International Journal of Research and Review
DOI: https://doi.org/10.52403/ijrr.20210758
Vol.8; Issue: 7; July 2021
Website: www.ijrrjournal.com
Original Research Article *E-ISSN: 2349-9788; P-ISSN: 2454-2237*

Assessment of Aberrant Promoter Hypermethylation of RASSF1a and P16 in Endometrial Carcinoma

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ABSTRACT

Endometrial carcinoma is one of the most common gynecological malignancies known to affect around 142000 women worldwide. Aberrant promoter hypermethylation of tumor suppressor genes is a common epigenetic alteration reported in cancers. In the present study we report the aberrant promoter hypermethylation of RASSF1a and p16 in 78 endometrial cancer patients.

Methods: Endometrial carcinoma samples were collected after pathological examination and DNA was extracted. The DNA was subjected to sodium bisulfite modification and then used to perform methylation specific PCR. The PCR was performed using a two step procedure of nested and methylation specific PCR. The PCR product was visualized using agarose gel electrophoresis. The results obtained were correlated with various clinicopathological parameters. Chi square test was used for statistical analysis and a p-value of <0.05 was considered statistically significant.

Result: 60 of the 78 (76.92%) samples showed methylation for RASSF1a gene. 28 of the 78 (35.8%) samples showed methylation for p16 gene. A higher methylation frequency was observed for RASSF1a in the endometrioid and poorly differentiated histological subtypes and stage I and II of the disease.

Conclusion: Aberrant promoter hypermethylation of RASSF1a and p16 is known to play an important role in endometrial cancer initiation and progression. The methylation patterns can be used as an important molecular marker for early diagnosis and prognosis of endometrial cancer.

Keywords: Epigenetic alterations, DNA Hypermethylation, Endometrial cancer, RASSF1a, p16, Methylation-specific PCR.

INTRODUCTION

Endometrial cancer (EC) is one of the most common gynecological cancers that are known to affect about 1,42,000 women worldwide. Most of the cases of EC are known to be diagnosed post menopause and is estimated to have a highest incidence in the seventh decade bout 75% of women are diagnosed in the early stage when the cancer is still confined to the uterus [1-3]. Various genetic and epigenetic alterations are attributed in the initiation and progression of EC.

DNA methylation is one of the well studied epigenetic alterations in cancers. The process of DNA methylation is primarily facilitated by the enzymes of the family of DNA methyltransferases (DNMT). DNMT 1, 3A and 3B are known to cause methylation of the cytosine bases in the DNA. These enzymes mediate the covalent addition of a methyl (-CH3) group in the fifth position of cytosine bases [4-7]. In cancer, the addition of methyl group to cytosine occurs in the region of CpG Island that is present in and around the promoter regions of various genes [8,9]. Methylation of the cytosine bases in the CpG islands of gene promoters prevents the association of transcription factors to the promoter sites, thereby resulting in transcriptional inactivation of genes. Tumor suppressor gene promoters are known to be methylated in various cancers and have been an important field of interest [10].

Damman et al., in 2000 cloned a gene in the 3p21.3 region and termed the gene as Ras association domain family 1(RASSF1) [11-13]. One of the isoforms of the RASSF1 is the RASSf1a, a tumor suppressor gene known to be involved in the regulation of various events of the cell cycle and the apoptosis pathway. RASSF1a lacks enzyme activity but serves a protein involved in various signalling pathways due to their ability to associate with Ras. RASSF1a can be silenced by genetic alterations such as mutation. But the most common mode of transcriptional silencing of the gene occurs by promoter hypermethylation [14].

p16, also known as Cyclin-dependent Kinase Inhibitor 2a (CDKN2A) is a tumor suppressor gene located on chromosome 9p21 [15]. It was discovered in 1993 as an inhibitor of cyclin dependent kinase (CDKI) [16]. p16 functions as a G1 specific cell cycle regulatory protein by binding to CDK4 or CDK6 and is also known to inhibit the function of cyclin D1 [17-20].

P 16 is known to be silenced by hypermethylation in cancers of liver, colon, lung etc [21-23].

