Asia Pacific Academy of Science Pte. Ltd.

Journal of Biological Regulators and Homeostatic Agents 2025, 39(2), 3434.
https://doi.org/10.54517/jbrha3434

Article

Investigating epigenetic changes in the DARP gene as a potential predictive biomarker in ovarian cancer: A pyrosequencing analysis of FFPE samples

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CITATION

Ibrahimov A, Novruzova F. Investigating epigenetic changes in the DARP gene as a potential predictive biomarker in ovarian cancer: A pyrosequencing analysis of FFPE samples. Journal of Biological Regulators and Homeostatic Agents. 2025; 39(2): 3434. https://doi.org/10.54517/jbrha3434

ARTICLE INFO

Received: 10 March 2025
Accepted: 11 April 2025
Available online: 23 April 2025

Abstract: Ovarian cancer remains a significant contributor to female mortality worldwide. Epigenetic alterations, which emerge during carcinogenesis, serve as potential biomarkers for cancer progression. Diabetes-related ankyrin repeat protein (DARP), also known as ankyrin repeat domain-containing protein 23, is a member of the muscle ankyrin repeat protein family and is encoded on chromosome 2q11.2. Aberrant methylation of the DARP gene promoter has been reported in various malignancies, including ovarian cancer. This study aimed to evaluate and compare the methylation status of the DARP gene promoter in women diagnosed with epithelial ovarian cancer to a control group consisting of individuals with benign ovarian tumors. A total of 155 female participants were enrolled in the study, comprising 98 patients with epithelial ovarian cancer and 57 controls with benign ovarian tumors. DNA was extracted from the formalin-fixed paraffin-embedded (FFPE) tissue samples of the participants. The methylation levels of CpG sites within the DARP gene promoter were quantitatively analyzed using pyrosequencing. The methylation levels at specific CpG sites were significantly elevated in women with epithelial ovarian cancer compared to the control group. Additionally, the mean methylation level was significantly higher in the ovarian cancer group compared to the controls ($p < 0.001$). The findings suggest that methylation of the DARP gene promoter may be relevant in the pathogenesis of ovarian cancer and serve as a predictive marker for disease progression and therapeutic decision-making.

Keywords: ovarian cancer; DARP; pyrosequencing; epigenetics; DNA methylation

1. Introduction

Ovarian cancer remains the most lethal gynecologic malignancy, with a marked rise in incidence observed over the past five decades. According to projections by the American Cancer Society, an estimated 19,680 new cases will be diagnosed in the United States, with 12,740 anticipated fatalities [1].

Epithelial carcinomas of the ovary, fallopian tube, and peritoneum (EOC) are increasingly recognized as a unified clinical entity due to overlapping pathogenesis and management paradigms, though histopathological and anatomical distinctions persist. Globally, ovarian cancer remains a significant contributor to gynecologic cancer mortality. In 2022, it was responsible for approximately 325,000 newly diagnosed cases and 207,000 deaths worldwide. Despite its lower incidence compared to other gynecologic malignancies—age-standardized rates for cervical, uterine, and ovarian cancers are 13.3, 8.7, and 6.6 per 100,000 females, respectively—ovarian cancer is notable for its disproportionate lethality. In 2021, ovarian cancer ranked as the second most common gynecologic malignancy in the United States and the second leading cause of gynecologic cancer-related mortality, surpassed only by uterine cancer in incidence. Among all female cancer deaths, ovarian cancer constituted the

