Assessment of DNA Methylation in p15, p16 and E-Cadherin Genes as a Screening Tool for Early Carcinoma Cervix

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Abstract Cancer cervix is diagnosed late in women due to anatomical inaccessibility of the area. Hence, a robust screening strategy will help detect carcinoma cervix early which will significantly decrease the mortality and morbidity due to this disease. We evaluated DNA methylation of three tumour suppressor genes p15, p16 and E-Cadherin on cervical smears to assess DNA methylation as a screening tool for detection of early cervical cancer in comparison to PAP smears. DNA was extracted from cervical smears of 20 cases and 30 controls. The DNA was bisulphite modified. Methylation specific PCR (MSP) was performed to assess the methylation status of the promoter region of each of the genes. MSP results were compared with PAP smears to assess the utility of DNA methylation of these genes in screening for cervical cancer. DNA methylation was detected in 55% subjects in p15 gene, 45% in p16 gene and 40% in E-Cadherin gene. This was statistically significant when compared to the controls. DNA methylation of E-Cadherin, and p15 genes as a panel has a sensitivity and specificity of 80% and 90% respectively, which is better than the sensitivity of PAP smear for detection of early cancer cervix. Increased DNA methylation is seen in p15, p16 and E-Cadherin genes in early cancer cervix. p15 and E-Cadherin in combination can be used as a screening tool for detection of early cancer cervix.

Keywords Methylation specific PCR · Methylation · Tumour suppressor · Dysplasia · PAP smear

Introduction

Cancer cervix is diagnosed mostly in advanced stages when the morbidity and mortality is high. This is due to the inaccessibility of the cervix which precludes regular visual examination. A robust screening test will help in detection of cancer cervix early which will lead to decrease in the mortality and morbidity.

DNA methylation is found as a covalent modification at the fifth carbon position of cytosine residues within CpG dinucleotides (regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide) [1]. The discussions for the role of DNA methylation in tumorigenesis are focused on aberrant DNA methylation events causing repression of tumor-suppressor genes [2]. The paradigm for epigenetic inactivation of a tumor suppressor gene is methylation of p16 gene. It leads to abrogation of cell cycle control, escape from senescence and induction of proliferation [3].

From the diagnostic point of view, because epigenetic abnormalities occur very early in the carcinogenic process they can potentially be exploited as molecular markers for early detection. In this sense, identification of a set of genes hypermethylated in cytologic smears could offer novel means for screening. Assessment of hypermethylated genes in primary tumor or in serum circulating DNA may serve as a prognostic factor or as a means of predicting response to radiation, chemotherapy and transcriptional agents.

Epithelial Cadherin (E-Cadherin) is a 120 kDa trans-membrane glycoprotein that mediates calcium-dependent intercellular adhesion. Mutations in this gene are correlated

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with cervical, gastric, breast, colorectal, thyroid and ovarian cancer. Loss of function is thought to contribute to progression in cancer by increasing proliferation, invasion and/or metastasis.

p15 belongs to a family of cyclin dependent kinase inhibitors that negatively regulate the cell cycle through competitive inhibition of the cyclin dependent kinase 4 and 6 involved in RB (retinoblastoma)—dependent cell cycle regulation.

p16 gene is an important tumor-suppressor gene, involved in the p16/cyclin-dependent kinase/retinoblastoma gene pathway of cell cycle control in which the p16 protein is considered to be a negative regulator involved in the inhibition of G1 phase progression [4]. Hypermethylation of the p16 gene promoter region has been reported in cervix, colon, bladder, breast, head and neck, lung carcinoma as well as in glioma, melanoma, leukemia and lymphoma [5, 6].

Materials and Methods

The present study was conducted in the Departments of Biochemistry and Gynaecology, of an urban tertiary care teaching hospital. The Institutional Ethics committee for human studies had approved the study. Informed consent was obtained from all patients participating in the study. Female patients above 35 years of age attending Gynaecology OPD for symptoms ranging from discharge per vaginum to post menopausal bleed were screened by cervical smears. 20 cases and 30 controls were selected based on the PAP smear findings.

The definition of case was given as any female with PAP smear report of low grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL) or Carcinoma. Control was defined as females reported as Negative for intra epithelial lesion or malignancy (NIELM) or any other infection/inflammation.

