-24). The detection of *BRCA1* promoter methylation in the WBCs of cancer-free (CF) females has raised the question of whether those individuals are at risk of developing breast and ovarian cancer (15,16,20,21,24,25). Our previous study demonstrated a strong association between *BRCA1* promoter methylation and cancer-associated molecular changes in WBCs of CF *BRCA1* methylation carriers (21). However, further studies are still needed to confirm the cancer risk of those individuals.

MicroRNA (miRNA/miR) is a type of cancer-associated molecule that regulates various cellular mechanisms, such as proliferation, differentiation and oncogenesis (26). These miRNAs are small 18-22 base-pair non-coding RNA molecules that play a crucial role in regulating gene expression by binding to the 3'-untranslated region of mRNA of the target gene. Long-lasting exposure to carcinogens results in miRNA alterations that activate carcinogenic mechanisms (27). The activated carcinogenic process, such as chromosome deletion and silencing of miRNA host genes, results in the irreversible loss of miRNA. Thus, miRNAs are a sensitive tool in detecting carcinogenic exposure and the pathological consequences induced by that exposure (27). Amongst the identified miRNAs is miR-126. The expression of miR-126 is increased as a defense mechanism to asbestos exposure. The subsequent loss of miR-126, due to the accumulation of DNA damage and chromosome deletion, leads to malignant mesothelioma (27).

miR-126 is one of several miRNAs that play critical roles in several human cancer types. miR-126 is located within the 7th intron of the epidermal growth factor-like protein 7 gene, and acts as a suppressor of metastasis in several cancer types. Loss of miR-126 expression in tumor tissue is associated with poor distal metastasis-free survival, and restoration of miR-126 reduces overall tumor growth and proliferation (28). Studies have shown that miR-126 differentiates malignant BC from benign BC (29,30). The patients with DCIS BC have a lower level of tissue and circulating miR-126 compared with normal adjacent tissue and healthy controls, respectively (31). Furthermore, downregulation of miR-126 is associated with aggressive OC with a poor prognosis (32,33). However, another study has observed upregulation of miR-126 in OC (34).

In contrast to the use of plasma miRNA, few studies have evaluated the use of miRNA in peripheral blood WBCs as a biomarker of cancer risk. In the present study, miR-126 in WBCs and plasma is investigated as a potential biomarker for the early prediction of BC and OC in CF *BRCA1*-methylated female carriers.

Materials and methods

Patient population. Fresh blood samples (10 ml) were collected from 502 patients with BC and 187 patients with OC who visited the Department of Oncology in King Faisal Specialist Hospital and Research Centre (Riyadh, Saudi Arabia) between November 2017 and November 2021. The age of the patients ranged from 20-83 years (median, 48 years) for BC and 18-88 years (median, 53 years) for OC. Age, histological grade, estrogen receptor status and progesterone receptor status were provided by the Department of Pathology. For the CF female group, 10 ml fresh blood was collected from 425 CF female volunteers with an age range from 15-50 years. For newborn females, 20 leftover WBC RNA samples from our previous study (24) were used. Ethical approval (approval no. RAC #2170017) was obtained from the Human Research Ethics Committee of King Faisal Specialist Hospital and Research Centre. All participants provided written informed consent. The guardian of the volunteers provided written informed consent for participants <18 years old.

DNA and RNA isolation from WBC. Each fresh blood sample was collected in two BD Vacutainer EDTA (Becton, Dickinson and Company) blood collection tubes. The tubes were centrifuged immediately at 4˚C for 10 min at 1,962 x g. The supernatants were frozen at -80˚C in Eppendorf tubes for subsequent circulating RNA extraction using the QIAamp Circulating Nucleic Acid Kit (Qiagen GmbH). The WBC layers were collected and transferred into two 2-ml Eppendorf tubes. One tube contained 900 ml RBC lysis solution for subsequent DNA extraction, and the other tube contained 1.2 ml RNALater solution for subsequent RNA extraction using the Gentra Puregene Blood Kit and RiboPure Blood Kit (Ambion; Thermo Fisher Scientific, Inc.), respectively (24).

