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ABERRANT PROMOTER HYPERMETHYLATION OF DEATH ASSOCIATED PROTEIN KINASE (DAPK) IN EPITHELIAL OVARIAN CARCINOMA

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ABSTRACT: PURPOSE: The aim of the study was to assess the promoter hypermethylation status of the apoptotic gene Death Associated Protein Kinase (DAPK) in epithelial ovarian cancer and its association with various clinicopathological parameters. **METHODS:** 110 epithelial ovarian cancers samples were assessed to study the hypermethylation by nested and Methylation Specific PCR. **STATISTICAL ANALYSIS:** All the available data was statistically analyzed using the Chi square test or Fisher Test on the SPSS software version 21.0. **RESULTS:** 31.2% and 14.2% methylation frequencies were observed for malignant and low malignant potential tumors respectively. A zero percent methylation was noted in benign and non-malignant samples. **CONCLUSION:** The observed promoter hypermethylation frequency for DAPK gene suggests that it plays a role in tumorogenesis. The methylation status along with biochemical parameters can aid in the early diagnosis of the disease and also serve as a prognostic tool. Due to Frequent hypermethylation of the DAPK promoter in several cancer types, studying the methylation pattern of the gene has been proposed as a diagnostic and follow-up test.
KEYWORDS: DAPK, Promoter Hypermethylation, Epithelial Ovarian Carcinoma, Nested PCR, Methylation Specific PCR.

INTRODUCTION: Ovarian cancer is one of the leading causes of gynecological cancer deaths among women. Most of the ovarian cancer patients are diagnosed at the advanced stages as they do not report with any major clinical symptoms in the early stages. Due to lack of precise screening strategies for ovarian cancer it is indispensable to come up with new surveillance modalities which are sensitive and specific enough to screen the disease at an early stage.

Ovarian cancer has the least survival rate of all female cancer. Statistical data have shown that 45% of women with ovarian cancer are likely to survive for 5 years in comparison to 89% of women with breast cancer.[1]

Aberrant promoter methylation of Tumor suppressor genes have been shown to play an important role in tumor initiation and metastasis. Death Associated Protein Kinase (DAPK) is one of the important tumor suppressor genes and therefore studying aberrant methylation pattern of this gene by MSP analysis can be used as biomarker for ovarian cancer.

DAPK gene is located on chromosome 9q 34.1. It is 160 KDa calcium/calmodulin regulated serine/threonine kinase protein with pro-apoptotic, tumor suppressor function. It induces apoptosis through IFN-γ signaling.[2] Human tumor biopsies and cell lines have shown the absence of DAPK protein expression indicating its role as a tumor suppressor gene.[3,4]

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Esteller et al have reported the occurrence of DAPK hypermethylation in many tumor types and pre-neoplastic lesions and therefore the clinical usefulness of this genes inactivation by epigenetic alterations is based on sensitive methods measuring the aberrant promoter hypermethylation status.[5]

Death associated protein kinase gene encodes for several domains including a death domain and is involved in both intrinsic and extrinsic apoptosis. Loss of DAPK protein expression has been observed in several cancers such as bladder, renal cell, breast, colon, lung etc. A detailed quest to find the reason for the loss of expression showed that it was not due to the rearrangement or deletion in the DAPK gene, but was due to the epigenetic silencing of the gene by hypermethylation of its promoter.[6] DAPK promoter is one of the genes involved in apoptosis that is most frequently hypermethylated from a range of 4-90% in cancers.[7,8]

MATERIALS AND METHODS:

Patient and Specimens: Patients diagnosed with EOC from the Department of Gynecology (2012-2015) at The Kidwai Memorial Institute of Oncology, Bangalore were considered for the study. Ethical clearance for the study was obtained from the Institutional Review and Ethics Board.

Tissue collection: 110 EOC tissues were collected after the surgical resection and confirmation by a senior pathologist. The samples were immediately stored at -80^0C until DNA extraction. The samples obtained were histologically classified according to the International Federation of Gynecology and Obstetrics (FIGO) classification. The 110 samples included 80 malignant, 14 low malignant potential tumors and 16 benign tumor samples. 10 normal ovary samples were collected from patients undergoing salphingo-oophorectomy for benign cancers and used as the control group.

DNA extraction: DNA was isolated from 30mg of the tumor tissue using the Qiagen DNA easy kit following the manufacturer's protocol. The extracted DNA was quantified spectrophoto-metrically using Eppendorf Biospectrophotometer Kinetics™

Sodium Bi-sulfite modification: 1µg of the DNA was subjected to sodium bisulfite treatment using the EZ DNA Methylation Lightening™ Kit (Zymo Research D5031, CA, USA) following the instructions by the manufacturer.

