Diagnostic Role of Aberrant DNA Promoter Methylation in Ovarian Cancer

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MS and AR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author AR performed the practical work. Authors MSM and MH managed the analyses of the study and literature searches. Author ME diagnose the samples. All authors read and approved the final manuscript.

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ABSTRACT

Background: Alteration of DNA methylation of CpG islands in the promoter regions of tumor suppressor genes is associated with cancer development. We aimed to examine the diagnostic efficacy role of promoter methylated tumor suppressor genes: *DAPK*, *OPCML* and *DLEC1* in ovarian cancer patients.
Materials and Methods: One hundred forty patients were enrolled (90 with epithelial ovarian cancer [EOC] while the remaining 50 were suffering from benign ovarian lesions. A group of healthy individuals (n=30) were included as control group. Methylation pattern were detected by methylation specific polymerase chain reaction (MSP) in serum samples from all individuals and protein based markers (CEA and CA125) were also estimated.
Results: Promoter methylation was significant for *DAPK*, *OPCML* and *DLEC1* in EOC as compared

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to benign ones while all control cases were unmethylated. Significant relation was reported between *DAPK*, *OPCML* hypermethylation and FIGO stage, histopathological type and histological grade while *DLEC1* hypermethylation was related to both FIGO stage (*P*= 0.03) and histological grading (*P*<0.0001). The correlation between promoter hypermethylation with CEA and CA125 showed significant differences. Both sensitivities and specificities for promoter methylation of investigated tumor suppressor genes were superior to CEA and CA125 for early diagnosis of ovarian cancer and detection of early ovarian stages while they were compatible with CA125 in detection of EOC low grades.

Conclusion: Promoter methylation of *DAPK*, *OPCML* and *DLEC1* genes in circulating blood serves as promising diagnostic approach for early detection of EOC particularly those with early stages, low - grade tumors.

1. INTRODUCTION

Ovarian cancer diagnosis is commonly delayed due to its ambiguous nature and non-specific symptoms that lead to high morbidity and mortality [1,2]. Epithelial ovarian cancer (EOC) is the most common ovarian cancer as it accounts for nearly 90% of this type of malignancies [1]. Laboratory and clinical efforts have tried to assess the efficiency of EOC screening and early detection using ultrasound imaging [3] and cancer antigen -125 (CA125); common tumor marker for ovarian cancer [4]. However, presently the screening methods do not improve the mortality of EOC due to their limitations [5]. Advances in molecular biology techniques allow the identification of new biomarkers that will enhance the sensitivity and the specificity of CA125.

Circulating DNA in body fluids shed from cancer cells have been reported to contain same mutations and aberrant methylation pattern of the solid tumors [6,7] and increased level of DNA methylation has been related to gene silencing which is common consequent in the onset of carcinogenesis [8,9]. Whole genome research has reported methylation signatures for several candidate genes that may be helpful as molecular markers for EOC characterization [8,9].

Utilizing DNA methylation status as a biomarker has several benefits such as its stability and capability to amplify which increases detection sensitivity, moderately decreases the cost of assessment and constrains to specific DNA regions [10,11]. Among these candidate genes, the current study focused on the *death associated protein kinase* (*DAPK*) gene; is a 160 KDa cytoskeletal-associated calcium/calmodulin dependent serine/threonine kinase that encodes for many domains including death domain and participates in intrinsic and extrinsic apoptosis [12], a previous study has reported *DAPK* gene hypermethylation in some gynecological malignancies such as ovarian cancer and cervical cancer especially squamous cell carcinoma (SCC) [13]. The second gene included in the current study is *opioid binding protein/cell adhesion molecule-like* (*OPCML*) gene; that is located at 11q25 and belongs to glycosylphosphatidylinositol (GPI)-anchored cell adhesion molecule and has been linked to tumorgenesis [14] with tumor suppressor activity [15], it has been reported that *OPCML* has been significantly increased in ovarian cancer tissues as compared to normal tissues as reported previously [16]. *Deleted in lung and esophageal cancer 1* (*DLEC1*) gene; a tumor suppressor gene, is the third gene assessed in the current study and its promoter methylation has been discovered in many types of cancer [17,18], and *DLEC1* silencing in ovarian cancer has been reported due to its promoter methylation [19].

We aimed to investigate the methylation status for the candidate tumor suppressor genes (*DAPK*, *DLEC1* and *OPCML*) in EOC patients as compared to those with benign ovarian lesions and healthy individuals, and other tumor markers (CEA and CA125). We also determined the correlation between these genes with each other and with clinico-pathological factors.

