The Journal of Obstetrics and Gynecology of India (November–December 2022) 72(6):522–528
https://doi.org/10.1007/s13224-022-01680-5

ORIGINAL ARTICLE

PAX1 Methylation Status in Cervical Scrapes as Novel Diagnostic Biomarker in CIN 2/3 and Invasive Squamous Cell Carcinoma

Bhavya[1] · Shalini Rajaram[2] · Bindiya Gupta[1] · B. D. Banerjee[3] · Vinod Kumar Arora[4] · Gaurav Thakur[3] · Sandhya Jain[1]

Received: 30 January 2022 / Accepted: 28 June 2022 / Published online: 1 October 2022

Abstracts

Objectives DNA methylation of paired box-1 (PAX-1) gene has been shown to be a potential biomarker for the detection of high-grade cervical intra-epithelial neoplasia (CIN) and invasive cervical cancer. The objective of this pilot study was to quantify and compare methylation percentage of PAX1 gene in benign cervical lesion, pre-invasive and invasive cervical cancer.

Methods A total of 200 screen positive women (VIA, VILI and Pap test) underwent colposcopy. Cervical scrapes taken were taken and stored for DNA analysis and PAX 1 methylation status. Women with Swede score of 5 or more ($n=98$) were biopsied. Cervical scrapes and biopsy were taken from women with obvious cervical growth ($n=14$), without prior colposcopy. Sixty women were recruited to the study and allocated into three groups on the basis of histopathology, i.e., benign cervix (Group 1; $n=20$), CIN 2/3 (Group 2; $n=20$) and invasive cervical carcinoma (Group; $n=20$). PAX 1 methylation percentage was calculated from the DNA extracted from the cervical scrapes of the women recruited.

Results The mean PAX1 methylation percentage in benign lesions, CIN 2/3 and invasive cancer was 9.58% (SD ± 2.37%), 18.21% (SD ± 2.67%) and 24.34% (SD ± 4.09%), respectively, with p-value of < 0.001.

Conclusions PAX 1 gene methylation has a promising role in identifying high-grade lesions and invasive cancer.

Keywords Cervical cancer biomarker · Cervical intra-epithelial neoplasia (CIN 2/3) PAX1 DNA methylation · Squamous cell carcinoma cervix

Introduction

Cervical carcinoma has now emerged as the eighth most common cancer worldwide. Globocan 2020 states that each year an estimated 604,127 cancer cases are detected, and 341,831 deaths occur due to cervical cancer worldwide. [1]

Cervical cancer has the unique feature of having well-defined pre-invasive lesions associated with progressive steps of carcinogenesis. The slow but steady progression of this disease from pre-malignant to malignant is induced by the persistent infection with high-risk human papilloma virus (HR HPV). Following HR HPV infection, high-grade precancerous lesions may develop within 3–5 years [2], whereas further evolution to full blown cancer requires another 10 years [3, 4]. Thus, cervical screening should aim at preventing carcinoma by identification and treatment of these high-grade lesions.

Epigenetic aberrations cause oncogene activation or tumor suppressor gene inactivation [5]. These epigenetic changes include histone modifications, nucleosome occupancy and positioning, protein and non-coding RNA interactions, as well as direct DNA modifications. The gradual

Dr Bhavya (MS) is a Senior Resident, Department of Obstetrics & Gynecology at UCMS & GTB hospital, Delhi, India; Shalini Rajaram (MD) is a Professor, Division of Gynecologic Oncology, AIIMS, Rishikesh, India; Bindiya Gupta (MD) is an Associate Professor, Department of Obstetrics & Gynecology at UCMS & GTB hospital, Delhi, India; B.D. Banerjee (MD) is a Professor, Department of Biochemistry, UCMS & GTB Hospital, Delhi, India; Vinod Kumar Arora (MD) is a Director Professor, Department of Pathology, UCMS & GTB Hospital, Delhi, India; Gaurav Thakur (Msc) is a Ph.D. Scholar, Department of Biochemistry, UCMS & GTB Hospital, Delhi, India; Sandhya Jain (MD) is an Associate Professor, Department of Obstetrics & Gynecology at UCMS & GTB hospital, Delhi, India.

✉ Bhavya
drbhavya.1992@gmail.com

Extended author information available on the last page of the article

buildup of these epigenetic modifications leads to development of the pre-invasive lesions and cancer [6].

Molecular biomarkers based on DNA methylation have gained the most attention and are well recognized in cervical cancer. Aberrant DNA methylation of promoters of upregulatory genes leads to inactivation of tumor suppressor genes like p53 and transcriptional gene inactivation. DNA methylation is also easier to detect in both histological and cytological specimens. Studies have shown that certain genes like paired box 1 gene (PAX1), SRY-box 1 (*SOX1*), LIM homeobox transcription factor 1α (*LMX1A*) and NK6 homeobox 1 (*NKX6-1*), the junctional adhesion molecule 3 (*JAM3*), *EPB41L3, TERC, TERT, C13ORF18*, and *CADM1* and *MAL* have been found to undergo aberrant DNA methylation during integration of HPV DNA in the host genome [7–9].

