

## A STUDY ON THE ABERRANT PROMOTER HYPERMETHYLATION OF RAR- $\beta$ GENE IN THE PLASMA OF EPITHELIAL OVARIAN CARCINOMA PATIENTS

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### HOW TO CITE THIS ARTICLE:

G. Ramesh, S. Sandeep Kumar, Shalini N. Swamy, C. S. Premalata, V. R. Pallavi. "A Study on the Aberrant Promoter Hypermethylation of RAR- $\beta$  Gene in the Plasma of Epithelial Ovarian Carcinoma Patients". Journal of Evidence based Medicine and Healthcare; Volume 2, Issue 21, May 25, 2015; Page: 3111-3118.

**ABSTRACT: PURPOSE:** The main aim of the study was to assess the hypermethylation pattern of the tumor suppressor gene RAR- $\beta$  in the plasma of patients with epithelial ovarian carcinoma and to study its role in the tumorigenesis of EOC and suggest its use as a biomarker in the early diagnosis of the disease by non-invasive methods. **METHODS:** 72 plasma samples, 10 corresponding matched tumor tissue specimens and 10 non-cancerous plasma samples were assessed for the aberrant hypermethylation of RAR- $\beta$  by nested and MSP. **STATISTICAL ANALYSIS:** The data was analyzed by Fischer test and Chi Square test. The analysis for the study was performed using SPSS software version 21.0 and a p-value <0.05 was considered significant. **RESULTS:** Of the 72 plasma samples included in the study 17 samples showed aberrant hypermethylation of RAR- $\beta$  promoter corresponding to a methylation frequency of 23.61%. Benign sample group in comparison to malignant and low malignant potential tumor groups showed a higher methylation percentage of 33.33% suggesting that hypermethylation may be an early event in ovarian cancer progression.

**KEYWORDS:** RAR- $\beta$ , plasma, cfc DNA, Aberrant Promoter Hypermethylation, Epithelial Ovarian Carcinoma, Nested PCR, Methylation Specific PCR.

**INTRODUCTION:** A major cause of gynecological death in women is attributed to ovarian cancer. The National cancer Registry Programme conducted by ICMR reported that ovarian cancer is the fourth leading cancer in women.<sup>(1)</sup> Ovarian cancer is asymptomatic at the early stages and has therefore been a challenging task to diagnose the disease and thereby increase the survival rate in patient presenting ovarian carcinoma.

Over 90% survival rate is noted in patients diagnosed at early stage (I/II) in contrast to the 11% survival when detected in the advanced stage of the disease.<sup>(2)</sup>

Epigenetic changes such as DNA hypermethylation in the promoter region of the tumor suppressor genes, is thought to be one of the earliest molecular changes during tumorigenesis and has been shown to find applicability in the detection of the disease in the pre malignant stages.<sup>(3)</sup>

Earlier studies have shown that the concentrations of circulating DNA are higher in the plasma of cancer patients than in healthy individuals.<sup>(4)</sup> This being suggestive that the release of circulating DNA is from the tumor sites and is hypothesized that it could be released by cells which undergoes apoptosis or necrosis.<sup>(5)</sup> Taking advantage of the released DNA in the plasma for the molecular diagnosis of the cancer can thus be used as a biomarker for the detection of the disease in the early stage. The prime benefit of using cfc DNA for diagnosis lies in the non-

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invasiveness and ease of detection and monitoring of the disease progression over the existing invasive methods.<sup>(6)</sup> Studying the aberrant promoter hypermethylation of TSG in circulating DNA can greatly help in developing diagnostic biomarker utilizing cancer specific alterations for early detection.

Retinoic Acid Receptor- $\beta$  is one of the important tumor suppressor gene located on chromosome 3p24.2 and codes for a 50.4KDa molecular weight protein. It belongs to the thyroid steroid hormone receptor super family and is localized in cytoplasm and sub nuclear region. The binding of Retinoic acid with RAR- $\beta$  mediates cellular signalling, cell growth and differentiation.<sup>(7)</sup> The key focus of this work is to study the aberrant hypermethylation signature of RAR- $\beta$  promoter in the cell free circulating DNA in the plasma of ovarian carcinoma patients.

## **MATERIAL AND METHODS:**

**Sample Collection:** Pre-operative Blood samples were collected from 72 patients diagnosed with EOC reporting to the Department of Gynaecology, Kidwai Memorial Institute of Oncology, Bangalore. Soon after the collection of blood, 1ml of plasma was separated by centrifugation at 3000rpm for 15 mins and the aliquots were stored at  $-80^{\circ}\text{C}$  until extraction of DNA.