2. MATERIALS AND METHODS

2.1 Clinical Features for Enrolled Individuals

The study was carried out in accordance with declaration of Helsinki and approved by the Medical ethical Committee, National Research Centre, Egypt. A total of 140 patients were enrolled from the National Cancer Institute after signing their informed consent, based on their

histopathological examinations they were divided into EOC patients (n=90) and patients with benign ovarian lesions (n=50). EOC patients were classified histologically according to the World Health Organization (WHO) criteria [20] into serous (n=54) and non-serous (n=36) as (19 endometrioid and 17 mucinous), tumor staging was carried according to International Federation of Gynecology and Obstetrics (FIGO) [21] into early stage (I-II) (n=42) and late stage (III-IV) (n=48), while tumors were graded into low grade (I-II) (n=29) and advanced grade (III) (n=61) [21,22]. Benign ovarian lesions were ovarian fibroma (n=17), mature cystic teratoma (n=15), serous cystadenoma (n=13) and mucinous cystadenoma (n=11). A group of healthy females (n=30) were included in the study as control group.

2.2 Sample Collection and Processing

Blood samples (3 ml) were collected from all individuals , after centrifugation at 4000xg for 10 minutes the serum samples were aliquoted and stored at –80°C till tumor markers (CEA and CA125) measurements and detection of methylation status was performed.

2.3 Tumor Markers Assessment

Both tumor markers (CEA and CA125) were quantitatively detected in serum samples based on enzyme immunoassay kit as per manufacturer' instructions (Immunospec-corporation, Netherland) and concentrations (ng/ml) were measured using multimode reader (GloMax- Multidetection system, Promega, USA).

2.4 Assessment of Methylation Status for Investigated Genes

DNA methylation assessment was achieved by the extraction of DNA from serum samples using QIAamp DNA Blood Mini kit (Cat no# 51104, QIAgen, Hilden, Germany). DNA purity and concentration were detected using Q-5000 Spectrophotometer (Quawell Technology, Inc., San Jose, USA), and then stored at –80°C for further bisulfite conversion.

To convert unmethylated cytosine to uracil; bisulfate treatment was applied to extracted genomic DNA was performed following the manufacturer instruction of EpiTect Fast Bisulfite Kit (Cat no# 59824 QIAgen, Hilden, Germany) then methylation specific polymerase chain reaction (PCR) was carried out with methylated and un-methylated primers listed in (Table 1)

using EpiTect MSP Kit (Cat no# 59305 QIAgen, Hilden, Germany) in thermal cycler (SureCycler, 8800, Agilent, USA), in brief: after HotStarTaq DNA activation with initial denaturation at 95°C for 10 minutes, 40 cycles were carried out with the following thermal profile: denaturation at 95°C for 15 seconds, annealing at suitable temperature depending on the used primer as reported in (Table 1) for 30 seconds and extension at 72°C for 30 seconds, then final extension at 72°C for 10 minutes. Finally the PCR products were separated on 2% agrose gel electrophoresis using vertical electrophoresis (SE300 miniVE, Hoefer, USA) loaded on a 2% Agarose gel to split DNA both methylated and unmethylated based on their size and compared them with bands from molecular weight marker, the gel was stained by ethidium bromide as previously reported [23], the gel images were captured using gel documentation system (SYNGENE G:Box F3, USA) as visualized in (Fig. 1) and analysis of the bands as was done using gene tool software.

2.5 Statistical Analysis

Univariate analyses were performed using a Chi-square test. All analyses were performed using Statistical Package for the Social Sciences software (SPSS Inc., Chicago, IL). *P* values less than 0.05 were considered as statistically significant.

3. RESULTS

3.1 Demographic and Clinicopathological Data

Serum samples from 170 individuals were examined and categorized as patients with epithelial ovarian cancer (EOC) (n=90), patients with benign ovarian lesions (n=50) and a group of healthy control (n=30). Relevant demographic and clinicopathological factors were statistically analyzed with investigated items; no significant differences were found among the investigated groups for the median age ; EOC (48 yrs, range 35 – 58), benign lesion (48 yrs, range 32 – 57) and control group (47 yrs, range 35 – 58) at (F=0.025, *P* = 0.975). Premenopausal status was identified in 105 females (19 controls, 31 with benign lesions and 55 with EOC) where as the remainder were postmenopausal (n=65) (11 controls, 19 with benign lesions and 35 with EOC) there was no significant difference between the investigated groups and the menopausal status (F=0.083, *P* = 0.959).

