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RESEARCH ARTICLE

GLOBAL DNA METHYLATION IN HUMAN PAPILLOMAVIRUS INFECTIED WOMEN OF REPRODUCTIVE AGE GROUP; AN EASTERN INDIAN STUDY

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Abstract

Purpose: DNA methylation is a novel biomarker for Human Papilloma Virus (HPV) infection amongst women to identify those progressing to pre-cancer. This study was done to see the HPV genotype distribution among the different population and find out the association between Global DNA Methylation and HPV infection.

Method:300 patients participated in the study. Cervical swab was collected and analysed by Real Time Polymerase Chain reaction (RT-PCR) for HPV Genotyping study. DNA was extracted from venous blood to estimate Global DNA methylation levels.

Results: Global DNA methylation levels were significantly lower in women with HPV infection than women without the infection. Global DNA hypomethylation was more predominant in unhealthy cervix group and cancer cervix and it was more prevalent in patients infected with HPV 16, HPV 18 and other high-risk strain.

Conclusion: In our study we found that other high-risk genotype of HPV had the highest rate of hypomethylation followed by HPV 18 and HPV 16 in cancer Cervix. Hence from a sample of whole blood of women infected with HPV, the Global DNA hypomethylation can be assessed and used as a predictive marker for progression to Cervical Cancer.

Introduction:-

Human Papilloma Viruses (HPVs) are icosahedral, circular, double stranded small DNA viruses without anenvelope. They are approx. 55 nm in diameter [1,2] and have a genome of approx. 8000 base pairs [3]. Over 100 HPV genotypes are found causing benign lesions or warts and infect cutaneous and mucosal epithelium. [4,5] Mostly it affects the basal cells of stratified squamous epithelium. It preferentially targets the metaplastic cells at the squamocolumnar junction of the cervix as well as the glandular epithelium of the endocervix, thus causing glandular neoplasms like adenocarcinoma in situ or invasive adenocarcinoma.[6]. HPV infection has a crucial role in mostly cervical cancer. Of the various genotypes of HPV identified, the anogenital region is infected by mostly 40 diverse types. Based on their oncogenic potentialthey are included in the following classes:[7]**High-risk group** which are - HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82. **Intermediate risk group** [7]-HPV 26, 53 and 66 types are included in this group.**Low-risk group** includes – HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81. [7]The

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genome has :Seven regulatory viral proteins acting in the infected epithelial cell (E1, E2, E4, E5, E6, E7, E8) encoded mostly by the Early (E) region. It also has a Late (L) region encodes two viral structural proteins L1 and L2, which form the viral capsid, and a long control region (LCR) is also called[Upstream regulatory region(URR)] [8]. This LCR domain contain the viral cis-acting regulatory sequences. The replication, transcription, and post-transcriptional modifications by the virus is done via LRE (late regulatory element). An icosahedral shell encapsulates the genome, which has 360 copies of the L1 protein with 72 pentameric capsomeres having one copy of L2 at the Centre.[9]. HPV enters epithelium by micro-abrasions. The High-Risk group (HR-HPV) infect the single layered squamous cellular junction between the endo- and ectocervix. [10,11]. The human papilloma virus first infects the basal epithelial stem cells but release of viral antigenic particles occurs only in the superficial surface squamous epithelium cells. This reduces the viral antigen exposure to immune system triggering a robust immune response.

Viral gene expression in the infected squamous epithelial host cells is mediated by DNA methylation. DNA methylation suppress the genes involved in cell cycle arrest and terminal differentiation in epithelial stem-cells.[12] DNA methyltransferases (DNMTs) transfers covalently a methyl group to the C-5 position of the cytosine ring of DNA. Methylation of distinct CpG dinucleotides in the E2-binding sites of the papillomavirus upstream regulatory region (URR) causes the HPV-mediated transformation of squamous epithelial cells.[12] DNA methylation is regulated by a family of DNMTs. Amongst them DNMT3A and DNMT3B have affinity for unmethylated CpG dinucleotides and can cause de novo methylation during development.[13,14] This pattern of DNA methylation during embryonic development, cellular differentiation and disease states are highly dynamic. Evidence have shown strong anti-correlation of DNA methylation with chromatin accessibility and Transcription Factor (TF) binding regions. Sites having no or low DNA methylation levels exhibit properties of good TF binding. Still anti-correlation is not indicative of which DNA methylation or TF binding comes first.[15] This promoter specific hypermethylation and global (genome-wide) hypomethylation, shows a different methylation pattern and might contribute to neoplasia and tumour growth.[16] Epigenetic hallmarks of cancer include global DNA hypomethylation and site-specific hypermethylation of CpG islands (CGIs). Tumour samples display global reductions of DNA methylation. Hypomethylation arises mainly from loss of methylation at normally heavily methylated sites, including satellite DNA and retrotransposons. This hypomethylation leads to genomic instability and oncogene activation causing cancer [16,17]

