Original Article

Aberrant promoter methylation and gene expression of H-cadherin gene is associated with tumor progression and recurrence in epithelial ovarian carcinoma

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ABSTRACT

Background: Loss of expression of cadherins by promoter hypermethylation has been described in many epithelial cancers, and it may play a role in tumor cell invasion and metastasis. Previously, we reported that E-cadherin gene is frequently methylated in epithelial ovarian cancer. **Aim:** The aim of this study was to compare the promoter hypermethylation of H-cadherin gene in ovarian epithelial neoplasms to better understand the role of epigenetic silencing in carcinogenesis. **Materials and Methods:** We examined the promoter methylation of the H-cadherin gene in 134 epithelial ovarian carcinomas (EOC), 23 low malignant potential (LMP) tumors, 26 benign cystadenomas and 15 normal ovarian tissues. Methylation was investigated by methylation specific polymerase chain reaction (MSP) and the results confirmed by bisulfite DNA sequencing. Relative gene expression of H-cadherin was done using quantitative reverse transcriptase PCR on 51 EOC cases, 9 LMP tumors, 7 benign cystadenomas with 5 normal ovarian tissues. **Results:** Aberrant methylation of H-cadherin was present in 20 of 134 (15%) carcinoma cases, 2 of 23 (09%) LMP tumors and 1 of 26 (4%) benign cystadenomas. No methylation was observed in any of the normal ovarian tissues. The mRNA expression level of H-cadherin was significantly down-regulated in EOC and LMP tumors than the corresponding normal tissues, whereas the expression level was normal in benign cystadenomas. A significant correlation of H-cadherin promoter methylation was observed with reduced gene expression in EOC. The prevalence of H-cadherin methylation was associated significantly with stage, histopathological grade, and menopausal status of the patient. H-cadherin methylation also had significant association with recurrence and differentiation of tumor. **Conclusion:** Our findings suggest an association between H-cadherin methylation, tumor progression and recurrence in EOC.

Key words: Bisulfite sequencing, epithelial ovarian carcinomas, gene expression, H-cadherin, low malignant potential tumors, promoter methylation

INTRODUCTION

Ovarian cancer is a leading cause of death from gynecologic malignancies due to its aggressive nature, and the fact that the majority of patients are diagnosed in advanced stages of the disease. 5-year overall survival is strongly stage dependent and is higher in Stage I ovarian cancer, who have a 5-year survival rate of over 90%,[1] however, only 25% of women with advanced ovarian cancer survive 5-year after diagnosis. More than 85% of patients with advanced disease relapse after cessation of primary therapy, despite an initially good response.

Evolving data support a significant role for epigenetic processes in the development of cancer. Epigenetic changes can predict tumor behavior and often distinguish between genetically identical tumors and present a new and entirely different mechanism for gene regulation. Several interrelated epigenetic modifications that are altered in abnormal growth state are DNA methylation changes, histone modifications and genomic imprinting.

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Aberrant methylation of normally unmethylated CpG islands, located in the 5′ promoter region of gene, has been associated with transcriptional inactivation of several genes in human cancer, and presents an alternative to mutational inactivation.[2] It has been increasingly shown over the past 10-year that the CpG islands in the promoter regions of a large number of genes, which are mostly unmethylated in normal tissues, are methylated to varying degrees in human cancers.[3-5] In ovarian cancer, a growing number of genes have been recognized as undergoing aberrant methylation at CpG islands, suggesting this to be an important molecular mechanism in the development of ovarian carcinoma.[6]

The cadherins are a family of cell surface glycoproteins responsible for selective cell recognition and adhesion.[7] Several family members, including E-cadherin (CDH1) and H-cadherin (CDH13) are located on the long arm of chromosome 16 (16q),[8] where loss of heterozygosity has been reported in several human cancers.[9-11] It has been found that a loss of cadherin expression led to transition from benign tumor to invasive tumor, and subsequent metastatic dissemination of tumor cells by causing changes in cell and cell-matrix adhesion.[12,13]

The H-cadherin gene (CDH13), another member of the cadherin superfamily, was isolated and has been mapped to 16q24. H-cadherin is a nonclassical cadherin, which lacks the transmembrane and cytoplasmic domains and is bound to the plasma membrane via a glycosylphosphatidylinositol anchor.[14] Its protein structure indicates that it may play a role in intracellular signaling and cell-cell adhesion.[14,15] Recent studies have found that H-cadherin is frequently under expressed in human cancers such as breast, lung, ovary, bladder, colorectal cancers and hematological malignancies and is able to inhibit tumorigenicity.[16-21] On the other hand, accumulating evidence has indicated that H-cadherin has positive roles on endothelial and vascular cells during atherosclerosis, neointima formation in experimental restenosis and tumor neovascularization.[22-24] These suggest that H-cadherin possesses multiple functions that may be different in different cell types.

