ORIGINAL ARTICLE

THE ABERRANT PROMOTER HYPERMETHYLATION PATTERN OF THE ANTI-ANGIOGENIC TSP1 GENE IN EPITHELIAL OVARIAN CARCINOMA: AN INDIAN STUDY

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ABSTRACT: PURPOSE: The promoter hypermethylation patterns of Thrombospodin-1 gene in 50 EOC patients were studied and the methylation pattern was correlated with various clinic pathological parameters. **METHODS:** The promoter hypermethylation pattern of the TSP-1 gene was assessed using nested PCR and Methylation specific PCR. **STATISTICAL ANALYSIS:** All the available data was statistically analyzed using the Chi square test or Fisher Exact Test on the SPSS software version 22.0 and a value <0.05 was considered statistically significant. **RESULTS:** Forty of the fifty ovarian carcinoma samples reported positive for methylation corresponding to a methylation frequency of 80%. A methylation frequency of 89.2%, 83.3% and 42.8% was observed in malignant, Low malignant potential (borderline) and benign sample cohorts. **CONCLUSION:** From the results drawn from this study, it clearly shows that the anti angiogenic protein TSP-1 is extensively hypermethylated in ovarian carcinoma and that it accumulates over the progression of the disease from benign to malignant. As previous reports suggest that there is no evidence of mutation of this gene, promoter hypermethylation may be a crucial factor for the down regulation of the gene. Further by clubbing together the promoter hypermethylation pattern of TSP-1 gene with hypermethylation patterns of other TSG may provide a better insight into the application of using methylation profiles of TSG as a biomarker in the detection of ovarian carcinoma.
KEYWORDS: Epithelial ovarian carcinoma, TSG, Thrombospodin-1, anti-angiogenic protein, Nested PCR, MSP.
ABBREVIATIONS: TSP-Thrombospodin, TSG-Tumor Suppressor Gene, EOC-Epithelial Ovarian Carcinoma, LMP-Low Malignant Potential, MSP-Methylation Specific PCR.

INTRODUCTION: One of the most lethal malignancies to affect the female reproductive system is ovarian cancer. The most prominent reason for this lethality may be attributed to the lack of early diagnosis of the disease as it is usually asymptomatic at the early stages. Epithelial ovarian cancer constitutes to about 85% of all ovarian cancers.[1] A report in 2012, stated that worldwide nearly 2, 39,000 women have been diagnosed with ovarian cancer.[2] In more than 80% of the cases, the tumor would have already spread beyond the ovaries by the time of diagnosis.[1]

Sustained angiogenesis is one among the major hallmarks of cancer. In normal cells, the angiogenic process is turned on only transiently. However in contrast, during tumor progression the "angiogenic switch" is almost always turned on causing the quiescent vasculature to continually produce new blood vessels to aid neoplastic growth.[3]

ORIGINAL ARTICLE

Thrombospodin-1 is an antiangiogenic protein with a molecular weight of 150kDa encoded by the TSP1 gene located on chromosome 15q15. It belongs to a family of glycosylated extracellular matrix protein modulating the interactions between cells and the microenvironment.[4,5] Lawler[6] et al reported that mice lacking TSP1 develop tumors earlier in a p53 null background suggesting its role as a potent tumor suppressor gene. So far there has been no report on the occurrence of mutation in the Thrombospodin-1 gene in cancers but, the loss of TSP1 expression has been attributed to the aberrant hypermethylation, transcriptional regulation by oncogenes or TSG or altered mRNA stability.[7] Hypermethylation of the gene promoter region is the most common mechanism of gene silencing in cancers and decreased expression of TSP1 has been known to occur in primary tumors.[8,9]

The aim of this work was to study the aberrant promoter hypermethylation pattern of the antiangiogenic gene TSP1 in epithelial ovarian cancer and its correlation with various clinicopathological parameters which could aid in the early detection of the disease. So far, to the best of our knowledge this is the first study on the promoter hypermethylation of TSP-1 in ovarian carcinoma from India. We have not been able to find any reports other than the 2012 report from M.Chmelařová [10]et al in ovarian carcinoma.