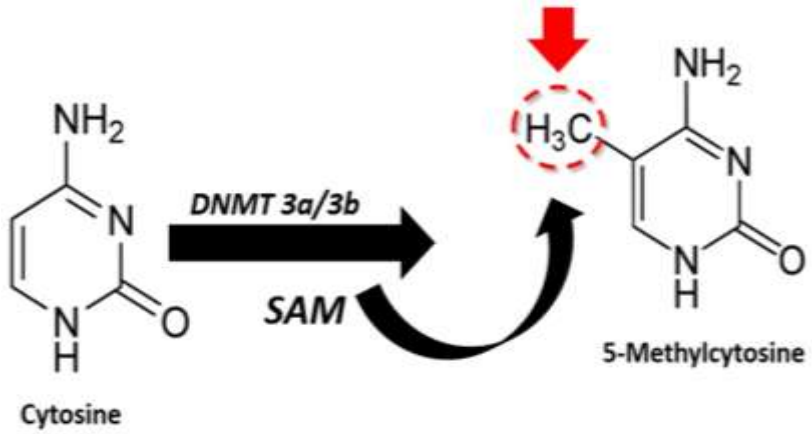


Fig. 1:- Action of DNA methyl transferases and S-Adenosyl Methionine(SAM) as methyl donor.

Materials And Methods:-

Sample Size Calculation

Sample size was calculated based on the 5year prevalence rate of cancer cervix in India which was 42 per 100,000. [18]