Methylation-specific polymerase chain reaction (PCR). Next, 2 μg WBC DNA was treated with sodium bisulfate and purified using the EpiTect Bisulfite kit (Qiagen GmbH) following the manufacturer's recommendations. The treated DNA was amplified using *BRCA1* PCR primers that distinguish between methylated and unmethylated DNA (Table I) (8). The PCR conditions used were an initial cycle at 95˚C for 1 min, then 40 cycles of 65˚C for 30 sec, 72˚C for 30 sec and a final extension at 72˚C for 7 min. All reactions were repeated at least twice.

Stem-loop PCR assay. Reverse transcription-quantitative PCR (RT-qPCR) for miR-126 was performed using a stem-loop RT primer and TaqMan miRNA RT kit (catalog no. 4427975; Applied Biosystems; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol (Table I) and using the thermocycling conditions stated below. The small nuclear RNA U6 (U6; assay ID: 001973) was used for normalization, and all primers

Table I. Methylation-specific PCR and RT-quantitative PCR primers.

Primer	Sequence (5'-3')	Annealing temperature, ˚C
M *BRCA1*		65
Forward	GGTTAATTTAGAGTTTCGAGAGACG	
Reverse	TCAACGAACTCACGCCGCGCAATCG	
U *BRCA1*		65
Forward	GGTTAATTTAGAGTTTTGAGAGATG	
Reverse	TCAACAAACTCACACCACACAATCA	
RT *BRCA1*		59
Forward	TGTAGGCTCCTTTTGGTTATATCATTC	
Reverse	CATGCTGAAACTTCTCAACCAGAA	
β-actin		59
Forward	TCCCTGGAGAAGAGCTACGA	
Reverse	TGAAGGTAGTTTCGTGGATGC	
miR-126 Stem-loop	CGGCCCAUUAUUACUUUUGGUACGCGCUAUGC CACUCUCAACUCGUACCGUGAGUAAUAAUGC	60
U6	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAA CGATACAGAGAAGATTAGCATGGCCCCTGCGCAAG GATGACACGCAAATTCGTGAAGCGTTCCATATTTT	60

PCR, polymerase chain reaction; M, methylated; U, unmethylated; RT, reverse transcription; *BRCA1*, breast cancer type 1 gene.

are stated in Table I. The expression level was calculated based on the threshold cycle value using the $2^{-\Delta\Delta Cq}$ method (35). The fold-change of miR-126 expression in patients and carriers was performed relative to controls.

RT-qPCR. cDNA was synthesized from WBC RNA using Superscript III, reverse transcriptase and random hexamers (High-Capacity cDNA Reverse Transcription Kit; cat. no. 4368814; Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR using specific primers for the BRCA1 transcript (Table I) was performed as described previously (24). β-actin was used as a housekeeping gene, and the primers for this and BRCA1 are stated in Table I. PCR was performed using the CFX96 Real-Time System (Bio-Rad Laboratories, Inc.) with SYBR Green (RT^2 SYBR Green Fluor qPCR Mastermix; cat. no. 330513; Qiagen GmbH). The qPCR thermocycling conditions were an initial cycle at 95˚C for 30 sec, followed by 44 cycles at 95˚C for 15 sec and 60˚C for 30 sec. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative BRCA1 expression. The fold-changes of mRNA expression were assessed relative to the unmethylated CF females, for patients with BC and OC, and CF female carriers.

Statistical analysis. Fisher's exact test was performed to determine the associations between *BRCA1* promoter methylation and age, miR-126 expression and clinicopathological features of BC and OC. The unpaired t-test was performed to determine the statistical significance between two groups for gene expression (adult CF carriers vs. controls and newborns carriers vs. newborn controls). One-way ANOVA with Dunnett's multiple comparison tests were performed for comparing multiple groups. GraphPad version 9.1.0 (GraphPad Software, Inc.) was used for all analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

WBC BRCA1 promoter methylation. Among the 502 patients with BC, 284 were aged <50 years and 218 were aged ≥50 years. There were 57 patients with methylated *BRCA1* in their WBCs, of which 45 were aged <50 years (15.8%) and 12 were aged ≥50 years (5.5%). There was a significant association between *BRCA1* methylation and the early onset of BC according to Fisher's exact test (P=0.0003; Table II). The clinicopathological characteristics of the screened patients with BC are shown in Table III. For the *BRCA1* methylation-positive patients, the clinicopathological parameters, other than age, were known for only 49 cases. Notably, 34.7% (17/49) of the methylated cases were TNG BC, compared to 17.4% (63/363) of unmethylated cases. There was a significant association between WBC *BRCA1* methylation and TNG BC according to Fisher's exact test (P=0.0066; Table III).