Table 1. Primer sequence, product size and annealing temperature used for MSP

Gene	Forward primer (F) (5\ ⟶ 3\)	Reverse primer (R) (5\ ⟶ 3\)	Annealing Temp. ˚C	Product size bp	Ref
DAPK	MF GGATAGTCGGATCGAGTTACGTC	MR CCCTCCCAAACGCCGA	56	98	[22]
	UF GGAGGATAGTTGGATTGAGTTAATGTT	UR CAAATCCCTCCCAAACACCAA	61	106	
OPCML	MFCGTTTAGTTTTTCGTGCGTTC	MRCGAAAACGCGCAACCGACG	65	129	[24]
	UFTTTGTTTAGTTTTTTGTGTGTTTG	UR CAAAACAAAAACACACAACCAACA	60	136	
DLEC1	MF GAT TAT AGC GAT GAC GGG ATT C	MR ACC CGA CTA ATA ACG AAA TTA ACG	60	197	[19]
	UF TTA TAG TGA TGA TGG GAT TTG	UR CCC AAC TAA TAA CAA AAT TAA CAC	60	197	
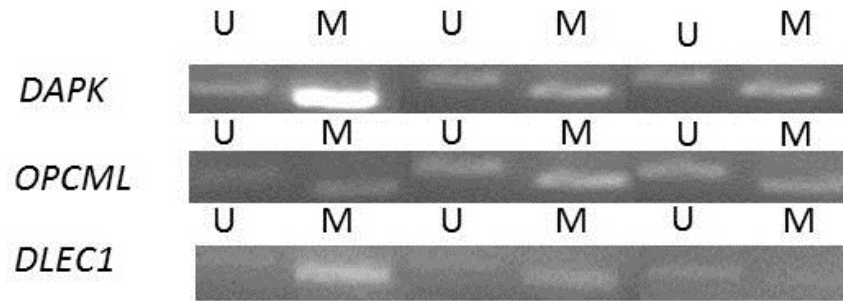
Fig. 1. Representative example for un-methylated (U) and methylated (M) forms for the investigated genes

3.2 Frequency of Promoter Methylation and Tumor Markers among Studied Groups

Promoter methylation status for investigated genes was assessed in the different groups as summarized in Table (2). Methylated pattern for investigated tumor suppressor genes were significantly higher in EOC patients as compared to benign ovarian patients while healthy group which served as control group reported unmethylated bands (100%). Among those cases reported methylation pattern some were homomethylated (M/M) other were heteromethylated (M/U), their distribution among the investigated tumor suppressor genes is represented in Fig. (2A-C). The diagnostic efficacy for these promoters was detected by using receiver operating characteristic (ROC) curve that was plotted between ovarian cancer and non-cancerous groups (benign and controls) in order to differentiate between them as illustrated in (Fig. 3A). The mean levels of tumor markers CEA and CA125 were significantly increased in EOC patients followed by benign and then the control individuals, the optimum cutoff value that discriminates between cancerous and non-cancerous groups were 5 ng/ml and 30 ng/ml for CEA and CA125, respectively, as shown in Fig. (3B), by using these cutoff points, positive cases (cutoff values) were significantly higher ($P<0.0001$) in EOC as compared to the other two groups (Table 2).

Table 2. Frequency of promoter methylation and positivity levels of tumor markers

| Investigated items	Healthy control (n=30)	Benign lesions (n=50)	EOC (n=90)	Statistics
CEA				
Mean ± SE	3 ± 0.2	3.9 ± 0.3	7 ± 0.2	F= 61, $P<0.0001$
≤ 5 ng/ml	30 (100%)	42 (84%)	17 (18.9%)	X^2=87.7, $P<0.0001$
> 5 ng/ml	0 (0%)	8 (16%)	73 (81.8%)	
CA125				
Mean ± SE	13 ± 0.3	3 ± 0.2	3 ± 0.2	F= 61, $P<0.0001$
≤ 30 ng/ml	30 (100%)	35 (70%)	5 (5.6%)	X^2=107, $P<0.0001$
> 30 ng/ml	0 (0%)	15 (30%)	85 (94.4%)	
DAPK				X^2=106, $P<0.0001$
Unmethylated	30 (100%)	30 (60%)	3 (3.3%)	
Methylated	0 (0%)	20 (40%)	87 (96.7%)	
OPCML				X^2=105, $P<0.0001$
Unmethylated	30 (100%)	26 (52%)	2 (2.2%)	
Methylated	0 (0%)	24 (48%)	88 (97.8%)	
DLEC1				X^2=102, $P<0.0001$
Unmethylated	30 (100%)	30 (60%)	4 (4.4%)	
Methylated	0 (0%)	20 (40%)	86 (95.6%)	