In the current study we have assessed the methylation of RASSF1a and p16 in endometrial carcinoma patients.

METHODS

Sample collection: 78 primary endometrial carcinoma samples were obtained from patients undergoing surgery for the malignancy. The samples were frozen at -80^0C until DNA extraction.

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: DNA was extracted from the cancer and normal samples using the DNAeasy mini kit (Qiagen, USA) following the manufacturer's instructions. The isolated DNA was quantified for all the samples and used for bisulfite modification.

***Sodium bisulfite modification*:** The DNA extracted from cancer and normal tissue was subjected to bisulfite modification using the EZDNA methylation kit (Zymo Research D5031, CA, USA).

Methylation specific PCR: The bisulfite modified DNA was used as a template to analyze the methylation frequency of the RASSF1a and p16 genes. The PCR was performed in two steps- first round of nested PCR which amplifies the genes irrespective of the methylation status and a second round of methylation specific PCR which can detect the methylated and unmethylated alleles of the genes. Specific primers were used for both round of PCR, the primer sequences and annealing temperatures are mentioned in the table 1 to 4.

Agarose gel electrophoresis: After the second round of methylation specific PCR, the amplified products were run on a 1.5% agarose gel to detect the amplicons for methylation and unmethylation.

Statistical analysis: Chi square test and Fischer test was used for statistical analysis

and a p value of <0.05 was considered to be statistically significant.

Ethics and approval: The study was approved by the institutional scientific review board and medical ethics committee and informed consent was obtained from all patients prior to sample collection.

RESULTS

Methylation of RASSF1a in Endometrial carcinoma:

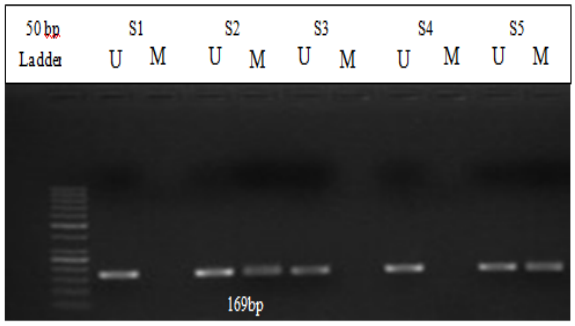

Fig1: Representative Agarose gel image of methylation frequency of RASSF1a in Endometrial carcinoma. S1, S2, S3, S4 and S5 represent the EC samples. U: Unmethylated allele. M: methylated allele. A few samples showed bands of both methylation and unmethylation and are called hemimethylated. These hemimethylated samples are considered to be methylated.

60 of the 78 samples analyzed for methylation showed a methylation for the RASSF1a gene accounting to about 76.9%. The Endometroid and poorly differentiated histological subtypes showed a methylation percentage of 78.33 and 87.5 % respectively. Stage I and II of the cancer showed 78.12 and 77.77% methylation frequency respectively. Of the three grades, 33/44 samples showed methylation (75%). 79.41% of the patients of the postmenopausal women with the disease showed methylation and 80.76% of samples were methylated where the invasion was <50%. Though a good percentage of methylation was observed, no statistical significant association with any clinicopathological conditions considered could be noted. The results are presented in Table 4 and the representative gel image is shown in fig 1.

Methylation of p16 in endometrial carcinoma:

A total of 28 samples of the 78 samples reported methylation of the p16 gene. Samples of stage I, II, III and IV showed a methylation frequency of 34.37%, 44.44%, 50% and 33.33% respectively. In the postmenopausal group with EC, a methylation frequency of 42.30% was observed. There was no statistically significant association that was noted for any of the clinicopathological parameters considered for the study. The result has been tabulated in table 4 and the representative image is shown in fig 2.