Among the 187 patients with OC, 70 were aged <50 years and 117 were aged ≥50 years. There were 30 patients with OC (16%) who tested positive for *BRCA1* methylation in their WBCs, with a significant association between WBC *BRCA1* methylation and the incidence of ovarian cancer according to Fisher's exact test (P=0.0266; Table II). However, unlike BC, there was no association between *BRCA1* methylation status and the onset of OC [20.0% aged <50 years (14/70) and 13.7% aged ≥50 years (16/117); P=0.3000]. The clinicopathological characteristics of the screened patients with OC are shown in Table IV. Most OC cases were of the serous OC subtype. Notably, 2 patients were positive for both BC and OC (Table IV).

Table II. Association between BC type 1 gene promoter methylation in the white blood cells of patients with OC or early onset BC.

Group	Patients with methylation, n (%)	Patients without methylation, n (%)	P-value
Control (n=425)	40 (9.4)	385 (90.6)	
BC (n=502)[a]	57 (11.4)	443 (88.2)	0.3890
Age, years			0.0003[b]
<50 (n=284)	45 (15.8)	238 (83.8)	
≥50 (n=218)	12 (5.5)	205 (94.0)	
OC (n=187)			0.0266[b]
Age, years			0.3000
<50 (n=70)	14 (20.0)	56 (80.0)	
≥50 (n=117)	16 (13.7)	101 (86.3)	

[a]Status of 2 patients was not determined. [b]P<0.05. BC, breast cancer; OC, ovarian cancer.

Table III. Clinicopathological features of screened BC cases.

Cancer subtype	Patients with methylation, n (%)	Patients without methylation, n (%)	ND, n (%)	P-value
BC (n=502)	57 (11.4)	443 (88.2)	2 (0.4)	
TNG (n=80)	17 (34.7)	63 (17.4)		0.0066[a]
IDC (n=264)	26 (53.1)	238 (65.5)		
DCIS (n=19)	3 (6)	16 (4.4)		
Other	3	46		
ND	8	80		

[a]P<0.05. For the *BRCA1* methylation-positive patients, the clinicopathological parameters, other than age, were known for only 49 cases. BC, breast cancer; TNG, triple-negative; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; ND, not determined.

In addition, among the 425 CF females who were screened, 9.4% (40/425) tested positive for the methylated *BRCA1* promoter (Table II).

miR-126 in WBCs of patients with BC. miR-126 was measured in 74 randomly selected patients with BC (age range, 29-82 years; median age, 47 years), of which 32 patients were positive for methylated *BRCA1* (median age, 44 years) (Table V). Based on a cut-off value of +3-fold relative to 17 age-matched female controls, there were 26 patients (35.1%) who had higher miR-126 expression, up to 18-fold, compared with the control (HBC group), and 48 patients (64.9%) who had unchanged miR-126 expression (UBC group) (Fig. 1A). The clinicopathological features, which were known for only 72 patients, showed that in the HBC group only 1 patient (3.8%) had distant metastasis compared to 11 patients (24%) in the UBC group. There was a significant negative association between miR-126 expression in WBC and the risk of distant BC metastasis according to Fisher's exact test (P=0.0452) (Table V). When the 32 patients with *BRCA1*-methylated BC were separately analyzed, similar results were observed where 9 patients (28%) had higher miR-126 expression, up to 13-fold, compared with the control (HBC group), and 23 patients (71.9%) had unchanged miR-126 expression (UBC group) (Fig. 1B). However, the negative association between miR-126 expression and the risk of distant BC metastasis was not statistically significant according to Fisher's exact test (P=0.3742) (Table VI).

Table IV. Clinicopathological features of screened OC cases.