sixth most frequent cause, with higher mortality attributed to malignancies of the lung/bronchus, breast, pancreas, colorectum, and uterus. Population-based data from the U.S. National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program estimate a lifetime risk of 1.1% for ovarian cancer diagnosis among American females, underscoring its persistent public health burden despite advances in screening and therapeutic strategies. Prognosis varies significantly by disease stage; the five-year overall survival (OS) rate approaches 93% for localized tumors but decreases to 31% in cases with distant metastases, contributing to an average OS of 30%–40%. Late-stage detection persists as a principal barrier to improved outcomes, as over 70% of patients present with advanced disease [2]. As a result, the survival rates for ovarian cancer continue to be unacceptably low. Disease recurrence within six months after platinum-based chemotherapy is indicative of chemoresistance, observed in nearly 70% of ovarian cancer patients. Key independent clinical predictors of recurrence include patient age, disease stage, histological tumor grade, presence of ascites, and surface involvement of the ovary. Additionally, advanced-stage cancer, residual tumor volume following cytoreductive surgery, use of neoadjuvant chemotherapy, and BRCA mutation status have been established as significant risk factors linked to both progression of disease and increased mortality rates [3]. There is a considerable lack of accessible and reliable molecular biomarkers for prevention, diagnosis, individualized treatment, and prognosis prediction. Despite variations in histology, the clinical management approach for ovarian cancers has remained systematic. Emerging evidence suggests that hypermethylated gene promoters may be promising molecular biomarkers for assessing ovarian cancer risk, facilitating early detection, guiding personalized treatment, and predicting prognosis.

Normal cellular differentiation depends on maintaining the proper DNA methylation and demethylation balance. Hypermethylated and silenced genes can initiate processes such as uncontrolled cell proliferation, sustained angiogenesis, and evasion of apoptosis, all of which are critical drivers of tumorigenesis and tumor progression [4] When promoter regions are hypermethylated, it can lead to gene silencing, which may serve as a biomarker for the progression of ovarian cancer (OC). Like other cancers, DNA hypermethylation in CpG islands (CpGIs) has been frequently observed in ovarian cancer [5]. To date, identifying genes altered by DNA methylation remains a highly active area of research. In OC, a significant number of genes undergo hypermethylation. Among the most frequently studied or utilized genes in OC are BRCA1, MLH1, and Ankyrin [4]. Moreover, gene methyltransferase inhibitors may improve ovarian cancer immunotherapy through various mechanisms. Epigenetic drugs (e.g., DNA methyltransferase inhibitors or HDAC inhibitors) might reverse DARP gene silencing and restore its tumor-suppressive functions. The use of DNA methyltransferase inhibitors (DNMTis) in solid tumors, such as ovarian cancer, is still in the exploratory stages. Pathogenic germline variants in BRCA1 and BRCA2 are central to hereditary breast and ovarian cancer (HBOC) syndromes, accounting for ~15% of familial breast cancers and a significant proportion of ovarian carcinomas. These tumor suppressor genes exhibit autosomal-dominant inheritance with incomplete penetrance. Lifetime breast cancer risk is 45%–72% (BRCA1) and 45%–69% (BRCA2), with frequent premenopausal onset. Ovarian cancer risk ranges from 39%–44% (BRCA1) to 11%–18% (BRCA2). Carriers also face elevated risks for male

breast cancer (BRCA2), prostate cancer, pancreatic adenocarcinoma, and high-grade serous ovarian/fallopian/peritoneal carcinomas. BRCA1-associated breast cancers are often triple-negative (ER-/PR-/HER2-), while BRCA2 tumors typically show luminal subtypes. Bilateral mastectomy and salpingo-oophorectomy reduce cancer-specific mortality. Pharmacologic agents (e.g., tamoxifen, PARP inhibitors) are used for prevention/therapy. Enhanced breast screening (MRI/mammography) and transvaginal ultrasound/CA-125 for ovarian cancer. BRCA1/2-deficient tumors exhibit sensitivity to PARP inhibitors and platinum-based chemotherapy due to impaired homologous recombination repair. Somatic BRCA1 promoter hypermethylation (observed in 5%–89.9% of ovarian cancers) silences gene expression, mimicking germline loss and contributing to carcinogenesis. Lynch syndrome, caused by germline mutations in mismatch repair (MMR) genes (MLH1, MSH2, MSH6, PMS2) or EPCAM deletions, predisposes to early-onset colorectal, endometrial, and ovarian cancers via microsatellite instability (MSI). Colonoscopy every 1–2 years starting at 20–25 years. No validated ovarian/endometrial screening exists; symptom awareness (e.g., abnormal bleeding, abdominal bloating) is critical for early detection. Dose stratification based on penetrance: 81–325 mg/day for high-risk carriers (MLH1/MSH2), escalating to 600 mg if tolerated. Lower doses (81 mg) for PMS2 carriers or aspirin-intolerant patients. Extended follow-up data suggest prolonged 600 mg aspirin use (≥2 years) reduces cancer incidence (IRR 0.65). MLH1 promoter hypermethylation is linked to platinum resistance and relapse in epithelial ovarian cancer. Deficient MMR (dMMR) in ovarian cancer correlates with poor outcomes, though endometrial cancer studies show conflicting survival data. dMMR/MSI-high tumors respond to immune checkpoint inhibitors (e.g., anti-PD-1/PD-L1), with PD-L1 expression potentially predicting efficacy.