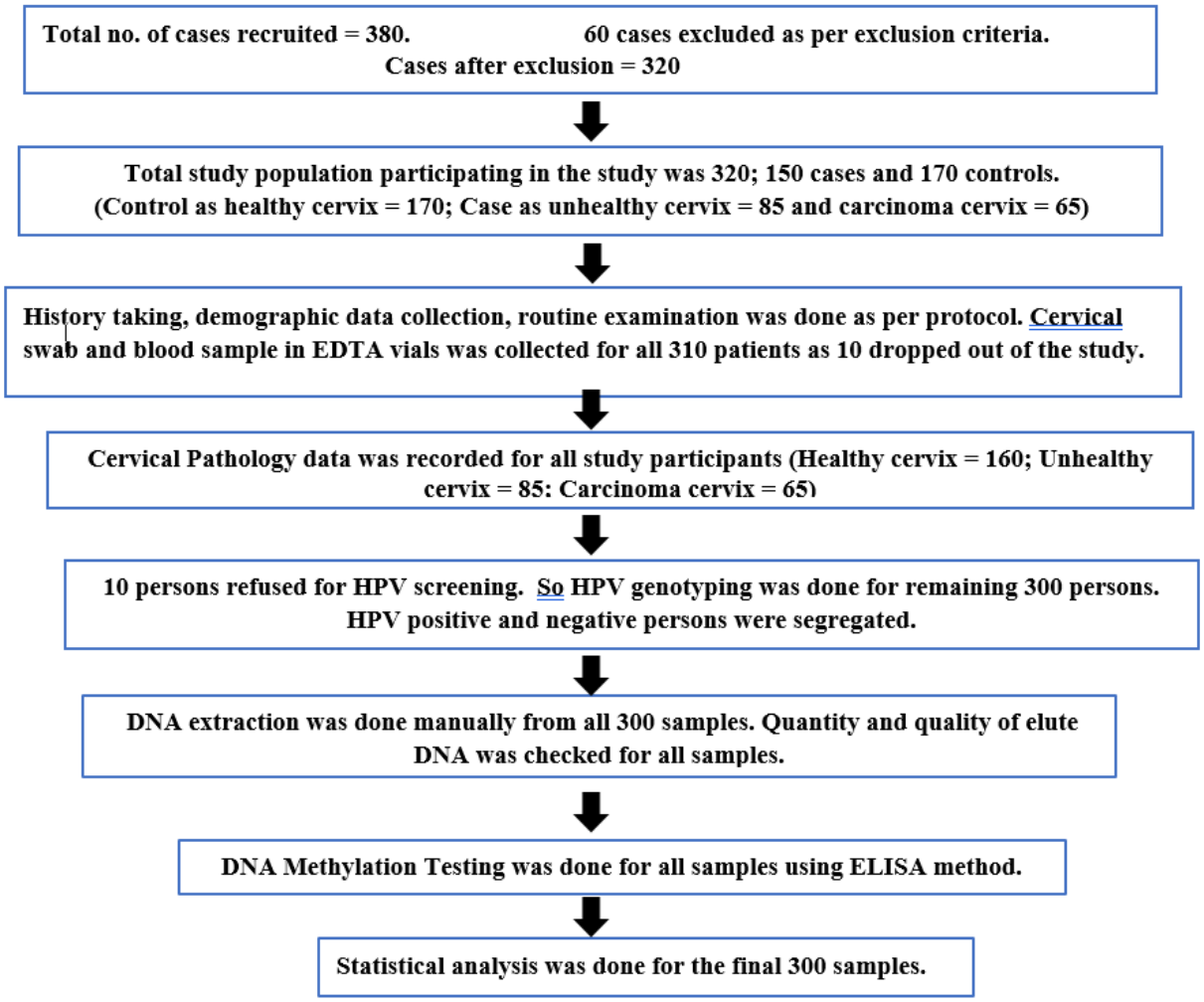
Selection of Cases

This study was conducted according to the standards of the Declaration of Helsinki and with approvalsof the Institutional Ethics Committee of S.C.B Medical College and Hospital, Cuttack. The study was conducted in Molecular Genomics Laboratory of Post-graduate Department of Biochemistry in collaboration with Department of Obstetrics and gynaecology. Those who had Diabetes, Chronic Kidney Disease, Chronic Lung and Cardiac Disease, Hypertension, Psychiatric Disorders and Previous history of sexually transmitted diseases were excluded from the

study. Women of reproductive age group (15-44 years) attending Obstetrics and Gynaecology OPD were included in the study after eliminating cases as per exclusion criteria. Informed consent from all participants were obtained as per the proforma. All the participants were subjected to histopathological tests (pap smear/biopsy) as per the standard protocol and were divided into age-matched 3 groups (healthy cervix, unhealthy cervix and cancer cervix) based on chief complains (post-menopausal bleeding, post-coital bleeding, cervical discharge), physical examination of cervix (cervicitis, cervical inflammation or hypertrophy or any growth) and documentation of cervical cancer by histopathological study reports. Women with none of the above complains (post-menopausal bleeding, post-coital bleeding, cervical discharge) or abnormal physical findings were considered in healthy cervix group. Similarly, women with any type of cervical discharge, postmenopausal bleeding or hypertrophied cervix were included in unhealthy cervix group. Cancer cervix included all diagnosed cases of cancer cervix.A case control study was designed with a total of 150 controls and 150 cases which included 65 cancer cervix cases and 85 unhealthy cervix cases and subjected to following tests.

1.HPV Genotyping

Cervical swab was collected in **Roche (Switzerland)** collection media using Roche cervical brush and processed for HPV Genotyping. The analysis was done by RT-PCR based method using **Cobas x-480 and Cobas z-480 instruments**. Two major processes were adopted for the HPV Test: (1) automated specimen preparation to simultaneously extract HPV and cellular DNA; (2) PCR amplification of target DNA sequences using both HPV and β-globin specific complementary primer pairs and real-time detection of cleaved fluorescent-labeled HPV and β-globin specific oligonucleotide detection probes. The test contained primer pairs and probes specific for 14 high-risk HPV types which were HPV16, HPV18 and 12 other high-risk HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). [19]



. **Fig. 2:-** Protocol followed for the present study.