Cancer type	Patients with methylation, n (%)	Patients without methylation, n (%)
OC (n=187)	30 (16.0)	157 (84.0)
HGSOC	24 (80.0)	93 (59.2)
OC + BC	2 (6.7)	5 (3.2)
Other	4 (13.3)	64 (40.8)

OC, ovarian cancer; BC, breast cancer; HGSOC, high-grade serous OC.

mRNA in WBCs of patients with BC. When *BRCA1* mRNA was measured in the WBCs of the patients with *BRCA1*-methylated BC, expression was significantly higher in the HBC group by up to 4-fold compared with the control group (P=0.0039). However, the UBC group did not significantly differ from the control group (P=0.4400) (Fig. 1C).

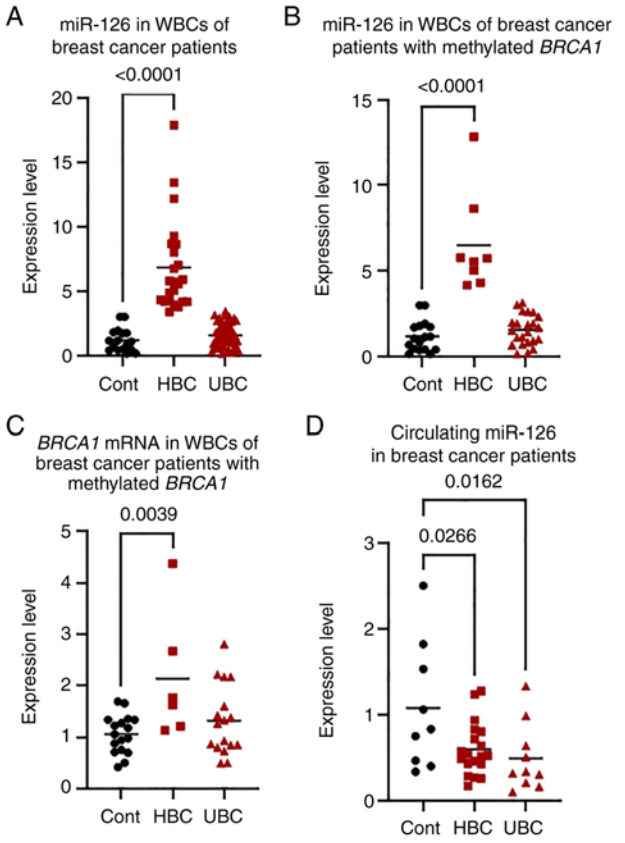

Figure 1. miR-126 is modulated in peripheral WBCs in BC. (A) Analysis of miR-126 expression in WBCs of patients with BC using stem-loop RT-qPCR. (B) Analysis of miR-126 expression in WBCs of the patients with *BRCA1*-methylated BC. (C) Analysis of *BRCA1* mRNA in WBCs of the patients with *BRCA1*-methylated BC using RT-qPCR. (D) Analysis of circulating miR-126 in patients with BC. Error bars represent the mean ± SD. BC, breast cancer; Cont, control; HBC, high expression BC; UBC, unchanged expression BC; *BRCA1*, breast cancer type 1 gene; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WBC, white blood cell; miR, microRNA.

Circulating miR-126 in patients with BC. When miR-126 expression was measured in the plasma of patients with BC, there was less circulating miR-126 in the HBC (P=0.0260) and UBC (P=0.0160) groups compared with that in age-matched controls (Fig. 1D).

miR-126 in WBCs of patients with OC. miR-126 expression in WBCs was measured in 46 randomly selected patients with OC (age range, 19-88 years; median age, 51.5 years), of which 21 were positive for methylated *BRCA1* (median age, 50 years) (Table VII). Similar to patients with BC, based on a cut-off value of +3-fold relative to 17 age-matched female controls, there were 11 patients (23.9%) who had higher miR-126 expression (HOC group), up to 7-fold, compared with the control, and 35 patients (76.1%) who had unchanged ImiR-126 expression (UOC group) (Fig. 2A; Table VII). In the HOC group, 8 patients (72.7%) had disease progression and 6 (54.5%) died, compared with the UOC group, where 10 patients (28.6%) had disease progression and 3 (8.57%) died. There was a significant positive association between the miR-126 expression in WBCs and the risk of OC disease progression and death according to Fisher's exact test (P=0.0029) (Table VII).

Table V. MicroRNA-126 expression in the white blood cells of patients with BC (n=74).