Fig. 2A

Fig. 2B

(C)
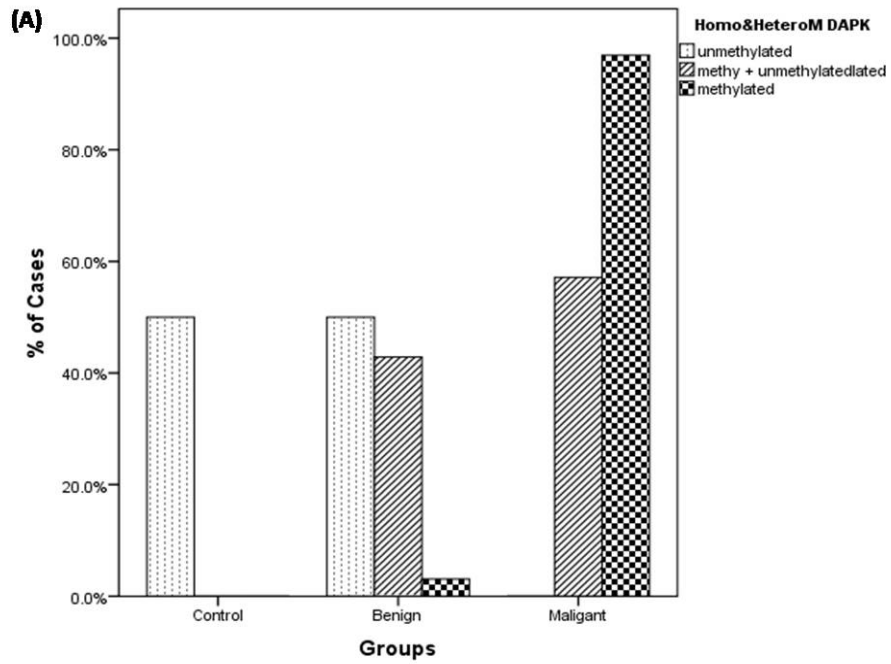
Fig. 2C

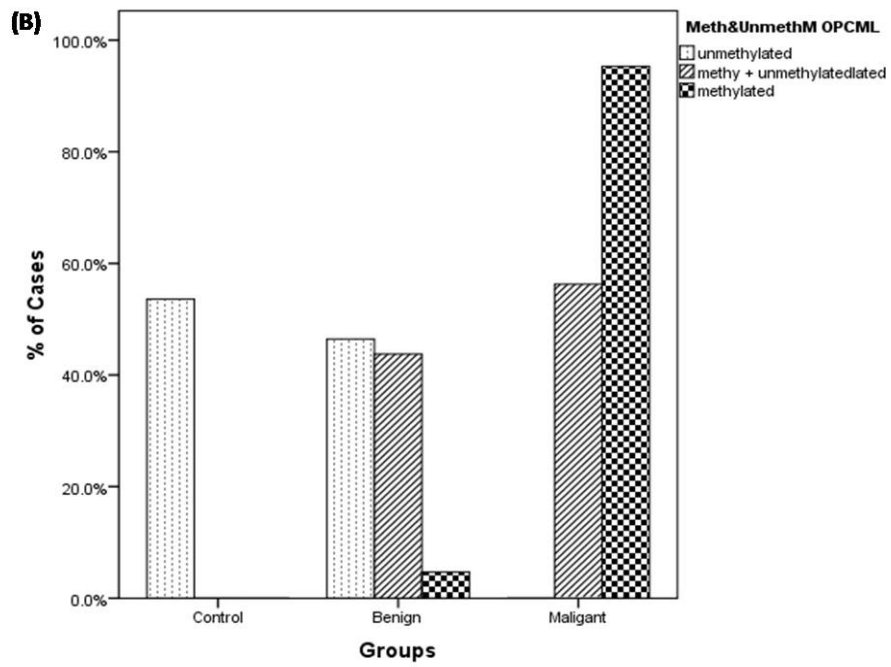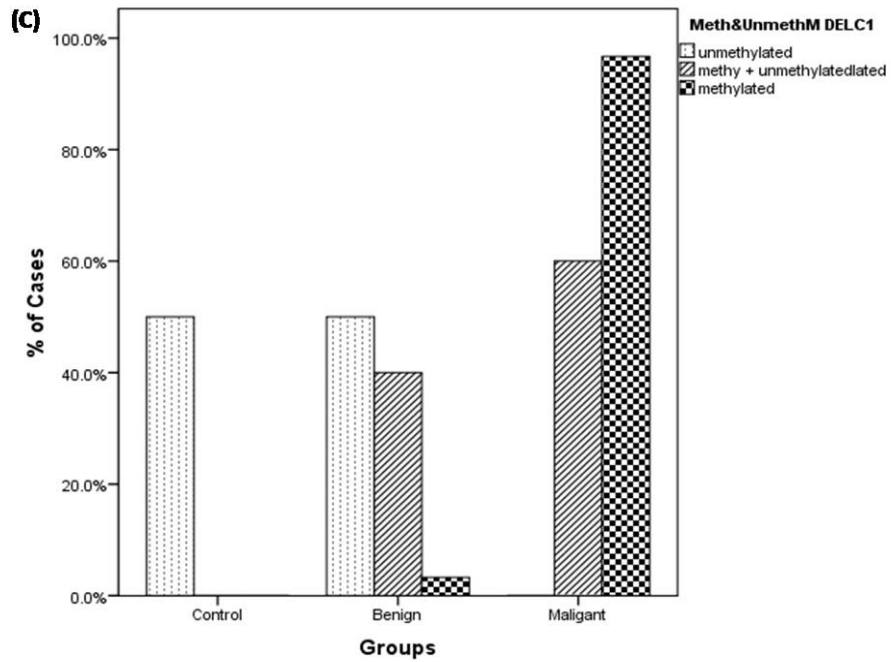**Fig. 2. Distributions of homomethylated and heteromethylated pattern for *DAPK* (A), *OPCML* (B), and *DLEC1* (C)**