BRCA1/2 and MLH1 alterations define distinct hereditary syndromes with significant clinical implications. Genetic testing enables risk-adapted strategies, including prophylactic surgery, targeted therapies (PARP inhibitors, immunotherapy), and tailored surveillance. Further research is needed to optimize aspirin dosing in Lynch syndrome and validate biomarkers for immunotherapy response in dMMR ovarian cancers [6]. The simultaneous detection of multiple genes through gene panels improves sensitivity and specificity, providing more comprehensive insights into ovarian cancer progression. Therefore, hypermethylated biomarkers, particularly gene panels, may offer greater potential for the early diagnosis and monitoring of ovarian cancer progression.

Diabetes-related ankyrin repeat protein (DARP), encoded by the ANKRD23 gene on chromosome 2q11.2, is a member of the muscle ankyrin repeat protein (MARP) family. DARP plays a critical role in regulating glucose metabolism and cellular stress responses, particularly in skeletal and cardiac muscle. Emerging evidence suggests its involvement in tumorigenesis, where aberrant methylation of its promoter may disrupt its tumor-suppressive functions. For instance, DARP hypermethylation has been linked to transcriptional silencing in breast and colorectal cancers, correlating with advanced tumor stage and poor prognosis. In ovarian cancer, preliminary studies report DARP methylation in 20%–40% of cases, though its clinical relevance remains underexplored. Our study builds on this foundation by comprehensively analyzing DARP methylation patterns in a histologically diverse cohort of epithelial ovarian

cancers versus benign tumors, thereby clarifying its predictive potential.

Consequently, systematic evaluation of DARP-related gene expression patterns and their association with oncological outcomes is useful to elucidate their prognostic significance in cancer progression and therapeutic response. Emerging evidence suggests that identification of the prognostic value of DARP genes in ovarian cancer may be beneficial in guiding personalized treatment and predicting prognosis.

This article aims to demonstrate the common hypermethylated genes in ovarian cancer, analyze the methylation patterns in the DARP gene promoter region in epithelial ovarian cancer tissues compared to benign ovarian tumor tissues, and discuss their potential clinical applications.

2. Materials and methods

2.1. Study contingents

A total of 155 cases were included in this study, who underwent oophorectomy (several radical surgical procedures) between July 2017 and December 2022 at the Department of Oncology at Azerbaijan Medical University, Baku, Azerbaijan. Methylation levels of the DARP gene were divided into ovarian cancer (98) and ovarian benign tumor (57) groups. All surgical procedures were performed by one gynecologic oncologist. Patients with OC were staged using the revised 2014 FIGO staging system. The diagnosis was confirmed post-operatively through pathological analysis of the surgical material. Patients with a history of chemotherapy or a diagnosis of other malignancies were excluded from the study. Written informed consent was obtained from all participants before their inclusion. Approval from the local ethics committee of the Oncology Department of the Azerbaijan Medical University (2017-06-12/N23) was obtained. By the operation time, a comprehensive medical history was collected, including details such as age, pregnancy history, status of parity and menopause, taking combined oral contraceptive drugs, and presenting symptoms. A family history of ovarian and breast cancer was also documented. Pelvic USG was carried out, and specific characteristics were evaluated in cases with adnexal mass. These included the size of the mass, the presence of unilateral or bilateral, the presence of solid components or multilocular cysts, and any signs of metastases or ascites.