Cancer subtype	HBC (n=26)	UBC (n=48)	P-value
Methylated *BRCA1*	9 (34.6)	23 (47.9)	
TNG	5 (19.2)	11 (23.9)	
IDC	20 (76.9)	39 (84.8)	
DCIS	4 (15.4)	3 (6.5)	
Metastasis	1 (3.8)	11 (23.9)	0.0452[a]
ND	0 (0.0)	2 (4.2)	

[a]P<0.05. For UBC, 2 cases were ND, so n=46 for TNG, IDC, DCIS and metastasis. BC, breast cancer; HBC, high expression BC; UBC, unchanged expression BC; TNG, triple-negative; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; ND, not determined; *BRCA1*, breast cancer type 1 gene.

Table VI. MicroRNA-126 expression in the white blood cells of patients with BC type 1 gene methylated BC (n=32).

Cancer subtype	HBC (n=9)	UBC (n=23)	P-value
TNG	3 (33.3)	9 (42.9)	
IDC	8 (88.9)	19 (90.5)	
DCIS	1 (11.1)	1 (4.7)	
Metastasis	1 (11.1)	7 (33)	0.3742
ND	0 (0.0)	2 (4.3)	

For UBC, 2 cases were ND, so n=21 for TNG, IDC, DCIS and metastasis. BC, breast cancer; HBC, high expression BC; UBC, unchanged expression BC; TNG, triple-negative; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; ND, not determined.

Table VII. MicroRNA-126 expression in the white blood cells of patients with OC (n=46).

Cancer outcome	HOC (n=11)	UOC (n=35)	P-value
Methylated *BRCA1*	4 (36.4)	17 (48.6)	
No recurrence	3 (27.3)	25 (71.4)	0.0138[a]
Progression	8 (72.7)	10 (28.6)	
Death	6 (54.5)	3 (8.6)	0.0029[a]

[a]P<0.05. OC, ovarian cancer; HOC, high expression OC; UOC, unchanged expression OC; *BRCA1*, breast cancer type 1 gene.

When the 21 patients with *BRCA1*-methylated OC were analyzed separately, similar results were found, with 4 patients (19%) exhibiting higher miR-126 expression (HOC group), up to 6-fold, compared with the control, and 17 patients (81%) with unchanged miR-126 expression (UOC group) (Fig. 2B;). However, the association between miR-126 expression and the risk of OC disease progression and death was not statistically significant according to Fisher's exact test (P=0.0797) (Table VIII).

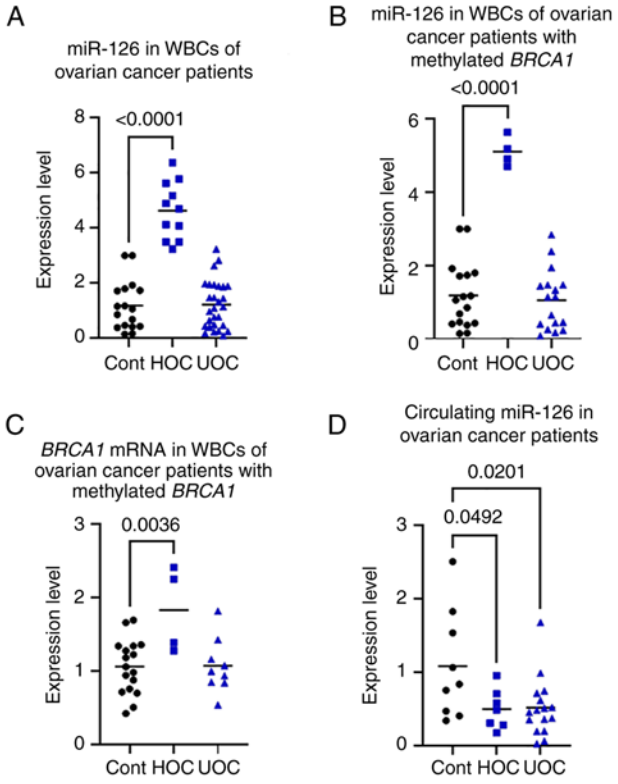

Figure 2. miR-126 is modulated in peripheral WBCs in OC. (A) Analysis of miR-126 expression in WBCs of patients with OC using stem-loop RT-qPCR. (B) Analysis of miR-126 expression in WBCs in the patients with *BRCA1*-methylated OC. (C) Analysis of *BRCA1* mRNA in the WBCs of the patients with *BRCA1*-methylated OC using RT-qPCR. (D) Analysis of circulating miR-126 in patients with OC. Error bars represent the mean ± SD. OC, ovarian cancer; Cont, control; HOC, high expression OC; UOC, unchanged expression OC; *BRCA1*, breast cancer type 1 gene; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WBC, white blood cell; miR, microRNA.