Genes	Area under Curve (AUC)	95% Confidence interval (SE)
DAPK	0.858	0.797 – 0.907 (0.02)
OPCML	0.839	0.777 – 0.892 (0.03)
DLEC1	0.841	0.775 – 0.891 (0.03)

Fig. 3A.

Tumor Markers	Area under Curve (AUC)	95% Confidence interval (SE)
CEA	0.884	0.826 – 0.928(0.03)
CA125	0.921	0.870 to 0.957(0.02)

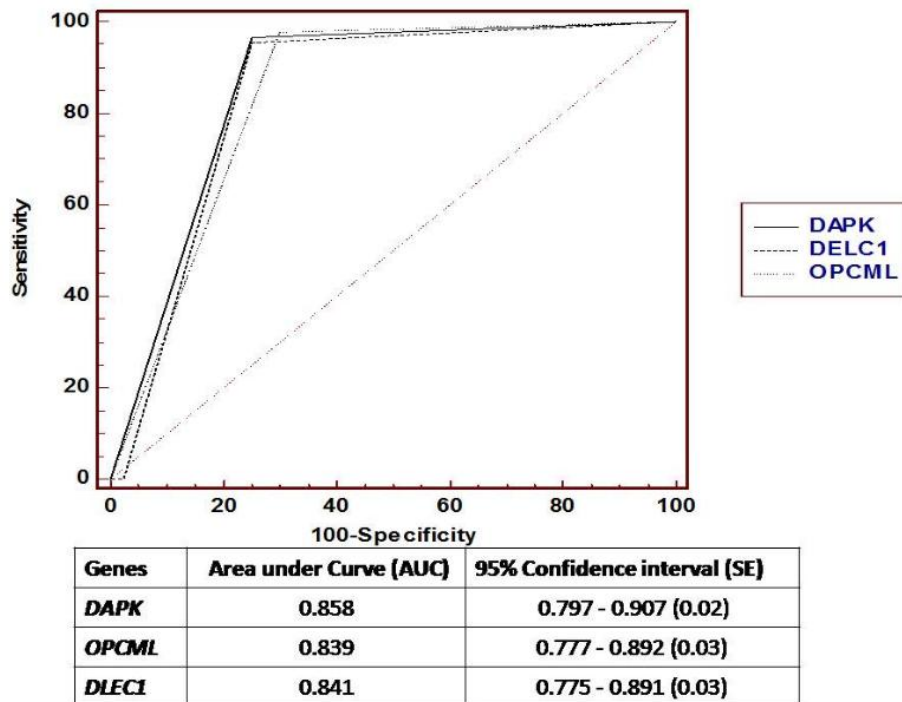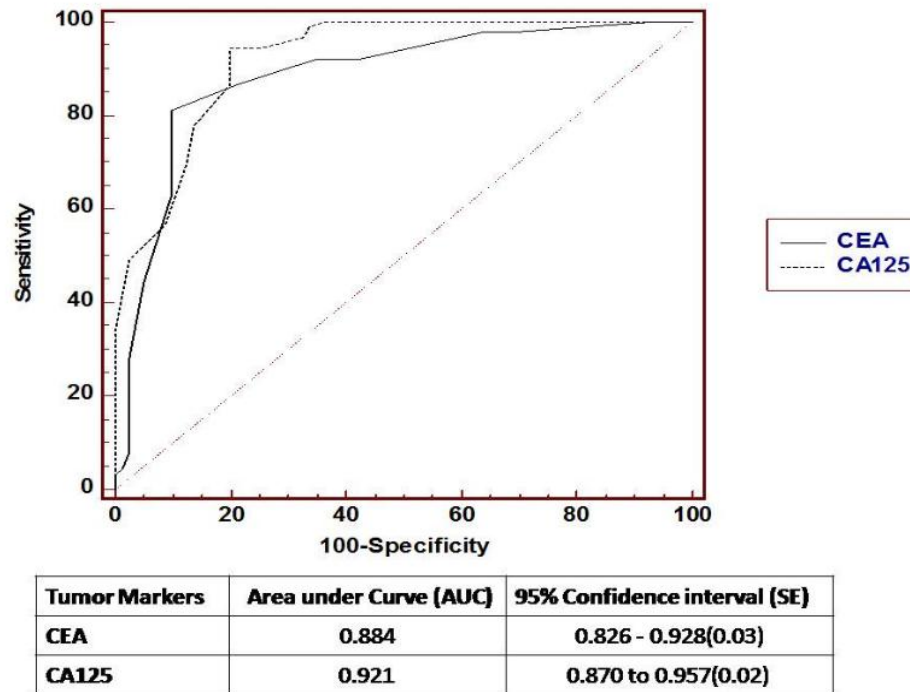**Fig. 3B.**

Fig. 3. Receiver Operating Characteristic Curve of methylation patterns of investigated gens (A) and tumor markers (B)

3.3 Distribution of Methylation Pattern and Positive Tumor Markers in Eoc Patients

As reported in Table (3), no significant difference was reported between tumor markers CEA and CA125 and clinicopathological data. Both hetero-methylated (M/U) and homo-methylated (M/M) patterns were investigated for the three promoters among EOC patients. Promoter methylation for *DAPK* gene significantly correlated with FIGO stage as methylation frequency (M/M) was elevated in late stage (39, 48.8% at *P*= 0.006), serous pathological type (42, 80.8% at *P*= 0.034) and high grade tumor (51, 86.4%, *P*<0.0001). Similarly *OPCML* homo-methylated frequencies were the highest with late FIGO stage (42, 89.4% at *P*<0.0001), serous pathological type (44, 83% at *P*= 0. 001) and high tumor grade (52, 86.7% at *P*<0.0001). For *DLEC1* promoter methylation, a significant relationship was found between methylation status and both FIGO staging and histological grading, as homo-methylation frequency was increased significantly in serous EOC and high grade tumors, the level of homo-methylation frequency was increased in serous pathological type (39, 75%) as compared to non-serous cancers (20, 58.8%) but did not reach significant levels.

3.4 Correlation between Promoter Methylation and Investigated Tumor Markers

When authors investigated the correlation between promoter methylation with CEA and CA125 in all enrolled individuals, significant level was observed (*P*<0.0001). Among the EOC patients only, statistical significance was found between the investigated genes apart from CEA and CA125. Similarly for the entire group of EOC patients reported hetero- and homo-methylation status for *DAPK* (n=87), *OPCML* (n=88) and *DLEC1* (n=86), significant difference was detected between them, and *DLEC1* showed significant correlation (R= 0.32, *P*=0.003) with CEA, as reported in Table (4).