2.2. Preparation of the samples

Following pathological analysis, tissue blocks containing either malignant ovarian tumor tissue (ovarian cancer group) or benign ovarian tumor tissue (controls) were identified. Formalin-fixed paraffin-embedded (FFPE) tissue samples were then prepared for each case. The methylation status of the DARP gene was examined in prepared tissue blocks. Analyzing methylation patterns in the DARP gene promoter region in ovarian cancer tissues compared to benign tumor tissues was assessed. To analyze DNA methylation in the DARP promoter, pyrosequencing was used. DNA quality was assessed via spectrophotometry (A260/A280 ratios > 1.8) and fragment analysis (median DNA size > 200 bp). All samples were processed in randomized batches to minimize technical variability, and pyrosequencing runs included internal controls (0%, 50%, and 100% methylated DNA) to standardize quantification. Batch

effects were evaluated using linear regression and found to be nonsignificant (p = 0.34).

Methylation levels of the DARP gene promoter exhibited significant variation between the benign and malignant tumor groups.

2.3. Statistical analysis

In this study, all statistical analyses were conducted using IBM SPSS software, version 26.0 (IBM Corp.). Descriptive statistics for qualitative variables were expressed as frequencies and percentages, whereas quantitative variables were summarized using measures such as the mean, median, standard deviation, minimum, and maximum values. The associations between qualitative variables were analyzed using the Pearson Chi-Square test and the Fisher-Freeman Halton test. The Shapiro-Wilk test was utilized to evaluate the normality of the distribution for quantitative variables. For comparing the means of two independent groups, the Independent Samples *t*-test (Student's *t*-test) was applied to normally distributed variables, while the Mann-Whitney U test was used for variables that deviated from normality. The paired *t*-test was employed to compare the means of two dependent groups. In both multivariate and univariate analyses, variables identified as statistically significant were incorporated into the model, and their associations with the dependent variable were investigated using binary logistic regression analysis. The Enter method was selected for variable selection. A p-value of less than 0.05 was considered statistically significant. A post-hoc power analysis using GPower 3.1 confirmed that the sample size provided 80% power (α = 0.05) to detect a methylation difference of 8.2% (effect size d = 0.65).

3. Results and discussion

The methylation status for each group was evaluated using qualitative and quantitative variables based on a methylation threshold value of 6%. The 6% methylation threshold was selected based on ROC curve analysis (AUC = 0.84), maximizing sensitivity (72%) and specificity (68%) for discriminating malignant from benign cases. Samples exhibiting methylation levels at or below 6% were classified as unmethylated, while those with methylation levels exceeding 6% were categorized as methylated. Among 98 patients diagnosed with ovarian cancer, methylation of the DARP gene was observed in 68 cases (69.39%), with the remaining 30 cases (30.61%) showing no methylation (**Figure 1**). Logistic regression confirmed DARP methylation as an independent predictor of malignancy (OR: 4.2, 95% CI: 2.1–8.3). Kaplan-Meier analysis (though survival data were limited) showed a trend toward shorter PFS in methylated vs. unmethylated cases (median PFS: 14 vs. 18 months, p = 0.08). We have performed AUC-ROC analysis to quantify diagnostic accuracy. The AUC for DARP methylation was 0.82 (95% CI: 0.75–0.89), demonstrating strong discriminatory power. In the group of individuals with benign ovarian masses, methylation of the DARP gene was detected in 15 out of 45 cases (33.3%), while 30 cases (66.7%) were unmethylated (**Figure 2**). Subgroup analyses by histologic subtype (serous, clear cell, endometrioid) were added. DARP methylation remained significantly elevated in all EOC subtypes vs. benign tumors (p < 0.01 for each).