mRNA in WBCs of patients with OC. When *BRCA1* mRNA was measured in the WBCs of the patients with *BRCA1*-methylated OC, expression was significantly higher by up to 2.5-fold in the HOC group compared with that in the control group (P=0.0056). However, the UOC group did not significantly differ from the control group (P=0.1120) (Fig. 2C).

Circulating miR-126 in OC patients. When miR-126 expression was measured in the plasma of patients with OC, there was less circulating miR-126 in the HOC (P=0.0110) and UOC (P=0.0170) groups compared with that in age-matched controls (Fig. 2D).

miR-126 is elevated in WBCs but unchanged in the plasma of CF BRCA1-methylated female carriers. To further appreciate the use of miR-126 as a biomarker for the early prediction of BC and OC, miR-126 levels were measured in the WBC and plasma from 10 *BRCA1* methylated CF female carriers (age range, 18-27 years; median age, 20 years). A greatly increased level of miR-126 expression, up to 27-fold higher, was noted in the WBCs of all the carriers compared with that in the age-matched control group. This result is similar to the miR-126 expression in the WBCs from the patients with BC and OC (Fig. 3A). In contrast to that in the patients with BC and OC, there was no change in the level of circulating miR-126 in the carrier group compared with the control group (Fig. 3C). This result revealed that, in the CF *BRCA1*-methylated carriers, miR-126 is altered in WBCs but not in the plasma.

Table VIII. MicroRNA-126 expression in the white blood cells of patients with breast cancer type 1 gene methylated OC (n=21).

Cancer outcome	HOC (n=4)	UOC (n=17)	P-value
No recurrence	1 (25.0)	11 (64.7)	
Progression	3 (75.0)	6 (35.3)	
Death	2 (50.0)	1 (5.9)	0.0797

OC, ovarian cancer; HOC, high expression OC; UOC, unchanged expression OC.