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Table 3. Relation between promoter methylation patterns for investigated genes and positivity rates for tumor markers with clinicopathological factors

Factors	Methylated *DAPK*		Methylated *OPCML*		Methylated *DLEC1*		CEA	CA125
	Hetero-	Homo-	Hetero-	Homo-	Hetero-	Homo-	> 5 ng/ml	> 30 ng/ml
Menopausal status								
Pre-menopause	17	38	18	38	19	35	44	53
Post-menopause	7	25	9	23	8	24	29	32
FIGO stage								
I –II	17	24	22	5	17	23	34	39
III – IV	7	39	19	42	10	36	39	46
	X^2=7.5, P= 0.006		X^2=19, P<0.0001		X^2=4.3, P= 0.03			
Pathological status								
Non-serous	14	21	18	17	14	20	30	34
Serous	10	42	9	44	13	39	43	51
	X^2= 4.5, P= 0.034		X^2=11.7, P= 0.001					
Histological grade								
Low grade	16	8	19	9	16	11	23	28
High grade	12	51	8	52	11	48	50	57
	X^2=18, P<0.0001		X^2=26, P<0.0001		X^2=14, P<0.0001			

Table 4. Correlation (R, *P*) between promoter methylation and tumor markers

Items	Enrolled groups (n=170)			EOC group (n=90)			EOC group with methylated promoter		
	DAPK	*OPCML*	*DLEC1*	*DAPK*	*OPCML*	*DLEC1*	*DAPK* (n=87)	*OPCML* (n=88)	*DLEC1* (n=86)
DAPK	---	R=0.9, P<0.001	R=0.95, P<0.001	---	R=0.61, P<0.001	R=0.73, P<0.001	---	R=0.55, P<0.001	R=0.67, P<0.001
OPCML	R=0.9, P<0.001	---	R=0.89, P<0.001	R=0.61, P<0.001	---	R=0.58, P<0.001	R=0.55, P<0.001	---	R=0.52, P<0.001
CEA	R=0.64, P<0.001	R=0.59, P<0.0001	R=0.66, P<0.001	R=0.12, P=0.256	R=0.43, P=0.687	R=0.296, P=0.005	R=0.1, P=0.32	R=0.06, P=0.9	R=0.32, P=0.003
CA125	R=0.75, P<0.001	R=0.72, P<0.001	R=0.74, P<0.001	R=0.07, P=0.47	R=0.069, P=0.519	R=0.054, P=0.614	R=0.01, P=0.9	R=0.03, P=0.8	R=0.03, P=0.78

Table 5. Assessment of promoter methylation *versus* tumor markers as screening test

Items	Ovarian cancer diagnosis		Early FIGO stage		Low grade	
	Sen.%	Spec.%	Sen.%	Spec.%	Sen.%	Spec.%
CEA	81.1	90	81	90	79.3	90
CA125	94.4	81.2	92.9	81.2	96.9	81.2
DAPK	96.7	75	97.6	75	96.6	75
OPCML	97.8	70	97.6	70	96.6	70
DLEC1	95.6	75	95.2	75	93.1	75

3.5 Assessment of Promoter Methylation versus Tumor Markers as Screening Test

The sensitivities and specificities of investigated methylated genes and tumor markers are reported in (Table 5). The highest sensitivity detected was 97.8% and it was reported for promoter methylation of *OPCML* followed by *DAPK, DELC1* then CA125 while CEA reported the lowest one (81.1%). For detection of early ovarian cancer, *OPCML* and *DAPK* were superior to *DLEC1* then CA125, while for detection of low grade tumors, the sensitivities of CA125, *DAPK* and *OPCML* were the same followed by *DLEC1* and CEA reported the lowest among the investigated markers

4. DISCUSSION

Among the common effect of death from gynaecological malignancies is ovarian cancer [1]. Early diagnosis of ovarian cancer patients especially those at stage I will improve their survival rates by 90% as compared to those identified with advanced stage as the survival rate decline to 40% [25]. Thus identification of markers with high sensitivity and specificity for early detection of ovarian cancer will improve the survival rate and even can be useful as prognostic markers. It has been reported earlier that promoter methylation pattern is linked to carcinogenesis and is cell and tissue type specific [26]. Aberrant methylation of genes have been reported to be linked to several types of cancer [27,28] as hypermethylation is linked to gene silencing due to aberrant promoter methylation of CpG islands while hypomethylation is linked to enhanced transcription and protein activation [29].