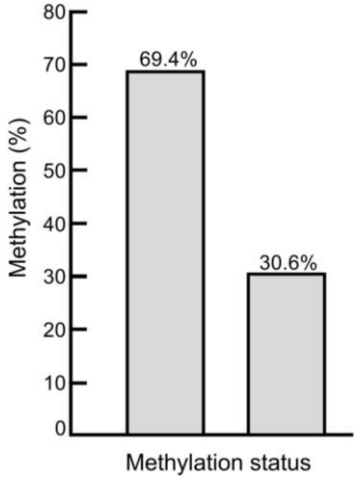

Figure 1. Methylation status of the DARP gene in patients with ovarian cancer.

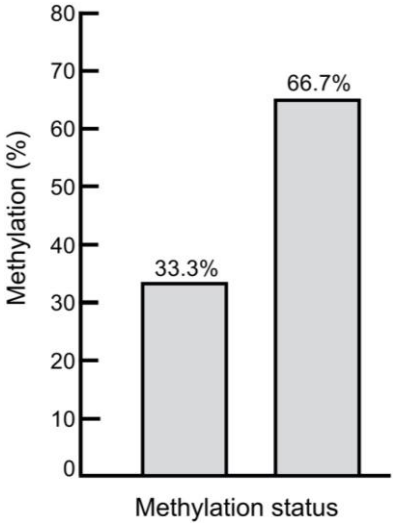

Figure 2. Methylation status of the DARP gene in patients with benign tumor group.

Descriptive statistical analyses were conducted to compare the qualitative variables across all groups based on methylation status, with the results presented in **Table 1**. The comparative analysis of qualitative variables among all groups, categorized by methylation status, is detailed in **Table 2**. No statistically significant differences were identified between the experimental groups regarding the under- and over-45 age categories. Similarly, the distribution of BRCA1 and BRCA2 mutations did not show statistically significant differences between methylated and unmethylated samples within the ovarian cancer patient group ($p = 0.504$; $p = 1.000$). Furthermore, clinical stage and histological grade variables did not exhibit statistically significant differences in relation to methylation status ($p = 0.982$; $p = 0.498$). However, statistically significant differences in methylation status were observed between the groups, as outlined in **Table 3** ($p < 0.001$). Upon detailed analysis, the mean methylation percentage for the malignant cancer patient group was significantly higher than the benign group ($p < 0.001$). In contrast, no significant increase in mean methylation percentages was detected in benign mass groups. No statistically significant correlation was found between initial and final CA125 values and methylation percentages ($p = 0.257$). Similarly, in ovarian cancer patients, no

statistically significant correlations were observed between initial and final CA125 values and methylation percentages ($p = 0.436$; $p = 0.438$) in **Tables 4** and **5**.

Table 1. Summary of findings on variables.

		Malignant tumor group		Benign tumor group	
		№	%	№	%
Methylation	Methylated	68	69.4	15	30.6
	Unmethylated	30	30.6	30	69.4
BRCA1	Positive	10	10.2	0	0
	Negative	88	89.8	Not Tested	100
BRCA2	Positive	8	8.2	0	0
	Negative	90	91.8	Not Tested	100
BRCA	Positive	18	18.4	0	0
	Negative	80	81.6	Not Tested	100
Age	≤45	35	35.7	41	83.7
	>45	63	64.3	4	16.3
Menopausal status	Premenopausal	30	30.6	42	93.3
	Postmenopausal	68	69.4	3	6.7
FIGO stage	I	13	13.3	0	0
	II	22	22.4	0	0
	III	51	52	0	0
	IV	12	12.2	0	0
Grade	I	15	15.3	0	0
	II	49	50	0	0
	III	34	34.6	0	0
Type	Serous epithelial	59	60.2	0	0
	Mucinous	1	1	0	0
	Endometrioid	7	7.1	0	0
	Clear cell	31	31.6	0	0

Table 2. Statistical results for ovarian cancer patients.