MATERIALS AND METHODS:

Patients and Samples: The studied cohort comprised of 50 women with Epithelial ovarian carcinoma who underwent surgery at the Department of Gyneconcology, Kidwai Memorial Institute of Oncology, Bangalore, India, between the years 2013 to 2015. Seven samples taken from normal ovaries were used as negative controls. Representative tumor samples were immediately stored at -80^{0}C until further processing. All specimens were subjected to histological examination in order to confirm the diagnosis of ovarian cancer by a senior pathologist. The histological type and grade of all tumors were classified according to the criteria of the World Health Organization (WHO). The clinical stage of each malignant tumor was established according to International Federation of Gynecology and Obstetrics (FIGO) criteria.

DNA Extraction: Genomic DNA was extracted from the tumor samples using the DNA easy Mini Kit (Qiagen, USA) according to manufacturer's protocol. The DNA concentration was estimated using Eppendorf Biospectrophotometer Kinetics.™ The eluted DNA samples were stored at -20^{0}C until sodium bisulfite modification.

Sodium Bisulfite Modification: 1ug of genomic DNA was subjected to sodium Bisulfite modification using the EZ DNA Methyl Lightening kit TM (Zymo Research D5031, CA, USA). The bisulfite modified DNA was eluted according to the manufacturer's protocol.

Methylation Specific PCR: To assess the methylation status of TSP1gene promoter, methylation specific PCR was used and was performed in two stages- a nested PCR followed by Methylation specific PCR. For the first step of nested PCR, 2ul of sodium bisulfite treated DNA was used as template. Primers were designed to amplify the DNA template irrespective of the methylation status as previously mentioned.[11] The PCR product from the first step was diluted 10-

ORIGINAL ARTICLE

folds and used as template for the second step where primers were designed to specifically amplify the methylated and the unmethylated regions of the TSP1 promoter. CpG Methylated HeLa Genomic DNA (New England Biolabs, USA) was used as a positive control for methylated allele and DNA obtained from the of normal ovary samples served as negative control. The Primer sequences (table-3) and the cycling conditions for MSP and USP are summarized table-4(a) and (b). The PCR products post 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 Estimation: Blood samples from 50 EOC patients were estimated using the Elecsys CA125 diagnostic kits procured from Roche, Germany and quantified on Cobas e411™ auto analyzer (Roche Diagnostics, Germany). A CA125 value of 0-35U/ml was considered normal.

STATISTICAL ANALYSIS: The data was assessed for statistical significance using the chi square test and Fischer exact test on the SPSS software version 22.0 and a p-value <0.05 was considered to be statistically significant.

RESULTS: Detection of aberrant promoter hypermethylation of TSP1: Assessment of aberrant promoter hypermethylation of TSP1 by MSP resulted in an 80% methylation where 40 of the 50 samples showed positive for methylation. 89.2% methylation was observed in malignant sample group and 83.3% methylation in low malignant potential tumor group. The benign sample cohort resulted in a 42.8% methylation whereas none of the normal samples showed methylation of the gene. In comparison with the normal sample, the malignant samples showed a good statistical significance with a p value of <0.001 and the LMP group showed a p-value of 0.05. The benign group did not show any statistical significance (p-value=0.192). The methylation frequencies and the p-values of the three tumor groups are enlisted in table-2.

Correlation of hypermethylation pattern with clinicopathological parameters: The methylation frequency was correlated with clinicopathological parameters such as menopausal state, pre-operative CA125 level, presence or absence of ascites, histological subtype, FIGO stage and grade of the tumors. Among the various parameters considered almost all the groups reported a characteristically high frequency of methylation but statistical correlation was noted only in the menopausal status of the patients (p-value=0.004). The other clinicopathological parameters did not show any significant statistical correlation.

