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Aberrant promoter hypermethylation of RAR-β in endometrial carcinoma- an Indian study.

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Endometrial cancer is the seventh most common cancer in women worldwide with an age-standardized rate of 8.4 per 100,000 women. Epigenetic alterations such as promoter hypermethylation of TSGs are known to be early events in carcinogenesis. The aim of the present study, we assessed the aberrant promoter hypermethylation pattern of RAR-β in 78 endometrial cancer samples. **Methods:** DNA was isolated from endometrial carcinoma samples and normal tissues and aberrant promoter hypermethylation was assessed using nested and methylation-specific PCR. The Chi-square test was used for statistical analysis and a p-value<0.05 was considered to be statistically significant. **Results:** 40 of the 78 (51.28%) endometrial carcinoma samples showed aberrant hypermethylation of the RAR-β gene. Methylation status in each histological subtype, grade and stage of the disease was also assessed. **Conclusion:** Aberrant hypermethylation is an important early epigenetic alteration that occurs during tumorigenesis. The Data shown here reports that promoter hypermethylation of RAR-β occurs in endometrial carcinoma and therefore could be used as a potential marker for early diagnosis and prognosis of the disease.

Keywords: DNA Hypermethylation, RAR-β, Endometrial cancer, epigenetic alterations, DNA hypermethylation, Methylation-specific PCR

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Introduction

Endometrial cancer (EC) is the seventh most common cancer in women worldwide with an age-standardized incidence rate (ASR) of 8.4 per 100,000 women. According to the 2018 Indian statistics, 13328 women were diagnosed with EC and an estimated 5010 women succumbed to the disease accounting for an ASR of 2.3 per 100,000 women [1-3]. Though most of the patients are diagnosed in the early stages when the disease is still confined to the primary site, about 30% of the patients are diagnosed in late stages where the disease would have disseminated from the uterus [4]. Various genetic and epigenetic alterations are known to be involved in the initiation and progression of EC. Aberrant promoter hypermethylation of Tumor suppressor genes (TSGs) is known to be an early event in many cancers including EC. Promoter hypermethylation is associated with transcriptional silencing of several tumour suppressor genes [5]. Retinoids are known to play a role in tumor suppression upon interaction with retinoic acid receptors. The retinoic receptors are nuclear receptors and include three retinoic acid receptors (RARs) - RAR-α, RAR-β, and RAR-γ [6,7]. Suppression of expression and function of RAR-β, have been reported in various cancers [8-10]. One of the mechanisms of suppressing the expression of RAR-β is by the epigenetic mechanism of promoter hypermethylation where a methyl group is added to deoxycytidine residues in the CpG islands of its promoter. This methylation of all or a few of the cytosine in the promoter region renders the closure of the chromatin structure eventually resulting in transcriptional silencing of the gene [11-13]. In the present study, we have attempted to analyze the methylation pattern of the RAR-β gene in 78 endometrial cancers of patients of the Indian cohort and associated the methylation patterns with various clinicopathological parameters.

Methods

Sample collection: 78 histologically confirmed endometrial cancer samples were collected post-surgery from patients undergoing surgery for endometrial cancer. All the samples were immediately stored at -80ºC until further processing.

Inclusion and exclusion criteria: Primary chemo naïve endometrial carcinoma patient samples were included in the study. Patients with secondary metastasis to the endometrium and chemotherapy-treated samples were excluded from the study.

DNA extraction: Genomic DNA was isolated from all the samples using DNA easy mini kit (Qiagen, USA) following the manufacturer's protocol. The DNA concentration was estimated after extraction using Eppendorf Biospectrophotometer Kinetics™. 1µg of the eluted DNA samples were used for bisulfite modification.

Bisulfite modification: Genomic DNA was subjected to sodium bisulfite modification using the EZ DNA Methyl Lightening kit TM (Zymo Research D5031, CA, USA) following the manufacturer's procedure. The bisulfite modified DNA was aliquoted and used for methylation analysis.

Methylation Specific PCR: To assess the methylation status of the RAR-β promoter, methylation-specific PCR was used and was performed in two stages-first a nested PCR followed by Methylation-specific PCR. For the nested PCR, 2µl of sodium bisulfite modified DNA was used as a template. The PCR product from the first step nested PCR was diluted 10-folds and used as a template for the second step to specifically amplify the methylated and the unmethylated regions of the RAR-β promoter. CpG Methylated HeLa Genomic DNA (New England Biolabs, USA) was used as a positive control for methylated allele and DNA obtained from the normal ovary samples served as a negative control. The Primer sequences (table-1) and the cycling conditions for MSP and USP are summarized in table-2. The PCR products of methylation-specific and unmethylation specific PCR 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.