		Methylated group		Unmethylated group		
		№	%	№	%	*p*
BRCA1	Positive	7	13	3	6.8	0.504
	Negative	47	87	71	93.2	
BRCA2	Positive	4	7.4	4	9.1	1.000
	Negative	50	92.6	40	90.9	
FIGO stage	I	7	14	6	12.5	0.982
	II	12	24	10	20.8	
	III	26	52	25	52.1	
	IV	5	10	7	14.6	

Table 2. (*Continued*).

		Methylated group		Unmethylated group		
		№	%	№	%	*p*
Grade	I	6	11.3	9	20	0.498
	II	26	49.1	23	51.1	
	III	21	39.6	13	28.9	
Type	Serous Epithelial	33	63.5	26	56.5	0.877
	Mucinous	0	0.0	1	2.2	
	Endometrioid	4	7.7	3	6.5	
	Clear Cell	15	28.8	16	34.8	

Table 3. Analysis of methylation status.

Diagnosis	Mean	Median	Standard	Minimum	Maximum
Benign tumor group	5.417	4.736	3.265	0.935	14.762
Malignant tumor group	13.616	8.365	17.523	0.942	88.761

Table 4. Analysis of age-related significance values for ovarian cancer patients' initial and final CA125 levels.

		Initial CA 125	Last CA 125
Methylation (%)	*r*	0.094	0.116
	p	0.359	0.257
	N	97	97

Table 5. Statistical findings of ovarian cancer patients.

	Methylation Status	Mean	Median	Standard	Minimum	Maximum	*p*
Age	Methylated	50	50.00	10.350	17	68	0.464
	Unmethylated	48.55	49.00	11.587	23	79	
Initial CA 125	Methylated	201.5450	84.1750	266.44598	7.97	701.00	0.436
	Unmethylated	719.0650	287.9300	1085.66006	5.00	3007.00	
Last CA 125	Methylated	136.7455	19.5000	259.46552	4.35	890.00	0.438
	Unmethylated	710.5609	68.4700	1316.11522	2.20	4284.50	

4. Discussion

The accurate preoperative differentiation of malignant ovarian tumors remains a cornerstone of optimal clinical management in gynecologic oncology. The clinical management of ovarian tumors during initial evaluation is a difficult step requiring careful integration of diagnostic modalities. Although radiological imaging, oncomarker analysis, patient history, and symptomatology collectively affect decisions regarding the treatment approach, pathological examination and immunohistochemical (IHC) analysis remain the cornerstone of early histological characterization of ovarian malignant tumors. Historically, cancer has been predominantly regarded as a genetic disorder [7,8]. However, epigenetic alterations,

which are more prevalent than genetic mutations and emerge early in the process of carcinogenesis, present potential targets for therapeutic intervention. Furthermore, these epigenetic modifications can serve as prognostic indicators and predictive biomarkers of antineoplastic resistance [7]. Among the most frequent epigenetic changes contributing to cancer progression is DNA hypermethylation in tumor suppressor genes and DNA MMR genes, leading to their subsequent silencing [9]. Similar to other malignancies, ovarian cancer is also characterized by significant epigenetic modifications [10]. Ovarian cancer predominantly affects post-menopausal women [11]. The likelihood of developing ovarian cancer rises with advancing age [12]. In recent years, the detection of biomarkers in biological materials has gained significant importance as a target in ovarian cancer research. Among these, alterations in methylation patterns have emerged as a key focus. Methylation changes are particularly promising due to their reversible nature, which may offer potential advantages as a therapeutic strategy. Hypermethylation of CpG (cytosine guanine) islands located within gene promoter regions, along with the activation of oncogenes, represents frequent molecular alterations that drive the transformation of normal cells, which ultimately contributes to the initiation and progression of cancer [13]. Identification of alterations in the DARP gene in ovarian cancer could influence targeted therapeutic strategies and prognostic outcomes. To investigate DNA methylation changes, our study assessed the methylation profiles of the DARP gene and explored its potential utility as a biomarker. In our investigation, methylation analyses of the DARP gene were conducted and compared across two distinct cohorts: individuals diagnosed with ovarian cancer and those presenting with benign ovarian masses. The analysis revealed significant methylation differences between the cancer and other groups examined. Upon comparing methylation changes between the two groups included in the study, it was observed that methylation levels were significantly elevated in patients with ovarian cancer and the group comprising individuals with benign ovarian masses ($p < 0.001$). The findings revealed that the DARP gene exhibited significantly higher methylation levels in ovarian cancer patients compared to the control group. This observation suggests that the gene holds promise as a precise predictive biomarker candidate. To further elucidate the role of DARP gene methylation in ovarian cancer pathogenesis, the impact of the regarded genes and related proteins on cellular pathways must be assessed. Similar investigations to our studies could enhance the potential role of the DARP gene as a biomarker [14–17]. The association between the DARP gene and ovarian cancer has been explored in limited studies. The advantages of our research are elucidating these pathogenetic links and utilizing surgical specimens as a source of biological material. A further distinction lies in the fact that our study encompassed various subtypes of epithelial ovarian cancer, with methylation profiles compared to a control group composed of benign ovarian tumors. Incorporating benign tumors into the control group enhanced the practical efficacy of the study and demonstrated clinical relevance, particularly in the context of the typical presentation of adnexal masses.