Our previous study demonstrated that loss of expression of E-cadherin correlates with the promoter hypermethylation of E-cadherin gene in epithelial ovarian carcinoma (EOC).[25] In this study, we investigated the aberrant methylation of H-cadherin promoter and gene expression of H-cadherin in EOC by, respectively, using methylation specific polymerase chain reaction (MSP) and quantitative reverse transcriptase PCR (qRT-PCR) to assess the clinicopathologic and prognostic significance of H-cadherin methylation.

MATERIALS AND METHODS

Sample collection and DNA extraction

Tissue samples were collected from a consecutive series of surgical excision specimens from 134 ovarian cancer patients, 23 patients with a low malignant potential (LMP) tumor and 26 patients with benign cystadenomas at Kidwai Memorial Institute of Oncology, Bangalore, India. The samples were snap frozen after surgical removal and stored at −80°C. All samples were verified by histology. Histological classification was established according to the WHO criteria, and tumor was staged following the International Federation of Gynecology and Obstetrics (FIGO) classification. 15 normal ovarian tissues were collected from patients without cancer undergoing bilateral salphingo oophorectomy at the time of surgery for benign gynecological disease. This study was approved by the Institutional Ethics Committee and all patients provided written, informed consent.

Postoperative follow-up was scheduled at 1 month, 2 months, and every 3 months during the first 2-year after surgery and every 6 months thereafter or more frequently if needed.

Genomic DNA was extracted from 25 mg of frozen tissue specimens using QIAamp DNA mini kit (Qiagen, CA, USA) following the manufacturer's instruction. The extracted DNA was examined by electrophoresis and the yield was measured spectrophotometrically using Eppendorf BioSpectrophotometer kinetis™ before use.

Bisulfite modification

600 ng of tissue genomic DNA was treated with bisulfite using the EZ DNA methylation kit™ (D 5001, Zymo Research, CA, USA) following the manufacturer's protocol. Bisulfite treatment of genomic DNA leads to the conversion of all unmethylated cytosine to uracil, while leaving methylated cytosine unaffected.

Methylation specific polymerase chain reaction

The methylation status of the H-cadherin gene was determined by MSP using a nested two-step approach to increase the sensitivity of detecting allelic hypermethylation at targeted sequences and to facilitate the examination of multiple gene loci.

The first step of MSP uses a primer set that recognizes the bisulfite modified template, but it does not discriminate between methylated and unmethylated alleles. The primers used to amplify flanking regions of H-cadherin were 5′-GTTTAAAGAAGTAAATGGGATGCCAC-3′ (sense) and 5′-CTACATTTTATCCYACTAGAAGC-3′ (antisense). In the first PCR step, we used ~ 100 ng of modified DNA in a 50 µl reaction mixture containing 5 µl of × 10 PCR

buffer (New England Biolabs Inc. MA, USA), 1.5 mmol/L of $MgCl_2$, 0.2 µmol/L of each primer, 0.2 mmol/L of dNTPs and 1 U of Taq polymerase (New England Biolabs Inc.). The PCR amplification was performed in a Veriti™ Thermal Cycler (Applied Biosystems) for 35 cycles, each of which consisted of an initial denaturation at 95°C for 10 min, denaturation at 95°C for 30 s, annealing for 30 s, extension at 72°C for 30 s followed by a final 7 min extension at 72°C. First step PCR products were diluted 20-fold and 2 µl were subjected to second step PCR in a 50 µl volume, using primers specific for the DNA that were either methylated or unmethylated at the promoter region of H-cadherin gene. The primers used for unmethylated H-cadherin were 5′-TTGTGGGGTTTGTTTTTTGT-3′ (sense) and 5′-ACATT TTCATTCATACACACA-3′ (antisense), and the primers for methylated H-cadherin were 5′-TCGCGGGGTTCGTTTTTCGC-3′ (sense) and 5′-GACGTTTTCATTCATACACGCG-3′ (antisense). The PCR amplification consisted of 35 cycles (at 95°C for 30 s, annealing at 57°C and 65°C for unmethylated and methylated alleles for 30 s, extension at 72 for 30 s) and a final extension at 72°C for 7 min. CpGenome Universal Methylated DNA (Zymo Research, CA, USA) was used as a positive control for methylated DNA. Lymphocyte DNA from healthy controls was used for unmethylated control and distilled water with no DNA was used as negative control.