**Tissue Specimens:** 10 EOC samples corresponding to the plasma samples were collected post the surgical resection of the tissue after confirmation from a senior pathologist. The DNA samples were stored at  $-80^{\circ}\text{C}$ .

**Extraction of cell free circulating DNA from Plasma:** The plasma samples were thawed on ice and incubated overnight at  $37^{\circ}\text{C}$  with Proteinase K (10mg/ml) and 0.5% SDS. Circulating cell free DNA was isolated from the plasma samples using the Phenol Chloroform isolation procedure and the DNA was precipitated with ammonium acetate and glycogen. The DNA was then cleaned with ethanol and then eluted with 3ul of TE buffer and used for Bisulfite modification.

**DNA extraction from matched tumor tissue sample:** 30mg of the tumor tissue was weighed and DNA was isolated using the Qiagen DNA easy kit following the manufacturer's protocol. Small aliquots of the eluted DNA were stored at  $-20^{\circ}\text{C}$  and subjected to sodium bisulfite modification.

**Sodium Bisulfite Modification:** The plasma DNA extracted was subjected to sodium Bisulfite modification using the EZ DNA Methyl Lightning kit<sup>TM</sup> (Zymo Research D5031, CA, USA). The bisulfite modified DNA was eluted according to the manufacturer's protocol.

**Methylation Specific PCR:** In order to assess the methylation status of RAR  $\beta$  gene promoter, the PCR was performed in two stages- a nested PCR followed by Methylation specific PCR.<sup>(8)</sup> 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-1 and 2A. 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

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RAR  $\beta$  promoter. CpG Methylated HeLa Genomic DNA (New England Biolabs) was used as a positive control for methylated allele and DNA obtained from the plasma of normal healthy subjects served as negative control. The Primer sequences and the cycling conditions for MSP and USP are summarized in benign samples. 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 72 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. A p-Value of less than 0.05 was considered to be statistically significant.

## RESULTS:

**Detection of RAR- $\beta$  Methylation analysis in Plasma samples:** In the malignant and low malignant potential sample cohort, a 22.6% (12/53) and 14.2% (1/07) hypermethylation was observed. Four of the twelve samples from the benign group showed hypermethylation corresponding to a frequency of 33.3%, however it failed to show any statistical significance (p-value=0.245). None of the 7 samples from the normal healthy controls showed methylation. An overall summary of the hypermethylation status of the gene in the study cohort of ovarian carcinomas, low malignant potential tumors, benign tumors and non-cancerous group has been listed in table-4 and the representative gel image is depicted in fig-1A.

**Correlation of plasma hypermethylation with tissue methylation:** We Determined of the hypermethylation status of RAR- $\beta$  in the matched tissue DNA and compared the pattern of the hypermethylation found to that of the corresponding plasma DNA. An identical pattern of promoter hypermethylation was observed (Fig-1B).

**Correlation of promoter hypermethylation with clinicopathological parameters:** Of the six clinicopathological parameters included in the study, there was no statistical association between the frequency of aberrant hypermethylation and four clinicopathological parameters viz. menopausal status, histopathological grade, tumor stage and CEA level. The aberrant promoter hypermethylation pattern however showed a statistically significant correlation between the CA125 levels and the presence of ascites with a p-value of <0.001 and 0.018 respectively. The detailed summary of the same along with the respective p- values are listed in the table-3.

**DISCUSSION:** Compared to other gynecological malignancies, ovarian cancer is the most lethal and accounted for more death than endometrial and cervical cancer combined. Existing screening modalities such as CA125 and trans vaginal ultrasonography together can detect only about 30-45% of the disease at the early stage.<sup>(9)</sup>

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RAR-  $\beta$  is known to suppress the activity of EGFR Activating Protein-1, COX2 and the phosphorylation of extracellular signal regulated protein kinase1 & 2 (ERK).<sup>(10,11)</sup> It was also reported that expression of Retinoid Receptor Induced Gene 1 (RRIG1) is seen in normal tissues but absent in many tumor cells and is known to mediate RAR-  $\beta$  in the process of regulating cancer growth and expression.<sup>(11)</sup>