All minors, post hysterectomy patients, pregnant women, critically ill patients, patients on radiotherapy and patients below 35 years of age were excluded. A PAP smear and an endocervical scraping with a cytobrush was collected for every patient and control. The cytobrush sample was collected in 1.5 ml of saline. DNA was extracted from the centrifuged deposit of the sample using the body fluid protocol of the QIamp DNA mini kit from Qiagen. The extracted DNA was quantified and bisulphite modification was performed using the MECOV 50 kit from Invitrogen. The optimal concentration of DNA was kept between 200 and 500 ng/μl for bisulphite modification. Hot start Methylation specific PCR was performed in a 25 μl reaction which comprised of water 19 μl, Primers U/M 1 μl, Axygen Qarta buffer mix 2.5 μl and DNA template

2.5 μl. Universal methylated and unmethylated DNA controls from Zymogen were bisulphite modified and run with each batch. Universal methylated and unmethylated commercially bisulphite modified DNA controls from Qiagen were also run with each batch. Thermal cycler settings were as follows: Initial denaturation for Hot start Taq activation was 95 °C for 14 min, Denaturation at 94 °C for 60 s, Annealing at 60 °C for 60 s, extension at 72 °C for 60 s for 40 cycles and final extension at 72 °C for 10 min. The annealing temperature for p15 gene was 61 °C.

2% agarose gel electrophoresis with Ethidium bromide was performed for detection of PCR products. Statistical evaluation was done with SPSS17.0.

Results

A total of 50 female patients with mean age of 44.68 years (35–72 years) were included in the study. The size of the amplified products were as depicted in Table 1. The cases comprised of 13 LSIL and 07 HSIL reported on PAP. One case diagnosed as ‘Inflammatory atypia but LSIL cannot be excluded’ was also taken up as a case in the study.

Biopsy reports of nine out of twenty cases were available. Out of the seven HSIL cases biopsy reports of five cases were available. Of these five, three were large cell keratinizing squamous cell carcinoma (LCKSCC), one was moderately differentiated adenocarcinoma and one was Cervical Intraepithelial Neoplasia II (CINII). Out of the thirteen LSIL cases, biopsy reports of four were available. Of these four, three were large cell non keratinizing squamous cell carcinoma (LCNKSCC) and one was LCKSCC.

Samples showing amplification with only ‘M’ primer were taken as ‘methylated’. Those showing amplification with both ‘M’ and ‘U’ primers were also taken as methylated. Samples showing amplification with only ‘U’ primer were taken as ‘unmethylated’. Table 2 depicts the results obtained in all 50 samples.

When analyzing MSP of p15 gene, 55% (11/20) cases had shown amplification with M primers. Out of the 11 cases which had shown amplification with M primer, 4

Table 1 Size of PCR products of E-cadherin, p15 and p16 genes

Gene	E-Cadherin		p15		p16	
	M	U	M	U	M	U
Primers						
Amplicon size in bp	212	206	205	203	193	195

M methylated primer, *U* unmethylated primer

Table 2 Results of MSP in samples and controls

S. no.	PAP report	HPE	Age	p15		p16		E-Cadherin	
				U	M	U	M	U	M
1	LSIL	LSNKCC	70	–	M	U	–	U	–
2	HSIL	CINII	66	U	–	U	–	U	–
3	HSIL	Mod Diff Adeno Ca	55	–	M	–	M	U	–
4	LSIL	–	48	U	M	U	–	U	M
5	LSIL	LCNKSCC	52	U	–	U	M	U	–
6	HSIL	LCKSCC	55	U	M	U	–	–	M
7	HSIL	LCKSCC	72	U	–	U	–	U	–
8	HSIL	LCKSCC	65	U	M	U	–	–	M
9	LSIL	LCKSCC	55	U	M	U	M	–	M
10	HSIL	–	43	U	–	U	M	–	M
11	LSIL	–	40	U	M	U	–	U	–
12	LSIL	–	40	U	M	U	–	U	–
13	LSIL	LCNKSCC	36	U	–	U	–	U	–
14	LSIL	–	40	U	–	U	–	U	–
15	LSIL	–	42	U	M	U	–	U	M
16	Inflammatory atypia. However, LSIL cannot be ruled out	–	38	U	–	U	M	U	–
17	LSIL	–	45	U	–	U	M	U	–
18	HSIL	–	46	U	M	–	M	U	M
19	LSIL	–	35	U	M	U	M	U	M
20	LSIL	–	38	U	–	–	M	U	–
21	NIELM		48	U	–		M	U	–
22	NIELM		42	U	–	U	–	U	–
23	NIELM		45	U	–	U	–	U	–
24	NIELM		45	U	–	U	M	U	–
25	NIELM		55	U	–	U	–	U	–
26	NIELM		38	U	–	U	–	U	–
27	Inflammatory smear		50	U	–	U	–	U	–
28	NIELM		36	U	–	U	–	U	–
29	NIELM		45	U	–	U	–	U	–
30	NIELM		38	U	–	U	–	U	–
31	NIELM		37	U	–	U	–	U	–
32	NIELM		42	U	–	U	–	U	–
33	NIELM		43	U	–	U	–	U	–
34	Inflammatory smear		39	U	–	U	–	U	–
35	NIELM		55	U	M	U	–	U	–
36	NIELM		49	U	–	U	–	U	M
37	NIELM		38	U	–	U	–	U	–
38	NIELM		49	U	–	U	–	U	–
39	Inflammatory smear		40	U	–	U	–	U	–
40	NIELM		36	U	–	U	–	U	–
41	NIELM		37	U	–	U	–	U	–
42	Inflammatory smear		48	U	–	U	–	U	–
43	Inflammatory smear		35	U	–	U	–	U	–
44	Inflammatory smear		45	U	–	U	M	U	–
45	Inflammatory smear		35	U	–	U	M	U	–
46	Normal PAP smear		40	U	–	U	–	U	–
47	Inflammatory smear		35	U	–	U	–	U	–