PCR products were loaded onto 2% agarose gels and visualized by ethidium bromide staining [Figure 1]. Samples which were negative for both methylated and unmethylated PCR were excluded from the study.

Bisulfite direct sequencing

A total of six samples were taken for bisulfite sequencing which included positive control for both methylated and unmethylated DNA and remaining four samples were tumor DNA chosen randomly. The sequencing was done using BigDye chemistry (Applied Biosystems) according to manufacturer's instructions.

The PCR products were sequenced from both ends. The sequenced region included 15 CpG sites [Figure 2].

RNA isolation, cDNA synthesis and quantitative PCR

Total RNA was available for 51 of the EOC, 9 LMP tumors, 7 benign cystadenomas and 5 normal ovaries. Total RNA was isolated from 30 mg of tissue preserved in RNAlater™ using RNeasy mini kit (Qiagen, CA, USA) following the manufacture's instruction. Subsequent cDNA synthesis was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) following the company protocol. The gene expression levels of H-cadherin were then quantified using TaqMan technology on a StepOnePlus™ real-time PCR system (Applied Biosystem, CA, USA). Gene specific primers and probe of H-cadherin (assay ID Hs01004530_m1) were available as TaqMan gene expression assays (Applied Biosystems). The 18S ribosomal RNA (18S rRNA) (assay ID Hs99999901_s1) was amplified and was used as an endogenous control in the quantification.

The real-time PCR was performed in 20 µl reaction containing 10 µl TaqMan gene expression mastermix, 1 µl TaqMan gene expression assay, 2 µl cDNA and 7 µl of nuclease free water. The thermocycling conditions were 50°C for 2 min, 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. All qRT-PCR experiments included a no template control and were performed in triplicate. Serial dilutions of cDNA from normal ovarian tissues were amplified in parallel as a control of amplification efficiency within each experiment and for the establishment of a standard curve for relative quantification. Expression of H-cadherin mRNA was

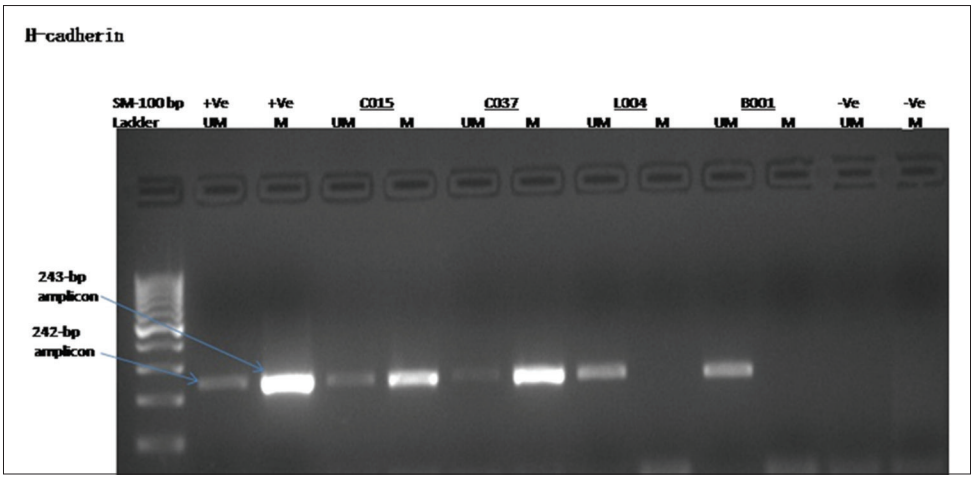

Figure 1: Representative MSP profile of H-cadherin gene promoter methylation in epithelial ovarian tumors. In each case, a universally methylated genomic DNA was used as a positive control and peripheral blood-derived DNA from normal healthy subjects as a negative control. PCR products in lane UM indicate the presence of an unmethylated allele, whereas PCR products in lane M indicate the presence of a methylated allele. C015, C037 are carcinomas, L004 is low malignant potential tumor, B001 is an benign adenoma, N001 is a noncancer tissue

normalized for 18S rRNA as an internal reference. Relative expression levels were calculated as H-cadherin/18S rRNA in tumor and normal tissues, respectively.