2.Global DNA Methylation

DNA was extracted manually from collected EDTA Blood by the Spin Column Based method using **Qiagen dna mini kit (Qiagen, Hilden Germany).** Its quality was checked using **Nanodrop Spectrophotometer (Thermofisher,Massachusetts,U.S.A)**.[20] and then was subjected to Enzyme linked immunosorbent Assay (ELISA) for quantifying the Global DNA methylation levels. Input DNA quantity was maintained between 10-20ng.

Results And Observations:-

Observations were recorded and following statistical calculations were done.

Table 1:- Age group distribution of the study population.

AGE GROUP (in years)	HEALTHY CERVIX (n = 150)	UNHEALTHY CERVIX (n = 85)	CARCINOMA CERVIX (n = 65)
18-30	48(32%)	13(15%)	3(5%)
31-42	26(17.3%)	26(30.80%)	22(33.7%)
43-55	76(50.70%)	46(54.2%)	40(61.3%)

Table 2:- Various HPV genotypes in the study groups according to cervical pathology:

	HPV 16(n=34)	HPV 18(n=8)	OTHER HIGH-RISK STRAIN (n=76)	NEGATIVE (n=182)
NORMALHEALTHY CERVIX	4(2.6%)	0	7(4.6%)	139(92.8%)
UNHEALTHY CERVIX	10(12.1%)	4(5.2%)	41(47.8%)	30(34.9%)
CARCINOMA CERVIX	20(31.4%)	4(5.7%)	28(42.8%)	13(20.1%)

Table 3:- Global DNA methylation levels in different study groups.

Descriptives								
METHYLATION								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Normal Cx	150	2.1024	.20292	.01657	2.0697	2.1352	1.31	2.72
Unhealthy Cx	85	1.0926	1.11372	.12080	.8524	1.3329	.20	11.00
CA Cx	65	.2293	.11697	.01451	.2003	.2583	.12	.76
Total	300	1.4105	.97168	.05610	1.3001	1.5209	.12	11.00

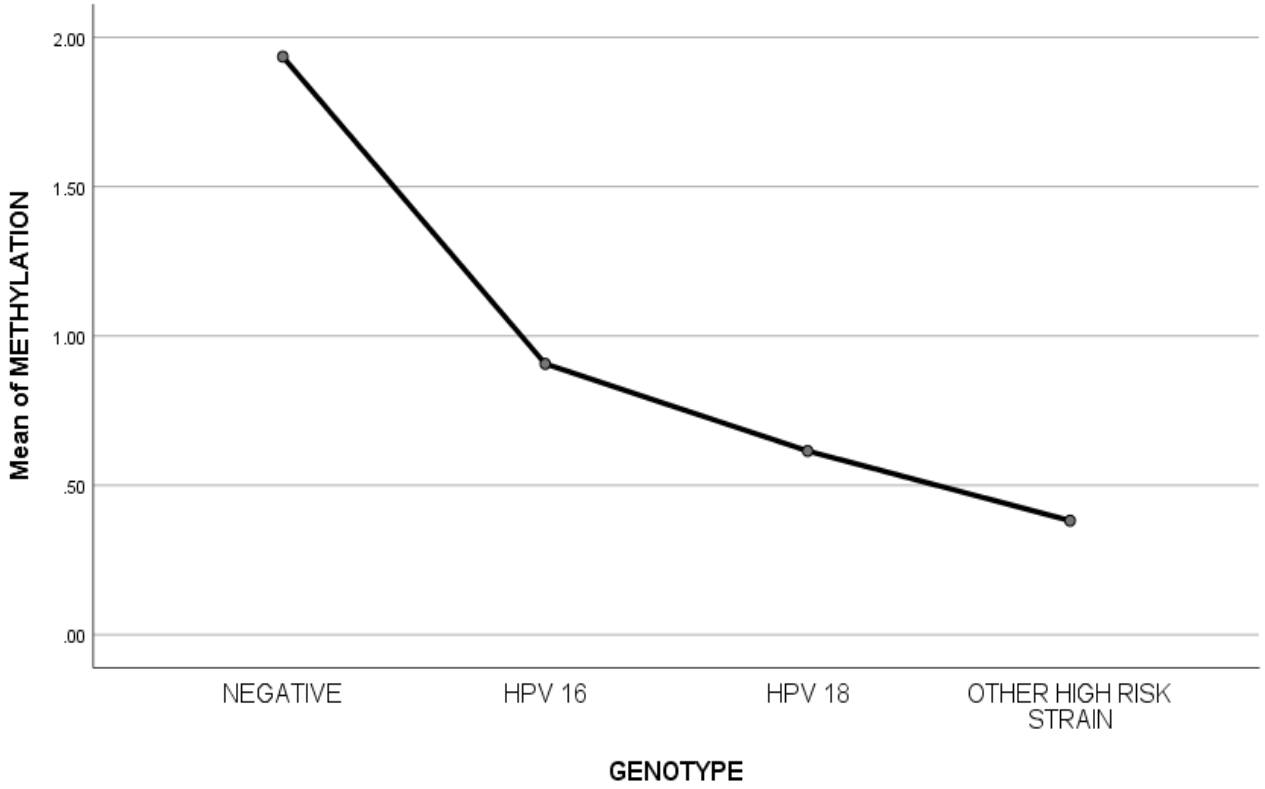
Global DNA Methylation levels among the three study groups was compared and ap<0.01was considered significant.