Earlier reports have shown that the RAR-  $\beta$  methylation is one of the most common means of silencing the RAR-  $\beta$  expression in SCLC, NSCLC and various other cancers.<sup>(12,13,14)</sup> A report by Letetia C Jones et al in patients with Myelofibrosis with Myeloid Metaplasia, showed an 89% hypermethylation of RAR-  $\beta$  gene.<sup>(15)</sup> Xiao-Chun Xu et al in their report suggests that loss of RAR-  $\beta$  correlates with the progression of malignancy in tissues and cells of Head and neck, breast, lung, esophagus, pancreas, cervix and prostate.<sup>(11)</sup>

The aberrant RAR- $\beta$  promoter hypermethylation patterns have been studied extensively in many cancer types and have been found to be methylated in the range of 30-70%.<sup>(16)</sup> In NSCLC 40% aberrant promoter hypermethylation of the gene was observed by three research groups.<sup>(13,17,18)</sup> Virmani et al reported 72% aberrant methylation of RAR- $\beta$  in SCLC and 41% in NSCLC respectively.<sup>(14)</sup>

In Breast cancer, Fackler et al reported a methylation frequency of 21% in Invasive Lobular Carcinoma (ILC) and 41% in Invasive Ductal Carcinoma (IDC).<sup>(19)</sup>

Khodyrev et al reported 46%, 59%, 30% methylation frequency in their study on Breast, Renal cell and ovarian cancer respectively.<sup>(16)</sup> As low as 18.6% methylation of RAR- $\beta$  gene promoter was observed in ovarian cancer by Dhillon et al.<sup>(20)</sup>

The current study on the plasma DNA methylation, we report a 23.61% methylation frequency and are concordant with the above reports.

R Lotan et al showed that when patients were administered with 13-Cis RA, there was an increase in the level of RAR-  $\beta$  protein expression and it also correlated with clinical response<sup>(21)</sup>.

**CONCLUSION:** From the above references it is clear that hypermethylation of gene promoters occur in cancer and is also one of the major causes of silencing of the TSG. Detecting these epigenetic changes by non-invasive techniques could therefore hold a high significance in the developing of biomarker to diagnose the disease at early stages. It could serve to be more promising when it is included with other biochemical markers.

**ACKNOWLEDGEMENT:** The authors thank The Department of Biotechnology, India, Grant No. BT/PR 13440/MED/30/267/2009 for the financial aid for this work. We also thank Dr. Shanmugam V (Retd. Research Assistant, NIMHANS) for his help in statistical analysis.

## REFERENCES:

1. Three-year report of population based cancer registries 2009–11: National Cancer Registry Programme (ICMR), Bangalore 2013.
2. K Stephen Suh Sang W Park, Angelica Castro, Hiren Patel, Patrick Blake, Michael Liang, Andre Goy. Ovarian cancer biomarkers for molecular biosensors and translational medicine. *Expert Rev Mol Diagn.* 2010; 10(8): 1069-1083.