Table 2 continued

S. no.	PAP report	HPE	Age	p15		p16		E-Cadherin	
				U	M	U	M	U	M
48	Inflammatory smear		37	U	M	U	–	U	–
49	Inflammatory smear with trichomoniasis		35	U	–	U	–	U	–
50	Normal PAP smear		36	U	–	U	–	U	–

were HSIL and 7 LSIL cases. 90% (18/20) of the cases also showed amplification with U primer. Two of the controls (2/30) 6.66% also showed amplification with M primer. The difference in methylation in the cases and controls was highly significant with a p value 0.0001 at 0.05 level of significance. The overall agreement between PAP smear results and MSP PCR was 78% (11 + 28)/50. 11 being no of cases showing amplification with M primer and 28 being no of controls showing amplification with U primers. Sensitivity and specificity to pick up cervical cancer using DNA Methylation in p15 gene is 55% and 93% respectively. Positive and Negative predictive value for picking up cervical cancer using DNA Methylation in p15 gene is 85% and 76% respectively.

On analyzing the results of p16 gene, 45% (09/20) cases had shown amplification with M primers. Out of these nine cases, three were HSIL and six were LSIL. 13.3% (4/30) controls had shown amplification with M primer. The difference in methylation in the cases and controls was highly significant with a p value 0.01 at 0.05 level of significance. Out of the total of 09 cases which had shown amplification with M primer, 06 cases also showed amplification with U primer. The overall agreement between PAP smear results and MSP PCR was 70%, (9 + 26)/50, 9 being no of cases showing amplification with M primer and 26 being no of controls showing amplification with U primers. Sensitivity and specificity to pick up cervical cancer using DNA Methylation in p16 gene is 45% and 87% respectively. Positive predictive value for picking up cervical cancer using DNA Methylation in p16 gene is 69.2% whereas Negative Predictive Value is 70%.

On analysis of E-Cadherin gene, 40% (08/20) cases had shown amplification with M primers. One of the controls (3.3%) also showed amplification with M primer. The difference in methylation in the cases and controls was highly significant with a p value 0.003 at 0.05 level of significance. Out of the total of 08 cases which had shown amplification with M primer, 04 cases also showed amplification with U primer.

The overall agreement between PAP smear results and MSP PCR was 74% (29 + 8)/50. 29 being no of controls showing amplification with U primer and 8 being no of cases showing amplification of M primer. Sensitivity and

specificity to pick up cervical cancer using DNA Methylation in E-Cadherin is 40% and 97% respectively. Positive and Negative predictive value for picking up cervical cancer using DNA Methylation in E-Cadherin gene is 89% and 71% respectively (Table 3).

We decided to undertake a panel approach to Methylation of the three genes in different combinations. This was done to increase the sensitivity and specificity of the genes to detect cervical cancer. Table 4 shows the methylation pattern of the three genes analysed in different combinations. When panel of or p15 genes were used to pick up the cases, 60% (12/20) cases had shown amplification with M primers of either of these genes. Sensitivity and specificity to pick up cervical cancer using DNA Methylation in p15 or E-cadherin gene is 80% and 91% respectively. Positive and Negative predictive value for picking up cervical cancer using DNA Methylation in p15 or E-Cadherin gene is 80% and 70% respectively.