The aberrant methylation patterns of genes in ovarian cancer is an area of great interest [11]. In our study promoter methylation patterns of three tumor suppressor genes were studied (*DAPK, OPCML* and *DLEC1*). *Death associated protein kinase* (*DAPK*) gene has an evident role in cell apoptosis [30] and due to aberrant methylation of *DAPK*, its protein expression is usually reduced in cancer cells and is linked to increase cancer invasiveness [31]. Among our studied cases (n=170) the frequency of *DAPK* promoter methylation was significantly higher in ovarian cancer than in benign ovarian lesion cases, while it was not detected in healthy control individuals. These results are in consistent with a previous study of Jiwani and his colleagues [32] as they reported increment of *DAPK* methylation in ovarian cancer tissues while it was not detected in normal ones. Thus our study suggest that hypermethylation of CpG islands cause gene silencing leading to cancer development. In a previous study [33] methylation was reported in pre-malignant gastric cancer cases, in ours *DAPK* methylation was detected in 20 cases (16 were hetero- and 4 cases homomethylated) of benign ovarian lesions which may point to the association between methylation and tumor initiation and hence these category of patients will need a close observation to prevent cancer development. The role of OPCML tumor suppressor gene silencing in EOC is resulted from CpG methylation has previously proved [14], we demonstrated significant promoter methylation in circulating blood of EOC patients as compared to benign ones while its mthylation was not reported in control individuals which direct to its efficacy as marker for detection of ovarian cancer. Currently *DLEC1* promoter hypermethylation was significantly reported in EOC patients, these results were confirmed by Kwong study as he reported that down-regulation of *DLEC1* is related to its hypermethylation in ovarian cancer cell lines [19]. In benign cases methylation was reported in 20 cases (18 were heteromethylated and 2 were homomethylated) which may predict onset of ovarian cancer and hence a close care is crucial.

The relationship between methylation pattern and clinicopathological factors was addressed in this study. A significant relationship was detected between *DAPK* methylation and FIGO stages

which emphasize a link between methylation and cancer progression, moreover the presence of methylation in early stages indicate that *DAPK* methylation plays a causative role in cancer development. The most frequent histologic ovarian cancer subtype is serous EOC as referred to 2003 World Health Organization while the other subtypes are categorized collectively as non-serous (e.g. endometrioid, clear cell, transitional, mucinous and undifferentiated subtypes) [34]. In our study EOC (n=90), 54 EOC patients were diagnosed as serous subtype (60%) while the remaining 36 (40%) were non-serous subtype, methylation frequencies for *DAPK* and *OPCML* were significantly related to histologic subtypes as homomethylated allele were highly detected in serous subtype (42 and 44, respectively) and for *DLEC1* methylation no significant level was reached although homomethylation was increased in serous subtype.

When authors tested the correlation between promoter methylation of *DAPK*, *OPCML* and *DLEC1* with preoperative biomarkers as CEA and CA125 levels, significant correlations were reported which indicates the independency of these methylated genes with biochemical markers in detection of EOC and they can give an additional value for early detection of ovarian cancer.

Epigenetic events, especially DNA methylation have a significant clinical role in cancer detection and can provide an estimate for cancer progression and treatment strategy response [35]. Searching for minimally non-invasive methods with satisfactory sensitivity and specificity will not only provide a good method for cancer detection but also enable its screening especially when it's a common type of cancer and its early detection will reduce patient's mortality. In the current study the sensitivities for *DAPK*, *OPCML* and *DLEC1* were superior to CEA and CA125 for detection of ovarian cancer and identify EOC patients at early stages, and were approximate with the sensitivity of CA125 for detection of low- grade tumors. These findings revealed the strong applicability of promoter methylation of *DAPK*, *OPCML* and *DLEC1*genes as diagnostic markers and in future study is in progress to detect their potential role as prognostic markers and treatment response.

In conclusion, it has been implicated that aberrant methylation of tumor suppressor genes cause is "the second hit" according to Knudson's two-hit hypothesis for cancer biology [35]. Hence detection of promoter methylation for individual genes will present an early event in diagnosis of ovarian cancer at early stages or premalignant disease and will be of great value if it can be detected in circulating blood as minimal-non-invasive method especially if superior sensitivity and specificity were detected.

To our knowledge this is the first analysis to combine the detection of the promoter methylation of these three tumor suppressor genes (*DAPK*, *OPCML* and *DLEC1*) in one study using less invasive sample type i.e., serum samples and our results were capable of prove early diagnosis of EOC from benign and control individuals. However it is still obstacle to apply these methods in clinical lab work until a reference value, applicable molecular technique with cost effectiveness is achieved.

CONSENT

Informed consent was obtained from all individual participants included in the study.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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