Statistical analysis

Chi-square and Fisher exact probability test was used to determine the significance of association between different variables. Mann-Whitney U-test was used to assess the association between methylation and gene expression. Disease - free interval and overall survival were assessed by Cox regression analysis. All statistical analyses were performed with SPSS 11.0 (SPSS Inc, USA) version statistical software. The level of statistical significance was $P < 0.05$.

RESULTS

H-cadherin promoter hypermethylation

The association between H-cadherin methylation in the 134 tumor samples and the clinicopathologic features of the patients are listed in Table 1. H-cadherin methylation was detected in 20 of 134 EOC samples (15%), 2 of 23 LMP tumors (9%) and 1 of 26 benign cystadenomas (4%). No methylation was observed in normal ovarian tissues. A significant association was found between H-cadherin methylation, clinical stage and histopathological grade of the disease. H-cadherin methylation was observed in 18 out of 93 patients with clinical Stages III and IV disease and 19 out of 91 in histological Grade 3 disease. 12 of the 20 patients with H-cadherin methylation (60%) had tumor recurrence within a period of 17.85 months.

Direct bisulfite sequencing

The methylation data of normal and tumor tissue DNA were concordant with bisulfite direct sequencing data.

H-cadherin mRNA expression

Reduced H-cadherin mRNA expression was seen in 26 out of 51 EOC cases with a mean expression level of 0.68 ± 0.20 whereas in LMP tumors the down-regulation was seen in 3 out of 9 cases with a mean expression level of 0.79 ± 0.40. In benign cystadenomas the down-regulation was not significant with only 1 out of 7 cases showing mild reduction in expression. The mean expression level in benign tumors was 1.51 ± 1.18 while the mean expression level in normal ovarian tissue was 1.0 ± 0.05 [Figure 3].

Table 1: Association of the methylation of H-cadherin gene in EOC tissues and clinicopathological parameters of tumors

Clinicopathological parameters	*N*	H-cadherin methylation proportion (%)
Ovarian tumors	134	20/134 (15)
Type of tumor		
Serous	76	12/76 (16)
Mucinous	17	03/17 (18)
Endometroid	08	01/08 (12.5)
Clear cell	08	00/08 (0)
Poorly differentiated	25	04/25 (16)
P value		0.918
FIGO stage		
1 and 2	41	02/41 (5)
3 and 4	93	18/93 (19)
P value		<0.05
Histopathological grade		
1	24	01/24 (04)
2	21	00/19 (00)
3	89	19/89 (21)
P value		<0.05*
Tumor recurrence		
Absent	86	08/86 (09)
Present	48	12/48 (25)
P value		χ^2=5.97; <0.05*
Menopausal status		
Premenopausal	49	02/49 (04)
Postmenopausal	85	18/85 (21)
P value		<0.05*
Ascitis		
Positive	81	09/54 (11)
Negative	53	11/53 (21)
P value		χ^2=0.29; 0.58
LMP tumors	23	02/23 (09)
Serous borderline	09	01/09 (11)
Mucinous borderline	14	01/14 (07)
Benign tumors	26	01/26 (04)
Serous cystadenoma	14	01/14 (07)
Mucinous cystadenoma	12	00/12 (0)
Normal control	15	00/15 (0)

Chi-square/Fisher exact test has been used to assess the significance. *P<0.05. FIGO: International Federation of Gynecology and Obstetrics, EOC: Epithelial ovarian carcinoma, LMP: Low malignant potential