Table 4:- Association of HPV status with Global DNAMethylation by chi-square test in the study population.

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	587.783a	382	.000
Likelihood Ratio	612.840	382	.000
Linear-by-Linear Association	180.891	1	.000
N of Valid Cases	300		

*p value < 0.05 was considered significant at 95% confidence interval.

Fig. 3:- Depicts mean of Global DNA Methylation Levels in different HPV Genotypes.



Discussion:-

Low socioeconomic status, very low literacy rate, poor personal hygiene and improper screening modalities contribute to high burden of HPV in India.Regular screening of all sexually active women and proper vaccination can provide adequate protection against cervical cancer. HPV vaccines provide very limited cross protection. So having a clear idea about the genotypic distribution among different population is essential in predicting the efficacy of current vaccine and devising new vaccine strategy [21]. Epidemiology of HPV infection and pattern of HPV genotype distribution are not well documented in the entire Indian subcontinent which limits the implementation of cervical cancer prevention programs.

Available reports from studies covering few parts of India show a wide variation in the prevalence of HPV infection and genotypes distribution which is probably due to diversified socio-economic and geo-climatic condition. [22,23]. Thus, a similar evaluation of the same in different geographical regions of the country is necessary. Hence this study has tried to show the presence of different genotype of HPV among women with and without cervical cancer.

Distribution in Table 1 depicts that of the total unhealthy cervix group 54.2% belonged to 43- 55 age group. Similarly, amongst the cancer cervix group ,43-55 years age group was (61.3%) predominant. This indicates that the age group of 43-55 are more vulnerable to abnormal cervical pathology which co-related with findings of Bobdey et al **[24]**. These authors reported that more than 85% of patients were from age group 40 years and above. This can be attributed to poor or moderate living standards, multiple sexual partners and a high prevalence of HPV due to lack of proper screening examinations.

Table 2 shows that normal healthy cervix group had the HPV- 16 strain positive to be 2.6%, and 4.6% positive of other high-risk strain. Among the unhealthy cervix group, 12.1% positivity was found for HPV 16, 5.2% for HPV 18 and 47.8% for other high-risk strain. Among carcinoma cervix group positivity found were, 31.4% for HPV 16, 5.7% for HPV 18 and 42.8% for other high-risk strain. Other high-risk strain genotype was prevalent in abnormal cervical pathology compared to normal cervix in our study.

This may be due to the fact that different geographical areas have different genotypic distribution of HPV. The HPV genotypes found in different regions of the world vary both in type and relative incidence. There is also evidence of the prevalence of specific variants of defined genotypes in certain ethnic groups.[25] But Senapati et al [26] in 2017 found in their study that the most commonly detected genotype was HPV16 (87.28%)followed by HPV18 (24.56%) Other detected genotypes in descending order were HPV 51(3.46%), HPV 39(3.17%), HPV 66(2.8%), HPV 68(2.3%), HPV 35(1.7%), HPV 45(1.7%), HPV 44(1.1%), HPV 58 (1.1%), HPV 52(.57%), HPV 6/11(.57%), HPV 42(1.1%) and HPV 43(.57%). Baloch et al [27] and Chopjitt et al [28] also found that the rate of single genotype infection especiallyHPV 16, was very high in the abnormal histopathological group as compared to the normal group.