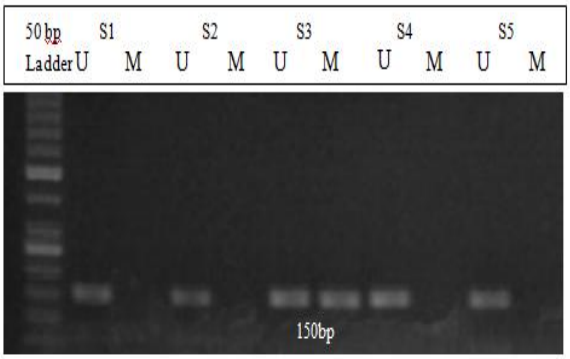

Fig 2: Representative Agarose gel image of methylation frequency of p16 in Endometrial carcinoma. S1, S2, S3, S4 and S5 represent the EC samples.

Table 1: Primer sequences for RASSF1a and p16

Gene	Forward (5'-3')	Reverse (5'-3')	Amplicon size
RASSF1a Nested	GGAGGGAAGGAAGGGTAAGG	GGGTTTTGCGAGAGCGCG	250bp
RASSF1a MSP	GGGTTTTGCGAGAGCGCGT	GCTAACAAACGAGAACCG	169bp
RASSF1a USP	GGTTTTGTGAGAGTGTGTTTAGT	CACTAACAAACACAAACCAAACA	169bp
p16 Nested	GAAGAAAGAGGAGGGGTTGG	CTACAAACCCTCTACCCACC	280bp
p16 MSP	TTATTAGAGGGTGGGGCGGATC	GACCCCGAACCGCGACCGTAA	150bp
p16 USP	TTATTAGAGGGTGGGGTGGATT	CAACCCCAAACCACAACCATAA	150bp

Table 2: RASSF1a and p16 gene Nested PCR Condition:

Gene	Initial denaturation	Cycling stage x 35cycles			Final extension
		Denaturation	Annealing	Extension	
RASSF1a	95°C	95°C	56°C	72°C	72°C
	7 mins	30 sec	30 sec	30 sec	7 mins
p16	95°C	95°C	57°C	72°C	72°C
	7 mins	30 sec	30 sec	30 sec	7 mins

Table 3: RASSF1a and p16 gene MSP/USP PCR Condition:

Gene	Initial denaturation	Cycling stage x 35cycles			Final extension
		Denaturation	Annealing	Extension	
RASSF1a	95°C	95°C	60°C/58°C (MSP/USP)	72°C	72°C
	7 mins	30 sec	30 sec	30sec	7 mins
p16	95°C	95°C	57°C	72°C	72°C
	7 mins	30 sec	30 sec	30sec	7 mins

Table 4: Association of RASSF1a and p16 methylation with clinicopathological parameters

Clinicopathological Parameters		N	Rassf1a methylation	p16 methylation
Endometrial tumors		**78**		
Type of tumor	Endometriod	60	47 (78.33%)	22 (36.66%)
	Serous	06	04 (66.66%)	02 (33.33%)
	Mucinous	02	01 (50%)	01 (50%)
	Clear cell	02	01 (50%)	01 (50%)
	Poorly differentiated	08	07 (87.5)	02 (25%)
	p-value		0.633876	0.939632
FIGO stage	I	64	50 (78.12%)	22 (34.37%)
	II	09	07 (77.77%)	04 (44.44%)
	III	02	01 (50%)	01 (50%)
	IV	03	02 (66.66%)	01 (33.33%)
	p-value		0.7891	0.91189
Histological grade	1	44	33 (75%)	13 (31.81%)
	2	19	17 (73.68%)	09 (47.36%)
	3	15	10 (66.66%)	06 (40%)
	p-value		0.263016	0.37387
Menopausal status	Premenopausal	10	06 (60%)	03 (30%)
	Postmenopausal	68	54 (79.41%)	25 (36.76%)
	p-value		0.173715	0.677134
Invasion	<50%	52	42 (80.76%)	12 (23.07%)
	>50%	26	18 (69.23%)	11 (42.30%)
	pValue		0.254213	0.079114

DISCUSSION

Epigenetic alterations are one of the most important that play a role in initiation of the tumorigenesis process by transcriptionally silencing tumor suppressor genes. The current study we have analyzed the aberrant promoter hypermethylation frequency of two crucial tumor suppressor genes that are known to regulate the process of cell proliferation- the RASSF1a and p16 genes.