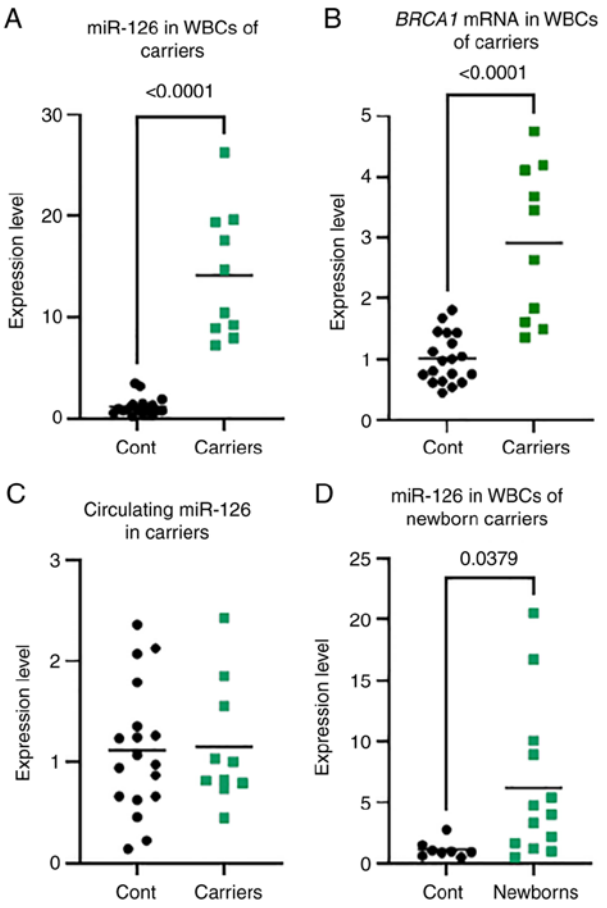

Figure 3. miR-126 is modulated in peripheral WBCs in cancer-free *BRCA1* methylation carriers. (A) Analysis of miR-126 expression in WBCs of adult female carriers using stem-loop RT-qPCR assay. (B) Analysis of *BRCA1* mRNA in WBCs of adult female carriers using RT-qPCR. (C) Analysis of circulating miR-126 in adult female carriers. (D) Analysis of miR-126 expression in WBCs of newborn female *BRCA1* methylation carriers. Error bars represent the mean ± SD. Cont, control; *BRCA1*, breast cancer type 1 gene; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WBC, white blood cell; miR, microRNA.

mRNA in WBCs of CF carriers. Similar to the results in patients with BC and OC, when *BRCA1* mRNA was measured

in the WBCs of the CF *BRCA1* methylation carriers, expression was significantly higher, up to 20-fold, in the carrier group compared with that in the age-matched control group (P<0.0001) (Fig. 3B).

miR-126 is elevated in the WBCs of CF BRCA1-methylated newborn female carriers. As *BRCA1* promoter methylation is detectable from early on in life in carriers (22,24), miR-126 was measured in the WBCs of 13 *BRCA1*-methylated newborn female carriers. A significantly higher miR-126 expression level, up to 20-fold higher, was observed in the newborn carrier group compared with that in the newborn non-carrier control group (P=0.0391) (Fig. 3D). This result indicates an alteration in miR-126 from early on in the life of the carriers.

Discussion

There is a well-established association between *BRCA1* promoter methylation in peripheral blood cells and the risk of BC and OC (20,22,23,36). However, less is known about its role in the cancer risk of CF *BRCA1*-methylated females. In the present study, *BRCA1* promoter methylation was detected in peripheral WBCs in 9.4% of 425 CF female controls. This result agrees with our previous findings (16,21). The detection of methylated *BRCA1* promoter in CF females raises the question of whether those individuals are at increased risk of developing BC or OC later in life. In the present study, miR-126 was investigated as a potential molecular biomarker for predicting BC and OC risk in CF *BRCA1*-methylated females.

In total, 502 patients with BC were screened for *BRCA1*-methylation in peripheral WBCs. *BRCA1* promoter methylation was significantly associated with early onset BC (P=0.0003), which agrees with the results of previous studies (16,17,21,36). Typically, TNG BC accounts for 10-20% of all BC cases (37). In the *BRCA1* methylated BC cases in the present study, TNG BC accounted for 34% compared with 17.4% of the unmethylated BC cases, which indicates enrichment of TNG BC in *BRCA1* methylation-positive cases. This finding agrees with a recent study (18), which investigated the association between the *BRCA1* promoter methylation in peripheral blood and the risk of TNG BC. The study reported that 30.2% of the TNG BC cases exhibited a methylated *BRCA1* gene status in the peripheral blood cells, indicating a significant association between constitutional *BRCA1* promoter methylation and TNG BC (18).

Furthermore, in the present study, 187 patients with OC were screened for methylated *BRCA1*. A significant association was found between WBC methylated *BRCA1* and OC incidence (P=0.0266). The vast majority of the methylated OC cases were HGSOC. These results agree with previously reported data, where it was shown that the frequency of *BRCA1* methylation in WBCs was higher in patients with OC than in controls and that it was associated with risk for HGSOC (22).

miR-126 is downregulated in tumor tissues compared with that in normal adjacent tissues in several cancer types, including BC, OC and colorectal cancer, and this decrease is associated with higher malignancy tumor grade and metastasis (31,32,38). The restoration of miR-126 has been demonstrated to inhibit metastasis properties in these cancer types (32,38).

By contrast, it has been reported that in BC, miR-126 is increased significantly in DCIS tissue compared with that in IDC and normal adjacent tissue. In addition, the downregulation of miR-126 is associated with the later onset of IDC (39). These findings suggest that non-invasive tumor cells inside DCIS may counteract the progression to an invasive lesion by increasing the level of miR-126 expression (39). In the present study, the results revealed a significant association between an increase in miR-126 expression in WBCs and a lower risk of distant metastasis in patients with *BRCA1*-methylated and unmethylated BC.