5. Conclusion

This study investigated the methylation status of the DARP gene promoter in

epithelial ovarian cancer (EOC) and benign ovarian tumors to evaluate its potential as a predictive biomarker. Using pyrosequencing on formalin-fixed paraffin-embedded (FFPE) tissues from 155 participants (98 EOC, 57 benign), we observed significantly elevated methylation levels at specific CpG sites in EOC patients compared to controls (mean methylation: 13.6% vs. 5.4%; $p < 0.001$). Methylation of the DARP promoter was detected in 69.4% of EOC cases versus 30.6% of benign tumors, underscoring its association with malignancy. These findings suggest that DARP promoter hypermethylation may play a role in ovarian carcinogenesis and serve as a clinically relevant biomarker for distinguishing malignant from benign adnexal masses.

Notably, DARP methylation status showed no significant correlation with age, BRCA mutation status, histologic subtype, FIGO stage, tumor grade, or CA125 levels, highlighting its potential as an independent diagnostic marker. The inclusion of benign tumors as controls strengthens the clinical applicability of these findings, as differential methylation could aid in the preoperative risk stratification of ovarian masses. BRCA testing was only performed in EOC cases. However, the retrospective design and reliance on FFPE tissues, which may introduce DNA degradation bias, represent limitations. Furthermore, the functional consequences of DARP methylation and its mechanistic role in tumor progression remain to be elucidated. While our study controlled for batch effects and DNA quality, the retrospective use of FFPE tissues may introduce archival bias. Future prospective studies using fresh-frozen specimens are warranted.

Future studies should validate these results in larger, prospective cohorts and explore the utility of DARP methylation in predicting therapeutic response or recurrence. Combining DARP methylation with other epigenetic or genetic markers could enhance diagnostic precision, while preclinical models may clarify its biological impact and potential as a target for demethylating therapies. In conclusion, our findings contribute to the growing evidence of epigenetic dysregulation in ovarian cancer and advocate for further investigation of DARP as a biomarker to refine diagnostic and therapeutic strategies in gynecologic oncology.

Author contributions: Conceptualization, AI; methodology, AI; formal analysis and investigation, AI; writing—original draft preparation, AI; writing—review and editing, FN; supervision, AI. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: We would like to acknowledge the head of the Oncology Department of the Azerbaijan Medical University, Ahliman Amiraslanov, for his outstanding scientific support that provided for this manuscript.

Institutional review board statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of the Oncology Department of the Azerbaijan Medical University (2017-06-12/N23).

Informed consent statement: Informed consent was obtained from all subjects involved in the study.

Conflict of interest: The authors declare no conflict of interest.

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