Methylation Analysis: The methylation status of DAPK was assessed using methylation specific PCR in two stages. For the first step of nested PCR, primers were designed to amplify the DNA template irrespective of the methylation status and the cycling conditions are mentioned in the table-4a. The PCR product from the first step was diluted 10-folds and used as template for the second step where primers were designed to specifically amplify the methylated and the unmethylated regions of the DAPK promoter. CpG Methylated HeLa Genomic DNA (New England Biolabs) was used as a positive control for methylated allele and DNA obtained from normal ovarian samples served as negative control. The Primer sequences and the cycling conditions for

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MSP and USP[8,9] are summarized in the table- 3 and 4b. The PCR products post the MSP and USP were loaded on a 2.5% Agarose gel and visualized by Ethidium Bromide Staining. The gel image was captured using Syngene G: Box gel Documentation system.

CA-125 and CEA estimation: Blood samples from 110 EOC patients and 10 healthy subjects were estimated using the Elecsys CA125 and CEA diagnostic kits procured from Roche, Germany and quantified on Cobas e411™ auto analyzer (Roche Diagnostics, Germany). A CA125 value of 0-35U/ml and a CEA value between 0-7.5ng/ml were considered normal

STATISTICAL ANALYSIS: To determine the significant correlation between different variables Chi-Square and Fisher Exact test was used. All the Statistical tests were performed using SPSS software 21.0. p-Value of less than 0.05 was considered to be statistically significant.

RESULTS:

Detection of Promoter methylation of DAPK: 27 samples of the 110 showed an aberrant hypermethylation for DAPK gene corresponding to a methylation frequency of 25.54%. Twenty five of the 80 malignant samples and 2 of 14 low malignant potential tumors showed promoter hypermethylation. However none of the benign samples showed hypermethylation suggesting that DAPK methylation may accumulate in the advanced stages of the disease. The methylation frequencies for the three tumor groups are shown in table-2.

Correlation with clinic pathological parameters: The methylation status of the gene was assessed for its association with 7 clinicopathological parameters enlisted in table 1. The DAPK methylation failed to show any significant correlation with clinicopathological parameters such as the menopausal state, histological subtype, stage or tumor grade. But the methylation status showed a significant association with the pre-operative CA-125 level and in patients with presence of ascites with a p value of <0.001 and 0.029 respectively. This being suggestive that the methylation status of the gene along with clinicopathological parameters could aid in a better diagnosis of the disease and serve as a prognostic tool for the same.

DISCUSSION: Despite the prevalent biochemical tests such as CA-125, CEA and screening techniques including ultra sound scanning etc. used to screen ovarian cancer, none have shown to have a cent percent accuracy in detecting the disease.

As it is possible to detect very minute quantities of methylated DNA molecules, therefore studying aberrant methylation status of TSGs can serve as a sensitive and promising biomarker in diagnosis of cancers.[10]

The results of the current study are consistent with the findings by other research groups across the world which have shown aberrant methylation pattern of DAPK at various cancer sites including ovarian.

Reports by Leung[11] et al and Narayan[12] et al showed a 56.08% and 43.3% methylation of the gene respectively in cervical cancer. Tang[13] et al reported a 44% methylation in NSCLC. A strikingly high methylation percentage of 84% in B cell malignancy was reported by Katzenellenbogen[14] et al.

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Hafner[13] et al reported a 50 % methylation of DAPK in ovarian cancer tissues. Collins[15] et al reported a even higher methylation percentage of 67 in ovarian cancer. Terasawa[16] et al and Ozdemir[8] et al reported a 13% (ovarian cancer cell lines) and 4% methylation respectively for the gene. The above results suggest that the DAPK promoter hypermethylation to have a tissue specific role.

The frequent methylation of the promoter region of DAPK has lead several research groups to propose the use of DAPK methylation as a diagnostic and follow-up test. Studying the epigenetic alterations such as promoter hypermethylation of TSG along with the existing biochemical markers can be a useful diagnostic tool in the early detection of ovarian cancer.

CONCLUSION: The underlying mechanism of silencing DAPK expression in tumor cells is attributed to aberrant promoter methylation of the gene.

Methylation pattern of a single gene promoter may not summarize the entire process of tumorogenesis; however studying the methylation signature of DAPK with other TSGs in a panel with biochemical markers can give a synergistic result to aid in disease diagnosis and prognosis.

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	Characteristics	N	DAPK (% methylation)
Menopause State	Pre Menopause	35	10/35(28.5%)
	Post Menopause	75	17/35(22.6%)
OVARIAN TUMORS	p-Value 0.140	80	
Histological type	Serous	68	19/68(27.9%)
	Mucinous	03	01/03(33.3%)
	Clear cell	05	03/05(60%)
	Endometroid	04	02/04(50%)
	p-Value 0.654		
FIGO Stage	I II	20 07	04/20(20%) 02/07(28.5%)
	III IV	51 02	17/51(33.3%) 02/02(100%)
	p-Value 0.133		
GRADE	I-II III-IV p-value 0.585	20 60	05/20(25%) 20/60(33.3%)