Statistical analysis: Chi-square test was used to analyze the statistical association of methylation with clinicopathological parameters. A p-value of <0.05 was considered to be statistically significant.

Ethical approval: The study was approved by the Institutional Scientific Review board and medical ethics committee and consent was obtained from patients before sample collection.

Results

RAR-β methylation was assessed in 78 endometrial cancer samples of 5 subtypes- endometrioid

(N=60), serous (n=06), mucinous (n=02), clear cell (n=02) and poorly differentiated carcinoma (n=08). Six normal endometrial samples were analyzed for RAR-β methylation as a control cohort. 40 of the 78 samples analyzed (51.28%) showed methylation of the RAR-β gene. Among the subtypes, 50 % of the Endometroid, mucinous, clear cell, and poorly differentiated samples showed methylation for the RAR-β gene and 66.6% of the samples of the serous subtype showed methylation for the gene. About 50% of the patient in Stage I and II and about 60% of the patients of stage III and IV showed methylation for the gene respectively. 20/44, 11/19 and 9/15 samples of grades 1,2,3 showed methylation respectively.

68 of the 78 patients (87.18%) included in the study posted menopausal women and 52 % of these postmenopausal patients showed methylation for the gene.

The results of the same are summarized in table 3. Though a good percentage of methylation was observed in various subtypes and subgroups of the clinicopathological parameters, there was no statistically significant association of the methylation status with these clinicopathological parameters.

Table 1: Primer sequence for nested and methylation specific PCR

Gene	Forward (5´-3´)	Reverse (5´-3´)	Annealing temperature	Product Size
RAR-β Nested	AAGTGAGTTGTTTAG AGGTAGGAGGG	CCTATAATTAATCCAA ATAATCATTTACC	520 C	276
RAR-β MSP	CCTATAATTAATCCAA ATAATCATTTACC	CCTATAATTAATCCAA ATAATCATTTACC	600 C	146
RAR-β USP	CCTATAATTAATCCAA ATAATCATTTACC	CTCAACCAATCCAAC CAAAACA	610 C	146

Table 2: PCR cycling conditions for nested and Methylation-specific PCR.

Gene	Initial Denaturation	Cycling stage			Final extension
		Denaturation	Annealing	Extension	
RAR-β Nested	950 C	950 C	520 C	720 C	720 C
	10 mins	30 seconds	30 seconds	30 seconds	7 mins
RAR-β MSP/USP	950 C	950 C	600 C/610C (MSP/USP)	720 C	720 C
	10 mins	30 seconds	30 seconds	30 seconds	7 mins

Table 3: Association of RAR-β methylation with clinicopathological parameters.

Clinicopathological Parameters		N	RAR-β methylation
Endometrial tumors		78	
Type of tumor	Endometriod	60	30 (50%)
	Serous	06	04 (66.6%)
	Mucinous	02	01(50%)
	Clear cell	02	01(50%)
	Poorly differentiated	08	04(50%)
	p-value		0.961293
FIGO stage	I	64	32 (50%)
	II	09	5 (55.5%)
	III	02	1(50%)
	IV	03	2 (66.6%)
	p-value		0.91598
Histological grade	1	44	20(45.45%)
	2	19	11(57.89)
	3	15	9(60%)
	p-value		0.499836
Menopausal status	Premenopausal	10	4(40%)
	Postmenopausal	68	36(52.94%)
	p-value		0.444594
Invasion	<50%	52	28(53.84%)
	>50%	26	12(46.15%)

	p Value	0.521702	
	II. Normal control	06	01

One of the six normal endometrial samples analyzed showed positive for both unmethylated and unmethylated alleles.