The mucosal types of HPV infect the lining of the cervical epithelium and present with a variety of clinical conditions that may range from innocuous lesions to cancer. During the HPV infection, the DNA undergoes mutation due to different cellular and environmental conditions leading to viral DNA integration with the host DNA synthesis machinery. As a result, virus can escape cellular and immune defence mechanisms and accelerates cell proliferation along with inhibiting cellular apoptotic mechanisms. Cervical carcinogenesis can be defined as the complex mechanism of uncontrolled cellular division that can involve HPV gene integration together with other cellular changes and epigenetic factors [29].

In Table. 3 the DNA methylation levels in different study groups are shown. Mean of the cancer cervix group and unhealthy cervix group were found to be lower ($p<0.001$) than normal healthy cervix group. This tallied with findings of Ehrlich et all [30] who reported that DNA of cancer cells lose their methyl groups. It was also in accordance with Kim et al [31] who has also indicated that epigenetic changes like global DNA hypomethylation play an important role in cervical carcinogenesis. Viral infections responsible for cervical neoplasia and further promoting cervical carcinogenesis might be due to such Global DNA hypomethylation. Thus, human papillomavirus (HPV) infection is a well-accepted causal factor in cervical carcinogenesis. Replication and cell cycle in cells are affected by DNA hypomethylation leading to tumour development. Besides, rRNA levels also increase while cervical cancer progresses and rDNA promoter decondensation and hypomethylation is found to be associated with epigenetic changes. As transcription factors cannot bind to methylated DNA initiation of transcription is inhibited. This can be observed in other cancers also, such as lung or ovarian cancers.[32]

Table 4 of our study shows that global DNA methylation levels of HPV positive genotype patients were found to be lower than HPV negative genotype ($p<0.001$) which is in accordance with studies by Kelly et al from tissue samples. [33] They reported that DNA methylation occur during HR-HPV infection and precancerous tissue lesions, leading to alterations in the functions of gene products which regulate tumour suppression. Such aberrant DNA methylation may help distinguish non-progressive HPV infections from those progressing to cancer.

From **Fig. 3** it is evident that Global DNA methylation levels also varied for different genotypes with max. hypomethylation seen in infection by Other High-Risk strain. This is evident from the fact that decreased DNA methylation has been shown to be associated with increasing persistence of HR-HPV genotypes,severity of CIN lesionsand risk of invasive cancer.[34,35]

Conclusion:-

From our studies we concluded that, Other high-risk strains were found to be more prevalent than HPV 16 and 18 particularly in unhealthy and carcinoma cervix group in this part of the country. Global DNA hypomethylation was seen in both unhealthy and cancer cervix participants amongst whom cancer cervix had higher hypomethylation levels compared to unhealthy cervix. Among the different HPV Genotypes Other high-risk strain genotype had the highest rate of hypomethylation followed by HPV 18 and HPV 16. Thus, global DNA hypomethylation may be used as predictive epigenetic marker in early diagnosis of cervical cancer.

HPV testing is the new upcoming modern technology and will gradually replace conventional means of testing like Pap smear. Low specificity of HPV DNA testing is surely a setback so finding a better alternative for HPV infected patients is very much required. Accurate molecular prognostic classifiers for people with High-Risk HPV-positive (HR-HPV+) test results, could be added to the screening protocols. This would reflexively indicate the future risk of progression of Cervical cancer. The risk of HPV infected lesions progressing to CIN3 or disappearing could be assessed from this epigenetic marker and would be a giant leap for HPV screening programs.

The use of global DNA methylation as a marker in evaluating the different stages of dysplasia is yet to be confirmed. More studies need to be done to find out about that. But Global DNA methylation can still be used in the study of potential of low-grade dysplasia and as a valuable end point in intervention studies to alter the course of cervical carcinogenesis.

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