CA125(U/ml)	0-35	21	0/21(0%)
	35-500	50	07/50(14%)
	500-1000	08	05/08(62.5%)
	>1000	31	15/31(48.3%)
	p-Value p<0.001*		
CEA (ng/ml)	0-7.5	58	16/58(27.5%)
	>7.5	52	11/52(21.1%)
	p-Value 0.509		
Ascites			
	Presence of ascites	57	19/57(33.3%)
	Absence of ascites	53	08/53(15%)
	p-Value 0.029 *		
LMP		14	
	SEROUS	02	01/02(50%)
	MUCINOUS	12	01/12(8.3%)
	P-VALUE 0.275		
BENIGN		16	
	SEROUS	10	0/10(0%)
	MUCINOUS	06	0/06(0%)
	P-VALUE --		
Table 1: Patients characteristics			

Note: *statistically significant. LMP-Low Malignant Potential.

Genes	Tumor type			
	Malignant (80)	LMP(14)	Benign (16)	Normal (10)
DAPK(M)	25/80(31.2%)	2/14(14.2%)	0/16(0%)	0/10(0%)
DAPK(U)	55/80(68.7%)	12/14(85.7%)	16/16(100%)	10/10(100%)
p Value	0.056	0.493	-	
Table 2: Methylation frequencies of study subjects for DAPK				

Gene	Forward (5´-3´)	Reverse (5´-3´)	Annealing temperature	Product Size
DAPK Nested	GGTTGTTTYGGAGTGTGAGGAGG	CTAAAAACTCCCCCRATCCCT	60^0 C	236
DAPK MSP	ATAGTCGGATCGAGTTAACGTC	AAAACTAACCGAAACGACGACG	60^0 C	152
DAPK USP	GGAGGATAGTTGGATTGAGTTAATGTTT	CAAATCCCTCCCAAACACCAA	60^0 C	80
Table 3: Primer sequences of DAPK gene				

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PCR Cycling Conditions:

Gene	Initial Denaturation	Cycling Stage x 35			Final Extension
		Denaturation	Annealing	Extension	
DAPK	95^0C	95^0C	56^0C	72^0C	72^0C
	10 mins	30 sec	30 sec	30 sec	7 mins
Table 4a: Nested PCR conditions					

Gene	Initial Denaturation	Cycling Stage x 35			Final Extension
		Denaturation	Annealing	Extension	
DAPK	95^0C	95^0C	60^0C	72^0C	72^0C
	10nims	30 sec	30 sec	30 sec	7 mins
Table 4b: Methylation and Unmethylation Specific PCR conditions					

Fig. 1: Agarose gel electrophoresis image as captured on the Gel Documentation System showing representative methylation-specific PCR result of the DAPK gene. The sizes of PCR products are listed in Table-3, along with the primers used for analysis. The presence of a visible PCR product in Lane U indicates the presence of unmethylated alleles; the presence of product in Lane M indicates the presence of methylated alleles. CpG methylated HeLa genomic DNA (+ve) was used as positive control for methylated alleles.
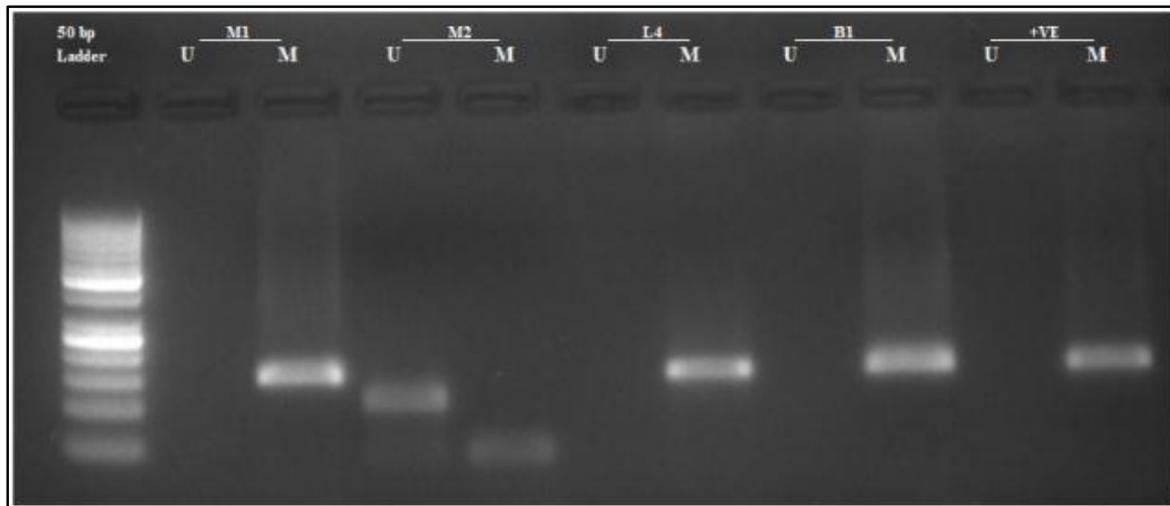

Fig. 1

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