DISCUSSION: The existing screening modalities though useful have not been able to show a high degree of specificity in detecting ovarian cancer at early stages. Epigenetic silencing in cancer occurs as frequently as mutation and deletion and leads to the inactivation of normal TSG.[12]

TSP1 has drawn attention as a potential regulator of tumor growth and metastasis due to its effect on tumor and endothelial cell behavior. Earlier reports have shown that the silencing of TSP1, an important TSG, is due to aberrant hypermethylation and not due to mutation.[7] Increased expression of the TSP1 protein has been reported to suppress the growth of tumor and inhibit angiogenesis.[13]

ORIGINAL ARTICLE

So far there have been several studies that have reported the patterns of hypermethylation of TSP1 gene in various types of cancers and have been shown to be methylated in the frequency of 2-43%.[14]

Guerrero[15] et al reported a 46% promoter hypermethylation in penile squamous cell carcinoma and suggested that it was associated with pathological variables and may have prognostic significance in the same. A 37% hypermethylation frequency was reported in a study on neurobalstoma.[16] In gastric cancer TSP1 was methylated in 35.4% of the total sample population studied.[17]

In primary Glioblastoma Multiforme a 33% hypermethylation was reported by Qing Li[18] et al and concluded that the de novo methylation plays an important role inactivating the TSP1 gene in human neoplasm. Megan. A. Healey[11] reported a 13% and 20% methylation of TSP1 in their study on primary melanoma and melanoma cell lines respectively. A 10 and 7% methylation was observed in esophageal squamous cell carcinoma and pancreatic adenocarcinoma respectively.[19,20]

The first ever report of the hypermethylation of TSP1 in ovarian cancer was reported by M. Chmelařová[10] et al in 2012, where they have reported a characteristic 50% methylation in Endometroid and clear cell subtypes of EOC. Post this report there are no reports on the hypermethylation pattern of TSP1 in ovarian cancer.

Our study is the second report after the report by M.Chmelařová[10] et al and in this study we intended at investigating the aberrant promoter hypermethylation signature of the TSP1 gene and correlating the methylation frequency with available clinicopathological parameters which thereby could serve as a prognostic marker for EOC. The frequency of hypermethylation observed in this study suggests that it may be potentially used in the screening of ovarian cancer in the early stage in combination with the existing biochemical screening modalities.

CONCLUSION: Studying the aberrant promoter hypermethylation patterns of TSG may help in detecting the disease at the early stages as methylated DNA represents a promising biomarker in the detection and monitoring the onset and progression of cancer[21]. Designing a robust panel constituting various TSG could serve as an effective biomarker and may also have implication for future chemotherapy based on epigenetic changes.

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ORIGINAL ARTICLE

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ORIGINAL ARTICLE

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	Characteristics	**N**	**TSP1 Methylation (%)**
Menopause State	Post Menopause	35	32/35(91.4%)
	Pre Menopause	15	8/15(53.3%)
	p-Value P=0.004*		
CA125(U/ml)	0-35	05	3/5(60%)
	35-500	21	15/21(71.4%)
	500-1000	08	8/8(100%)
	>1000	16	14/16(87.5%)
	p-Value P=0.197 NS		
Ascites			
	Presence of ascites	31	28/31(90.3%)
	Absence of ascites	19	12/19(63.1%)
	p-Value P=1.000 NS		
Ovarian tumors			
Histological type	Serous	28	25/28(89.2%)
	Mucinous	04	2/4(50%)
	Clear cell	03	3/3(100%)
	Endometriod	02	2/2(100%)
	p-Value P=0.136 NS		
FIGO Stage			
	I	11	11/11(100%)
	II	2	2/2(100%)
	III	22	17/22(77.2%)
	IV	2	2/2(100%)
	p-Value P= 0.262 NS		
Histopathological grade			
	I	09	7/9(77.7%)
	II	03	3/3(100%)
	III	21	18/21(85.7%)
	IV	04	4/4(100%)
	p-Value P=0.890 NS		
Table1: Clinicopathological Parameters			

Note: 'NS'- not significant; '*'-significant.