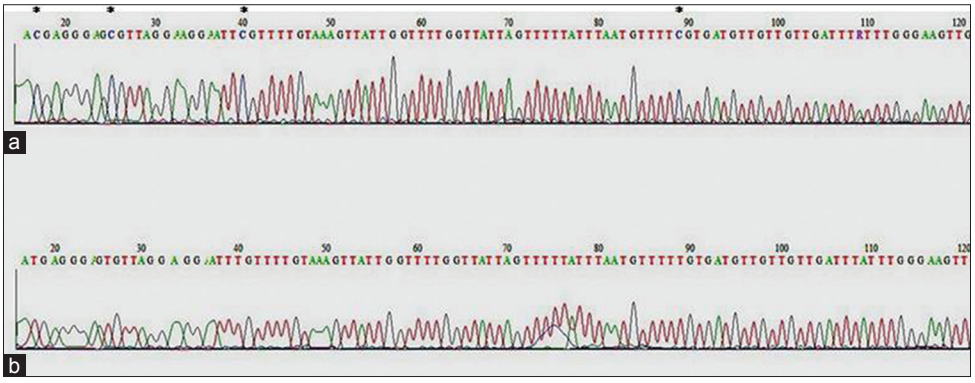

Figure 2: (a) Part of the promoter of H-cadherin gene, which is methylated heterozygously in epithelial ovarian carcinoma samples; (b) the same region of unmethylated promoter in the normal tissues. Methylated nucleotides are indicated with asterisk

Down-regulation of H-cadherin gene significantly correlated with promoter hypermethylation of H-cadherin gene ($Z = -3.394$; $P < 0.05$) [Table 2].

DNA methylation as prognostic factor

The prognostic value of promoter methylation of H-cadherin was analyzed with regard to recurrence and overall survival. FIGO stage and histopathological grading were significantly related to recurrence [Table 3] and overall survival [Table 4]. Univariate analysis showed a significant correlation between presence of methylation of H-cadherin and recurrence [Table 3].

For overall survival, the presence of promoter methylation of H-cadherin gene did not correlated with survival [Table 4].

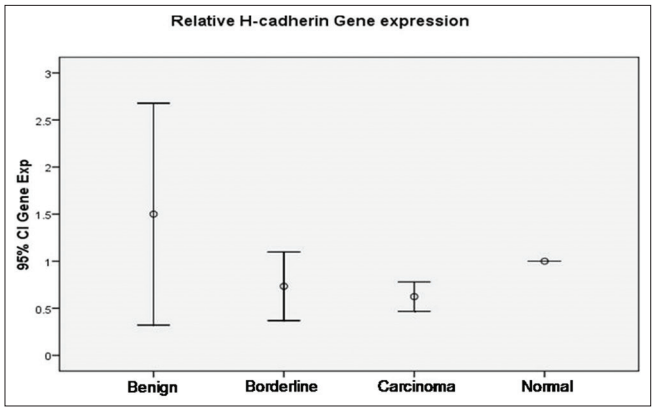

Figure 3: Interval plot demonstrating relative H-cadherin gene expression in epithelial ovarian carcinoma (EOC), low malignant potential (LMP) tumors and benign cystadenomas. 51 EOC, 9 LMP tumors and 7 Benign cystadenomas were compared with normal ovarian tissues. The endpoints of the interval represent the 95% confidence interval for the mean and the dot corresponds to the mean value

Table 2: Correlation of H-cadherin methylation with H-cadherin gene expression

Tumor samples	*N*	Mean H-cadherin expression	*Z* score	*P* value
Unmethylated	43	1.02±0.84	−3.394	<0.05
Methylated	08	0.106±0.104		
Total	51	0.68±0.20		

DISCUSSION

Several tumor suppressor genes (TSGs) contain CpG islands in their promoters, prompting many studies investigating the role of methylation in silencing these genes. Many TSGs show evidence of methylation silencing, providing a new potential pathway for the deactivation of TSGs. Aberrant methylation of H-cadherin promoter is one of the major mechanisms for the inactivation or down-regulation of H-cadherin expression in a number of tumor types, including breast cancer, lung cancer and colorectal cancer.[26-29]

Our study indicated that in epithelial ovarian cancer, aberrant methylation of H-cadherin promoter was present in 20 of 134 cases. Out of 20 EOC cases with H-cadherin promoter methylation, 18 EOC cases with promoter methylation were seen in patients with advanced disease (17 cases with Stage III and 1 case with Stage IV cancer). LMP tumors and benign cystadenomas cases presented with low percentage of H-cadherin promoter methylation suggesting that H-cadherin promoter hypermethylation is associated with tumor progression, whereas normal ovarian tissues did not reveal any hypermethylation. Aberrant methylation of H-cadherin promoter region was significantly correlated with down-regulation of H-cadherin mRNA. H-cadherin is a truncated cadherin that plays an important role not only in cell-cell adhesion but also in maintaining the normal cellular phenotype.[14] Recent studies revealed the re-expression of H-cadherin in breast cancer cells and glioma cells has inhibited the invasive potential and cell growth of tumor cells *in vitro*.[30,31] Previous studies have suggested that methylation profiles of cancers are tumor type - and ethnicity specific.[32,33]