Considering a panel of p15 or p16 genes used to pick up the cases, 80% (16/20) cases had shown amplification with M primers of either of the genes.

Sensitivity and specificity to pick up cervical cancer using DNA Methylation in p16 or p15 gene is 50% and 86% respectively. Positive and Negative predictive values for picking up cervical cancer using DNA Methylation in p16 or p15 gene is 76% and 65% respectively.

When we considered p16 or E-Cadherin as a panel to pick up early cervical cancer, 65% (13/20) cases had shown amplification with M primers of either of these genes. Sensitivity to pick up cervical cancer using DNA Methylation in p16 or E-Cadherin gene is 45% whereas Specificity is 86%. Positive and Negative predictive value of p16 or E-cadherin gene panel is 72% and 65% respectively.

When considering a panel of either of the three genes, 80% (16/20) cases had shown amplification with M primers of either of these genes. Sensitivity and specificity of a panel of p16, p15 or E-Cadherin genes is 48% and 81% respectively. Positive and Negative predictive value of this panel is 70% and 64% respectively.

MSP results were correlated with respect to grades of dysplasia in p15 gene, 54% of LSIL and 57% of HSIL patients showed positivity ($p = 0.74$). In p16, 46% of LSIL and 43% of HSIL ($p = 0.74$) showed positivity and in

Table 3 Correlation of PAP report and methylation specific PCR

Pap results	p15 M	p15 U	p16 M	p16 U	E-Cadherin M	E-Cadherin U
Cases (HSIL) (n = 7)	4	6	3	5	4	4
Cases (LSIL) (n = 13)	7	12	6	12	4	12
Controls/NIELM (30)	2	30	4	29	1	30

Table 4 Diagnostic utility of panel of markers

Gene	Sensitivity	Specificity
p15	55	93
p16	45	87
E-Cadherin	40	97
p15 or p16	50	86
p16 or E-Cadherin	45	86
p15 or E-Cadherin	80	91
p15 or p16 or E-Cadherin	48	81

E-Cadherin 31% of LSIL and 57% of HSIL ($p = 0.5$) showed positivity.

Discussion

This study identified 40% (8/20) methylation in E-Cadherin gene and 45% (9/20) in p16 gene and 55% (11/20) in p15 gene in the cervical dysplasia/carcinoma in situ/carcinoma cases. Attaleb et al. used MSP to evaluate the promoter methylation status of p16 and E-cadherin genes in 22 specimens of cervical carcinomas, four cervical cancer cell lines (HeLa, SiHa, Caski, C33A) and 20 HPV negative specimens, obtained from normal cervical swabs. Hypermethylation of the 5' CpG island of the p16 and E-cadherin genes were found in 13 (59.1%) and 10 (45.5%) of 22 cervical cancer samples, respectively which is similar to our study [7]. Ren et al. used Methylation-specific PCR (MSP) to examine CpG island methylation of the FHIT and E-cadherin genes in 151 pre-treatment plasma samples and 30 tumour tissue samples from cervical cancer patients. CpG island methylation of the FHIT and E-cadherin genes were found in 30.46% and 39.74% of plasma samples, respectively and in 53.33% and 60.0% of tissue samples, respectively in cervical cancers [8]. The MSP positivity was 60% for E-Cadherin from tissue samples whereas in our study it was 40% from cervical smears. This can be explained as in cervical smears the sample is contaminated with blood, mucus etc. which is responsible for a lower rate of positivity in our study. Nayera et al. detected aberrant p15 promoter methylation in 49% (29/59) of the patients. In 4 of these patients, no DNA could be amplified by the p15 unmethylated reaction showing a complete

methylation of both alleles in the examined region. In the remaining 25 cases both methylated and unmethylated DNA could be amplified [9]. In our study, out of the 11 cases which showed positivity with p15 gene, two cases did not show amplification with U primers thus signifying that both the alleles were methylated in these two cases.