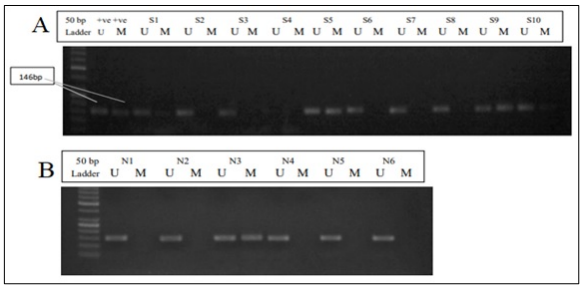

Figure 1: (A) Representative Agarose gel image of the RAR-β methylation pattern observed in the endometrial carcinoma samples and (B) in normal endometrial samples.

(S: Endometrial carcinoma sample; N: Normal; U: Unmethylated; M: Methylated allele)

Discussion

Cancers are known to evolve in a stepwise manner accumulating several genetic and epigenetic alterations [14,15]. One of the most well studied epigenetic alterations in the promoter methylation which plays significant roles in both normal and cancer cells. In normal cells, it is involved in processes such as chromatin organization, X-chromosome inactivation and genetic imprinting. On the contrary, in cancer cells aberrant hypermethylation is observed in the CpG islands of gene promoters which are involved in the regulation of cell cycle and apoptosis. This type of aberrant promoter hypermethylation leads to transcriptional gene silencing of several important tumour suppressor genes. Reports have also shown that the methylation patterns are gene-specific and cancer-specific and therefore specific methylation patterns may provide insights in unravelling specific molecular signatures of cancer [14-17].

The RAR-β gene is known to exhibit its tumour suppressor function by having significant roles in several important cellular pathways such as the apoptotic pathway and is known to inhibit cellular proliferation [18]. Aberrant hypermethylation of RAR-β has been studied across various cancer types.

Letetia C Jones et al reported 89% hypermethylation of RAR-β in patients with Myelofibrosis with Myeloid Metaplasia [19]. Yousseff EM et al have reported a 67% methylation frequency of RAR-β in head and neck cancer [20]. Other reports have shown that loss of RAR- β correlates with the progression of malignancy in tissues and cells of the Head and neck, breast, lung, esophagus, pancreas, cervix and prostate [21]. Around 40% methylation of RAR- β was reported by three independent studies in NSCLC [22-25]. Methylation of 23.61% was reported in ovarian carcinoma and 10% methylation was reported in breast cancer [26,27]. However, studies in other cancers have reported much higher frequencies of *RAR*-β methylation such as, cervical cancer (40%) and ovarian cancer (31%) [28,29].

44% methylation was reported for *RAR*-β in endometrial cancer of the endometrioid subtype [30]. A 75% and 92.2% methylation was reported in the endometrial hyperplasia and carcinoma respectively [31]. suggesting that the hypermethylation of *RAR*-β is an early epigenetic alteration in the endometrial carcinogenesis. Two independent studies have reported a methylation frequency as low as 2.3% and 1% methylation (in Endometroid and serous subtype) for the *RAR*-β gene [32,33]. These findings suggest that epigenetic silencing of the *RAR*-β gene promoter through promoter hypermethylation is a common event in the initiation and progression of several cancers and thus can be used as a marker for diagnosing the disease at the early stages. The methylation analysis also has the benefit of serving as a molecular marker for the prognosis of cancers. In addition methylation patterns can also be detected in circulating cell-free DNA of cancer patients thereby making it a promising minimally invasive marker of diagnosis and prognosis.

Conclusion

Promoter hypermethylation of tumor suppressor genes is an early event in cancer. Analysis of promoter hypermethylation with a larger cohort of samples will aid in the development of a methylation-based biomarker for endometrial cancer which could potentially aid in the diagnosis and management of the disease along with existing clinical modalities.

What this study adds to the existing knowledge

Analysis of promoter hypermethylation pattern of tumor suppressor gene panels in larger sample cohorts will provide a deeper understanding of the underlying epigenetic alteration that contributes to endometrial carcinogenesis. These hypermethylation patterns can aid in the development of methylation-based biomarker assays that could aid in better diagnosis and management of the disease.

Author Contributions

Nagaratna Shivanandappa: designed the study, Experimentation and data collection, manuscript writing, statistical analysis and manuscript editing **Shalini N Swamy:** experimental design, manuscript writing and editing, statistical analysis, **Sandeep Kumar S**: manuscript writing and editing, statistical analysis, **Suma Sheshadri:** data collection and sample screening, **Pallavi V R:** data collection and sample selection, **Ramesh Gawari:** designed the study, manuscript writing, editing, statistical analysis.

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