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3. Montavon, C., Gloss, B. S., Warton, K., Barton, C. A., Statham, A. L., Scurry, J. P., . & O'Brien, P. M. (2012). Prognostic and diagnostic significance of DNA methylation patterns in high grade serous ovarian cancer. *Gynecologic oncology*, 124(3), 582-588.
4. Luke B. Hesson, Wendy N. Cooper and Farida Latifa. The role of RASSF1A methylation in cancer. *Disease Markers* 23 (2007) 73–87 73.
5. Levenson, V. V., & Melnikov, A. A. (2012). DNA methylation as clinically useful biomarkers—light at the end of the tunnel. *Pharmaceuticals*, 5(1), 94-113.
6. Schwarzenbach, H., Hoon, D. S., & Pantel, K. (2011). Cell-free nucleic acids as biomarkers in cancer patients. *Nature Reviews Cancer*, 11(6), 426-437.
7. Sun, S. Y. (2004). Retinoic acid receptor beta and colon cancer. *Cancer biology & therapy*, 3(1), 87-88.
8. Cirincione, R., Lintas, C., Conte, D., Mariani, L., Roz, L., Vignola, A. M, & Sozzi, G. (2006). Methylation profile in tumor and sputum samples of lung cancer patients detected by spiral computed tomography: a nested case–control study. *International journal of cancer*, 118(5), 1248-1253.
9. Nguyen, L., Cardenas-Goicoechea, S. J., Gordon, P., Curtin, C., Momeni, M., Chuang, L., & Fishman, D. (2013). Biomarkers for early detection of ovarian cancer. *Women's Health*, 9(2), 171-187.
10. Thomas W. Grunt, Klaudia Puckmair, Katharina Tomek, Birgit Kainz, Alexander Gaiger. An EGF receptor inhibitor induces RAR-b expression in breast and ovarian cancer cells. *Biochemical and Biophysical Research Communications* 329 (2005) 1253–1259.
11. Xu, X. C. (2007). Tumor-suppressive activity of retinoic acid receptor- $\beta$  in cancer. *Cancer letters*, 253(1), 14-24.
12. Baylin, S. B., & Ohm, J. E. (2006). Epigenetic gene silencing in cancer—a mechanism for early oncogenic pathway addiction?. *Nature Reviews Cancer*, 6(2), 107-116.
13. Zöchbauer-Müller, S., Fong, K. M., Virmani, A. K., Geradts, J., Gazdar, A. F., & Minna, J. D. (2001). Aberrant promoter methylation of multiple genes in non-small cell lung cancers. *Cancer research*, 61(1), 249-255.
14. Virmani, A. K., Rathi, A., Zöchbauer-Müller, S., Sacchi, N., Fukuyama, Y., Bryant, D., & Gazdar, A. F. (2000). Promoter methylation and silencing of the retinoic acid receptor- $\beta$  gene in lung carcinomas. *Journal of the National Cancer Institute*, 92(16), 1303-1307.
15. Jones, L. C., Tefferi, A., Idos, G. E., Kumagai, T., Hofmann, W. K., & Koeffler, H. P. (2004). RAR $\beta$ 2 is a candidate tumor suppressor gene in myelofibrosis with myeloid metaplasia. *Oncogene*, 23(47), 7846-7853.
16. Khodyrev, D. S., Loginov, V. I., Pronina, I. V., Kazubskaya, T. P., Garkavtseva, R. F., & Braga, E. A. (2008). Methylation of promoter region of RAR- $\beta$ 2 gene in renal cell, breast, and ovarian carcinomas. *Russian Journal of Genetics*, 44(8), 983-988.
17. Kim, J. S., Lee, H., Kim, H., Shim, Y. M., Han, J., Park, J., & Kim, D. H. (2004). Promoter Methylation of Retinoic Acid Receptor Beta 2 and the Development of Second Primary Lung Cancers in Non–Small-Cell Lung Cancer. *Journal of clinical oncology*, 22(17), 3443-3450.

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18. Esteller, M., Sanchez-Cespedes, M., Rosell, R., Sidransky, D., Baylin, S. B., & Herman, J. G. (1999). Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer research*, 59(1), 67-70.
19. Fackler, M. J., McVeigh, M., Evron, E., Garrett, E., Mehrotra, J., Polyak, K., & Argani, P. (2003). DNA methylation of RASSF1A, HIN-1, RAR- $\beta$ , Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. *International journal of cancer*, 107(6), 970-975.
20. Dhillon, V. S., Young, A. R., Husain, S. A., & Aslam, M. (2004). Promoter hypermethylation of MGMT, CDH1, RAR- $\beta$  and SYK tumour suppressor genes in granulosa cell tumours (GCTs) of ovarian origin. *British journal of cancer*, 90(4), 874-881.
21. Lotan, R. (1997). Roles of retinoids and their nuclear receptors in the development and prevention of upper aerodigestive tract cancers. *Environmental health perspectives*, 105(Suppl 4), 985.

## SUPPLEMENTARY DATA:

Gene	Forward (5' -3')	Reverse (5' -3')	Annealing temperature	Product Size
RAR- $\beta$ Nested	AAGTGAGTTGTTTAGAGGTAGGAGGG	CCTATAATTAATCCAAATAATCATTTACC	52° C	276
RAR- $\beta$ MSP	CCTATAATTAATCCAAATAATCATTTACC	CCTATAATTAATCCAAATAATCATTTACC	60° C	146
RAR- $\beta$ USP	CCTATAATTAATCCAAATAATCATTTACC	CTCAACCAATCCAACCAAAACA	61° C	146

Table 1: Primer sequences of RAR- $\beta$  gene

## Table 2: PCR Cycling Conditions:

Gene	Initial Denaturation	Cycling Stage x 35			Final Extension
		Denaturation	Annealing	Extension	
DAPK	95°C	95°C	53°C	72°C	72°C
	10 mins	30 sec	30 sec	30 sec	7 mins

Nested PCR conditions

Gene	Initial Denaturation	Cycling Stage x 35			Final Extension
		Denaturation	Annealing	Extension	
DAPK	95°C	95°C	60°C/61°C (MSP/USP)	72°C	72°C
	10mins	30 sec	30 sec	30 sec	7 mins