In p15 gene, 54% of LSIL and 57% of HSIL patients showed positivity ($p = 0.74$). In p16, 46% of LSIL and 43% of HSIL ($p = 0.74$) showed positivity and in E-Cadherin 31% of LSIL and 57% of HSIL ($p = 0.5$) showed positivity. Hence, in E-Cadherin and p15 genes the trend of increased methylation with severity of lesion is seen although it is not statistically significant. Jo-Heon Kim et al. observed that there was a trend toward increasing methylation of HIN-1, MGMT, RAR- β and SHP-1 with increasing severity of cervical squamous lesions. They also studied E-Cadherin and p16 gene methylation with severity of lesion. However, they did not report increasing methylation with higher grades of severity of lesions in these two genes [10]. In our study, we observed a trend of increased methylation with higher grade of severity in p15 and E-Cadherin genes although statistically not significant due to our small sample size. This could be attributed to a higher rate of infection with HPV 16 and 18, high risk HPVs, in our population as compared to the other study. Wen et al. evaluated the frequency of promoter hypermethylation in p16 gene and demonstrated an increasing trend from normal to dysplastic to invasive squamous cancer specimens and had a statistical significance ($p < 0.0001$) [11]. Xu et al. investigated the change of aberrant methylation of p16, CDH1 and TIMP3 in cervical carcinoma using methylation-specific PCR (MSP). They examined the aberrant promoter hypermethylation patterns in 140 samples of cervical intraepithelial neoplasia (CINI, $n = 40$), CINII-III ($n = 40$), cervical carcinomas (CC, $n = 40$), and normal cervical tissue as a control group ($n = 20$). Methylation for at least one gene was a frequent event. These figures in carcinoma 90% (36/40) were significantly different from CIN II-III 55% (22/40; $p < 0.05$) [12]. In our study, 80% (16/20) of the cases showed methylation of any one of the three genes. 77% of LSIL and 71.4% of HSIL showed positivity with any of the three genes. 67% of histopathologically proven cases showed positivity with any of these three genes.

In our study, there were amplifications by ‘U’ primers along with ‘M’ primers in the methylated cases. Methylation patterns are heterogeneous by nature and therefore cannot be assumed to be either completely methylated or unmethylated, at any one particular site. If all the Cs are not methylated then it will result in both M and U bands.

Tien Tien et al. had shown that in addition to the amplified bands shown by PCR using p15 M primers, samples from 9 of the 12 patients with p15 methylation could also be amplified with p15 U primers specific for unmethylated DNA. The authors suggested that this might be as a result of sample contamination by normal cells or the presence of unmethylated alleles [13]. Cervical smear samples are always contaminated with blood and mucus which has resulted in amplification with both U and M primers to a certain extent.

The sensitivity and specificity of PAP smear for the detection of CIN is 51% and 99% respectively whereas the best combination in our panel approach is that of a combination of p15 and E-Cadherin which has a sensitivity of 80% and a specificity of 90%. By using this combination screening for early carcinoma cervix will yield much better results.

In this age of personalized medicine, detection of DNA methylation should be of immense help as patients showing evidence of hypermethylation in the tumour suppressor genes could be exhibited demethylating agents since the process of methylation is reversible. Demethylating agents like Azacytidine as well as Decitabine have become the standard of care in Myelodysplastic syndromes and AML [14]. The antihypertensive drugs procainamide and hydralazine have been found to reverse the process of methylation of RAR β and p16 genes in cell culture lines as well as mice [15]. The day is not far when patients of cervical cancer will also be exhibited demethylating agents.

The study should be done on more number of patients to get a better picture of assessment of DNA methylation as a screening tool for cervical cancer. Another interesting fact would have come to light if the controls which showed positivity could be followed up over years to see if their PAP smears eventually showed changes. However, since they had a normal PAP smear report they were lost to follow up.

In conclusion, DNA methylation in the promoter regions of tumour suppressor genes does lead to cervical cancer as has been proven by the increased levels of DNA methylation in cases as compared to controls in our study. DNA methylation occurs early as has been seen by 77% cases of LSIL. It also has a correlation with severity of lesion as evidenced in E-Cadherin and p15 genes although not found to be statistically significant due to the small sample size. The combination of DNA methylation

in E-Cadherin and p15 gene to detect cancer cervix has a sensitivity of 80% and specificity of 90% which is better than the sensitivity and specificity of PAP smear alone. This fact needs to be validated on a large cohort. DNA methylation is a reversible process hence if demethylating agents are used these tumour suppressor genes could become upregulated. Large multi centric studies on a much larger panel of tumour suppressor genes are required to be done with high throughput systems like real time PCR and pyrosequencing on cervical smears to establish MSP as a screening tool for detection of early cervical carcinoma.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed Consent The research was performed on human cervical smear samples after taking due consent from each patient and control after informing them about the study.

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