Ozdemir *et al*., have reported a methylation frequency of 16% for H-cadherin gene promoter in a cohort of 75 ovarian cancer patients.[34]

There are numerous studies addressing the hypermethylation status of H-cadherin in breast and lung cancer;[35] however, there is a dearth for data concerning the same in ovarian cancer reporting a methylation frequency of 13-67%.[36-40]

Table 3: Univariate and multivariate analysis of recurrence

Variable	No. of patients with recurrence/total no.	Univariate analysis crude RR (95% CI)	Multivariate analysis crude RR (95% CI)
Stage			
1	03/29	1.0 (reference)	1.0 (reference)
2	01/12	1.069 (0.178-6.416)	1.226 (0.184-8.148)
3	44/89	4.69 (1.453-15.133)	4.631 (1.264-16.967)
4	03/04	6.014 (0.996-36.325)	5.494 (0.788-38.318)
Grade			
1	05/24	1.0 (reference)	1.0 (reference)
2	06/21	1.108 (0.357-3.445)	1.468 (0.406-5.306)
3	40/89	2.045 (0.862-4.851)	1.411 (0.568-3.509)
H-cadherin			
Unmethylated	39/114	1.0 (reference)	1.0 (reference)
Methylated	12/20	1.811 (0.928-3.535)	1.280 (0.585-2.799)

CI: Confidence interval, RR: Relative risks

Table 4: Univariate and multivariate analysis of overall survival

Variable	No. of patients who died/total no.	Univariate analysis crude RR (95% CI)	Multivariate analysis crude RR (95% CI)
Stage			
1	02/29	1.0 (reference)	1.0 (reference)
2	00/12	0.00 (0.00)	0.00 (0.00)
3	20/89	3.137 (0.730-13.488)	3.215 (0.574-18.022)
4	01/04	4.53 (0.362-45.336)	6.573 (0.468-92.231)
Grade			
1	01/24	1.0 (reference)	1.0 (reference)
2	04/21	4.686 (0.523-42.002)	4.508 (0.405-50.172)
3	18/89	5.720 (0.760-43.080)	3.673 (0.469-28.786)
H-cadherin			
Unmethylated	21/114	1.0 (reference)	1.0 (reference)
Methylated	02/20	0.629 (0.147-2.685)	0.551 (0.114-2.661)

CI: Confidence interval, RR: Relative risks

However, the methylation frequency in our study was not high as the other studies. This difference may reflect population differences, since epigenetic alterations may be different in different ethnic groups.

Although there is a clear distinction between metastasis-promoting and growth-transforming genes, there is increasing evidence that some genes, such as integrins, mediate signals that affect both processes. Several groups have suggested the involvement of H-cadherin in the regulation of tumor growth and progression. It has been reported that the introduction and over expression of H-cadherin in human breast carcinoma cells (MDA-MB-435) markedly inhibit tumor growth and invasiveness.[16,17] Zhong *et al*. also reported that the loss of H-cadherin expression is associated with tumorigenicity in nude mice transplanted with nonsmall cell lung cancer tumors and that it is more prevalent in larger local tumors.[41] The mechanism by which H-cadherin functions as cell growth regulator is not clear; however, Huang *et al*., recently reported that H-cadherin regulates cell growth by inducing p21CIP1/WAF1 expression and G2-phase arrest. H-cadherin over expression results in the suppression of C6 glioma cell growth by inducing G2-phase arrest, and the growth arrest mediated by H-cadherin is associated with p21CIP1/WAF1 expression, but not with p27Kip1 expression.[30] Zhong *et al*., also reported that H-cadherin is involved in contact inhibition by inducing p21CIP1/WAF1 expression in Chinese hamster ovarian cells.[42] These observations suggest that H-cadherin may be involved in two processes, that is, tumor growth and progression.

Our study showed that all patients with hypermethylation of H-cadherin gene promoter had advanced disease and 60% of the patients with H-cadherin methylation presented with recurrence of the disease. Given these observations our results strongly suggest that promoter methylation of H-cadherin plays a role in the molecular pathogenesis of epithelial ovarian cancer and are associated with disease progression. The results of our study suggest a role of H-cadherin in cell proliferation and tumor growth along with cancer cell metastasis.

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