In patients with OC, the expression of miR-126 in WBCs was significantly associated with a higher risk of disease progression and death in the present study. These results suggest that miR-126 is a dual-functional miRNA, functioning as a tumor suppressor in BC and as an oncogene in OC, which may indicate different targets and mechanisms of action in the two types of cancer. Indeed, it was previously reported that the abundance of miRNAs and their targets could contribute to their contradictory roles in cancer (40). Notably, as WBCs are considered normal cells with the body tissues, the present findings might suggest an increase in miR-126 in the whole body. However, further studies are needed to address these findings.

Similar to the findings in patients with BC and OC, increased miR-126 expression was observed in the WBCs of CF *BRCA1*-methylated carriers compared with that in age-matched controls. Unlike that in patients with cancer, miR-126 expression was not decreased in any of the carriers. Notably, the increase in miR-126 appears to occur from early on in the life of the carriers, as significant upregulation of miR-126 expression was observed in newborn female carriers. As *BRCA1* epimutation is present from early on in the life of the carriers (24), we hypothesize that the increase in miR-126 could be a protective mechanism activated by the whole body, from the start of life, as a response to the epigenetically altered cancer suppressor gene, *BRCA1*. This claim is supported by the increase in *BRCA1* mRNA expression in the patients and carriers despite the methylation status of the *BRCA1* promoter. It has been reported that some hypermethylated genes are overexpressed due to the interaction with other factors (41). Based on the present findings, it is tempting to speculate that the increase in *BRCA1* mRNA, which occurs from early on in the life of the carriers (24), could be the result of the interaction with other factors regulated by miR-126. The inevitable activation of the carcinogenic mechanisms derived by the constitutional methylation of the *BRCA1* promoter, such as genomic instability, gross chromosomal rearrangements and generation of mutations, may result in the loss of miR-126 that leads to carcinogenesis (42). We therefore hypothesize that the upregulation of miR-126 could be part of a mechanism linking constitutional *BRCA1* promoter methylation with the pathological consequences induced by this epigenetic defect. Indeed, it has been reported that miR-126 is reversibly increased in response to short-term exposure to asbestos as a defensive process activating detoxifying mechanisms (42). However, long-lasting asbestos exposure results in the irreversible downregulation of miR-126 due to asbestos-induced DNA damage. The reduction in miR-126

activates the IRS1/PI3K/AKT pathway leading to the development of malignant mesothelioma (42). Thus, the expression of miR-126 links asbestos exposure to malignant mesothelioma.

Circulating miR-126 has been reported to differentiate patients with cancer from controls (43). In the present study, decreased circulating miR-126 was observed in the patients with BC and OC compared with that in healthy controls, regardless of its level in the WBCs, which agrees with previously reported data (27,29). Notably, the fact that there was no derease in the level of circulating miR-126 in the CF *BRCA1* methylated carriers, despite the increase in the WBCs, suggests the use of miR-126 as a prognosticator for BC and OC risk for those carriers.

The present study has certain limitations. For example, the physiological association between miR-126 and BRCA1 in WBCs has not been explored. Further studies are needed to investigate if there is any transcriptional regulation between BRCA1 and miR-126. Additionally, future studies are needed to search for the different targets of miR-126 in BC and OC cancer that contribute to its contradictory actions in these two types of cancer.

In conclusion, the present study revealed the likely involvement of miR-126 in the constitutional methylation of *BRCA1* promoter-related malignancies. Significant upregulation was observed in the level of miR-126 in WBCs, not only in patients with BC and OC, but also in CF *BRCA1* methylated carriers. Overall, the increase in miR-126 could be a mechanism activated by the body in response to the aberrantly methylated cancer-suppressor gene *BRCA1*, which has different pathological consequences according to cancer type.

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

MAS, NAY and NAM performed the data analysis. MAS, WA, SA and AA contributed to the sample and data collection. OA and HA permitted sample collection and contributed to data acquisition. NAM conceived and designed the study and drafted the manuscript. All authors read and approved the final manuscript. OA, HA and AA confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Ethical approval (approval no. RAC #2170017) was obtained by the Human Research Ethics Committee of King Faisal Specialist Hospital and Research Centre. All participants provided written informed consent. The guardian of the patient provided written informed consent for participants <18 years old.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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