Methylation and Unmethylation Specific PCR conditions

	Characteristics	N	RAR- $\beta$ (% methylation)
<b>Ovarian tumors</b>		72	
<b>Menopause State</b>	Pre Menopause	17	2/17 (11.7%)
	Post Menopause	55	15/55 (27.2%)
	p-Value 0.327		

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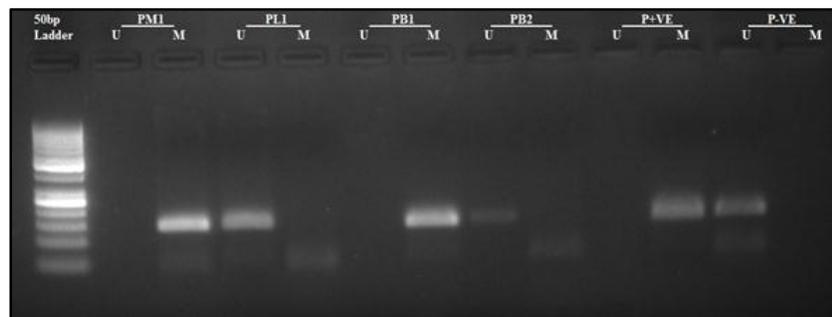
<b>Histological type</b>	Serous	36	07/36 (19.4%)
	Mucinous	10	04/10 (40%)
	Clear cell	3	1/03 (33.3%)
	Endometrioid	4	0/04 (0%)
	p-Value 0.314		
<b>FIGO Stage</b>	I-II	15	3/15 (20%)
	III-IV	38	09/38 (23.6%)
	p-Value 1.000		
<b>CA125(U/ml)</b>	0-35	13	0/13 (0%)
	35-500	16	11/16 (68.7%)
	500-1000	17	4/17 (23.5%)
	>1000	26	2/26 (7.6%)
	p-Value <0.001*		
<b>CEA (ng/ml)</b>	0-7.5	67	14/67 (20.8%)
	>7.5	5	3/5 (60%)
	p-Value 0.082		
<b>Ascites</b>			
	Presence of ascites	24	10/24 (41.6%)
	Absence of ascites	48	7/48 (14.5%)
	p-Value 0.018*		

Table 3: Clinicopathological parameters and Patients characteristics

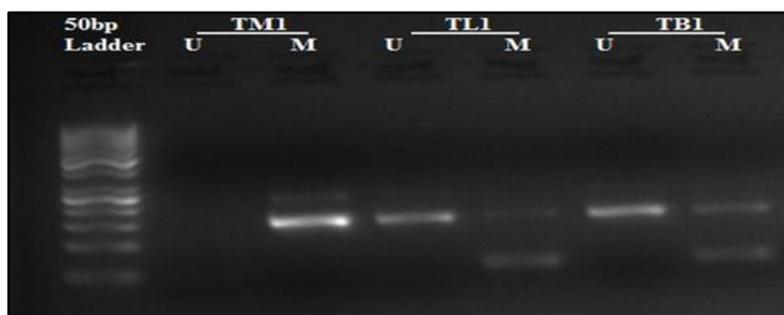
**Note:** \* indicates statistical significance.

Genes	Tumor type			
	Malignant (53)	LMP(07)	Benign (12)	Normal (07)
<b>RAR (M)</b>	12/53(22.6%)	01/7(14.2%)	04/12(33.3%)	0/7 (0%)
<b>RAR(U)</b>	41/53(77.3%)	06/7(85.7%)	08/12(66.6%)	7/7 (100%)
<b>pValue</b>	0.326	1.000	0.245	

Table 4: Methylation frequencies of study subjects for RAR -beta



**Figure 1A**



**Figure 1B**

Agarose gel electrophoresis images as captured on the Gel Documentation System showing representative methylation-specific PCR result of the RAR- $\beta$  gene. The sizes of PCR products are listed in Table-1, 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 and DNA from non-cancerous patients (-ve) control. Fig1A indicates the results obtained in plasma DNA and Fig1B denote the results obtained in corresponding tumor tissue DNA.

'PM'- Plasma Malignant sample, 'PL'- Plasma Low Malignant Potential Sample, 'PL'-Plasma Benign samples, 'TM'-Tissue Malignant sample, 'TL'- Tissue Low Malignant Potential Sample, 'TB'-Tissue Benign samples.

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Date of Submission: 13/05/2015.  
Date of Peer Review: 14/05/2015.  
Date of Acceptance: 16/05/2015.  
Date of Publishing: 20/05/2015.