RASSF1a promoter hypermethylation has been one of the most studied epigenetic alterations in various cancers including ovarian cancers. The methylation analysis by Fiolka R et al., reported a methylation frequency of 84.5% in EC. *RASSF1A*-promoter methylation was very frequent in endometrial carcinoma 74% and 51.42% methylation was reported by Pallares J and Jo H et al., respectively [24-26]. Pijnenborg et al., have reported a methylation of 70% in endometrial cancer and a 33.33% frequency was reported by Kang et al., [27,28]. Several studies in breast cancer have reported a methylation frequency of 85%, 83.6, 82.6, 70% and 49% [29-33].

In lung cancer, RASSF1a methylation frequency of 80.36%, 55%, 42%, 33.8% frequency was observed [34-37]. 50%, 42%, 40%, 34%, 31.9%, methylation was reported in ovarian cancers for the RASSF1a gene [38-42].

The p16 gene encoded protein is known to be an inhibitor of cyclin dependent kinases (regulators of cell cycle). The p16 protein interacts by binding to CDK4/6. This binding inhibits two important events in cell cycle- inhibits the cyclinD-CDK complex formation and also inhibits the phosphorylation of pRb. These two inhibitory events caused due to p16 bring about the arresting of cells in the G1 phase of the cell cycle and thus acts as a tumor suppressor protein. The transcriptional silencing of p16 gene has been reported to be an underlying mechanism of tumorigenesis. The silencing of p16 by promoter hypermethylation has been reported in breast, lung, ovary and endometrial cancers.

The p16 gene was found to be methylated in a range from 75%, 41%, 20%, 17.4%,4.2%, 0% methylation in endometrial cancers [43-48].

Lee JJ et al., have reported 52.8% and 57.8% methylation in invasive and intraductal tumors of the breast respectively [49]. A 54.1% methylation was reported in a study on triple negative breast cancer [50]. A 23% and 14% methylation frequency was reported by Silva LM et al., in the tumor and plasma of breast cancer patients included in their study [51]. Another study by Goyal et al., have reported a methylation frequency as high as 71% for p16 in breast cancer [52].

A 40% hypermethylation frequency in the p16 promoter was observed in epithelial ovarian cancer in a study involving 249 patients [53]. Another study reported a 50% methylation of p16 in ovarian cancer [54]. The study by Bhagat et al., which included 134 invasive cancers of the ovary, reported a methylation of 43% [55].

The above mentioned data for the methylation frequency of RASSF1a and p16 in various cancer types are in concordance to the results presented in the current study. The data obtained in the current study suggests that RASSF1a and p16 genes undergo promoter hypermethylation and this may serve as a means to transcriptionally silence these genes in endometrial carcinoma. The hypermethylation data can be used for diagnosis and prognosis of the patients with endometrial carcinoma.

CONCLUSION

Aberrant promoter hypermethylation is an important epigenetic alteration which occurs in the early stages of the cancer initiation and prevails as the cancer progresses. Assessment of methylation frequency of tumor suppressor genes such as RASSF1a and p16 can serve as useful molecular markers for diagnosis and prognosis of endometrial carcinoma.

What this study adds to the existing knowledge: Assessment of aberrant promoter hypermethylation frequency of RASSF1a and p16 gene can serve as important molecular marker of cancer. This could also aid in developing a molecular based diagnosis and prognostic strategy.

Authors' Contributions

Nagaratna Shivanandappa: Study design, Experimental design, Experimentation, data collection, manuscript writing, statistical analysis and manuscript writing and 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:** Study design, experimental design manuscript writing, editing, statistical analysis.

Acknowledgement: None

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

Source of Funding: None

Ethical Approval: Approved

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How to cite this article: Shivanandappa N, Swamy SN, Sandeep Kumar S et.al. Assessment of aberrant promoter hypermethylation of RASSF1a and p16 in endometrial carcinoma. *International Journal of Research and Review*. 2021; 8(7): 402-409. DOI: *https://doi.org/10.52403/ijrr.20210758*

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