ORIGINAL ARTICLE

Genes	Tumor type			
	Malignant (37)	LMP(06)	Benign (07)	Normal (07)
TSP1 (M)	32/37(89.2%)	05/06(83.3%)	03/07(42.8%)	0/7 (0%)
TSP1(U)	5/37(13.5%)	01/06(16.6%)	04/07(57.1%)	7/7 (100%)
pValue	P<0.001*	P=0.005	P=0.192 NS	
Table 2: Methylation frequencies of study subjects for TSP1				

Gene	Forward (5´-3´)	Reverse (5´-3´)	Annealing temperature
TSP1 Nested	GGGTTTTTGTYGTTTTTTAGGAGTAATT	CTCCAAATAAATATCCCRAACAACTTTA	57°c
TSP1 MSP	TCGGACGTATAGGTATTTTTCGC	ATCCTCGACGACCGCCG	60°c
TSP1 USP	TTTTTATTTTGGATGTATAGGTATTTTTTGT	CAACTTTAATCCTCAACAACCACCA	60°c
Table 3: Primer sequences and annealing temperatures			

Table 4: PCR Cycling Conditions:

Gene	Initial Denaturation	Cycling Stage x 35			Final Extension
		Denaturation	Annealing	Extension	
TSP1	95^0C	95^0C	57^0C	72^0C	72^0C
	5 mins	30 sec	30 sec	30 sec	7 mins
Table a: Nested PCR conditions					

Gene	Initial Denaturation	Cycling Stage x 35			Final Extension
		Denaturation	Annealing	Extension	
TSP1	95^0C	95^0C	60^0C (MSP/USP)	72^0C	72^0C
	5mins	30 sec	30 sec	30 sec	7 mins
Table b: Methylation and Unmethylation Specific PCR conditions					

ORIGINAL ARTICLE

Fig. 1: Representative gel image: Representative gel image of the promoter hypermethylation pattern of TSP-1 gene observed. 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 and DNA from non-cancerous patients (-ve) control.
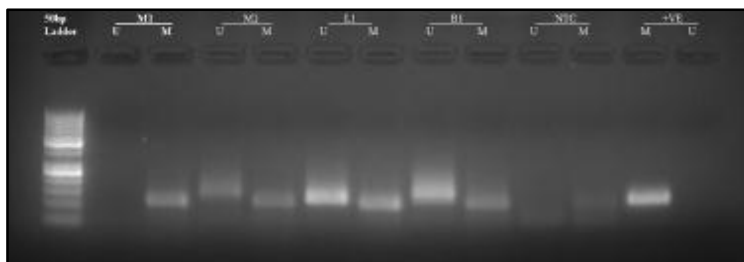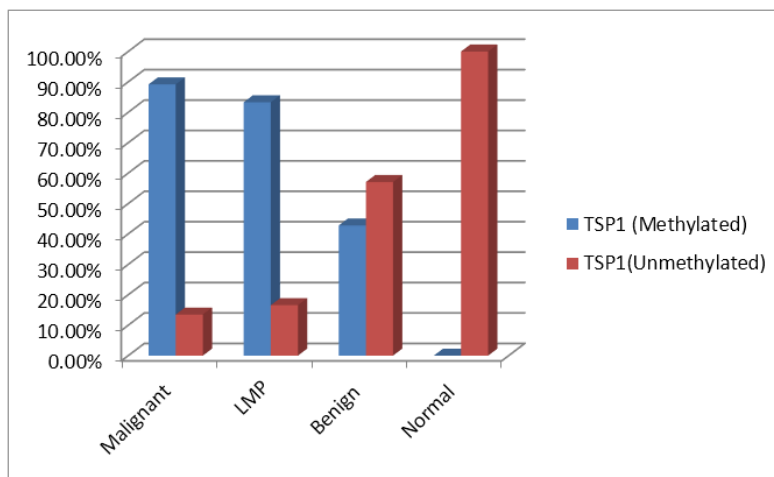

Fig. 1

M –Malignant sample; L- LMP sample; B- Benign sample; NTC-Non Template Control; +ve – Positive control (universally methylated HeLa genomic DNA).

Graphical representation of the methylation percentages in the three tumor groups:

Graph 1

Graph depicting the percentages of methylation and unmethylation in the 3 tumor groups Y-axis: percentage; X-axis: tumor groups.

ORIGINAL ARTICLE

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