A recent study conducted by PH Su et al. has shown the role of PAX 1 gene in maintaining the on–off homeostasis between the kinases and phosphatases in the cervical epithelium. Epigenetic transformation through hypermethylation of PAX1 gene disturbs this equilibrium and leads to cancer. The authors further suggest that therapies targeting these epigenetic transformations and the resultant reactivation of the tumor suppressor may present with new possibility of dealing with chemo- and targeted therapy resistance [10].

The aim of the present study was to evaluate methylation status of paired box 1 gene (PAX 1) in cervical intraepithelial neoplasia and cervical cancer. The objective was to quantify and compare the status of methylated PAX1 gene in benign lesions, pre-invasive and invasive cervical cancer. The secondary objective was to compare the DNA methylation level with cytopathology report.

Material and Method

This was a pilot study, performed at University College of Medical Sciences, Guru Tegh Bahadur Hospital, Delhi, a tertiary care hospital catering to lower-middle-class population from November 2016 to April 2018. A written informed consent was taken from all patients, and ethical clearance was taken from Institutional Ethics Committee.

Sample size was calculated with 80% power and 5% level of significance using F-test for fixed affect one-way ANOVA. Three groups were made including women with chronic cervicitis ($n=17$), CIN 2/3 ($n=17$) and invasive cancer ($n=17$), such that 51 women were included in the study [11]. Women with biopsy proven diagnosis of CIN 2/3 and squamous cell carcinoma were recruited in the study. Pregnant women were excluded in this research study to avoid bleeding associated with cervical scrapes using a cytobrush. Women with HIV coinfection were excluded from the study as methylation of host genes may be disrupted by HIV infection and cross reactivity between HPV and HIV is still under research. Women with prior surgery on the cervix, previously treated CIN and any other coexisting cancer were also excluded.

A total of 1680 sexually active women > 21 years of age attending gynecology OPD were screened in the gynecology OPD using Pap test followed by visual inspection tests. Two hundred colposcopies were conducted on screen positive women and 98 biopsies were taken when calculated Swede score was more than or equal to 5 [12]. On histopathology 59 women were found to have chronic cervicitis or squamous metaplasia, 13 women had diagnosis of cervical intraepithelial neoplasia 1, 17 women had CIN 2/3, and 6 women were diagnosed to have squamous cell carcinoma. Out of the 59 women having chronic cervicitis on biopsy, samples of 17 women were selected on a random basis for further DNA analysis and taken as controls. CIN 1 lesions are known to be transient lesions, while CIN 2/3 are the transforming lesions; hence, being true pre-invasive lesions, women with CIN 2/3 were recruited for the study. Eleven women having cancer cervix had presented with an obvious growth (screening tests not done and did not undergo colposcopy) were also included in the study and biopsy was sent from the growth. Thus, 17 women in each group were recruited namely Group 1 (benign/ chronic cervicitis), Group 2 (CIN 2/3) and Group 3 (invasive SCC) using colposcopy and/or direct biopsy.

Cervical scrapes for PAX 1 methylation analysis were taken from all women who underwent colposcopy before which a written informed consent was taken. Cervical scrapes for PAX 1 methylation analysis were also taken from women presenting with invasive cancer and frank cervical growth.

Cervical scrapes were then stored at a temperature of—20 deg till it was further processed for DNA analysis and PAX -1 methylation status. The DNA was extracted from the cervical scrapings and purified using the QIA amp DNA Mini Kit (QIAamp^{R}). The purified DNA was subjected to Bisulfite conversion using the EZ-DNA methylation kit (Zymo Research Corp.). PAX 1 gene promoter primers of 140 base pairs were obtained for carrying out the polymerase chain reaction.

The bisulfite-modified DNA obtained from cervical scrapes underwent Sanger sequencing according to the manufacturers protocol (Eurofin services). Comparison was made between Sanger-sequenced untreated DNA and bisulfite-modified DNA enabling the detection of the methylated cytosines. During the process of sequencing, instead of labeling the products of all 4 sequencing reactions with the same radioactive deoxynucleotide; each dideoxynucleotide was labeled with a different fluorescent marker. When excited with a laser, four different

kinds of products were detected and the fluorescence intensity translated into a data "peak". The results thus produced were in the form of graphs. These sequencing graphs were further processed for quantification of methylation status, by the BISMARK software. This software captured the snap shot of a cell's epigenomic state by revealing its genome-wide cytosine methylation at single base resolution. The output from this software discriminated between cytosines in CpG, CHG and CHH context and enabled interpretation of their methylation data soon after the sequencing run was completed, thus giving the results in form of methylation percentages, after comparing untreated PAX 1 promoter region primer and the DNA extracted from the cervical scrapings.

Data were recorded using Microsoft Excel and analyzed using MedCalc Statistical Software version 18.2.1. Quantitative data were represented as mean and standard deviation and qualitative data as number and percentage. Kruksal–Wallis test was applied to compare quantitative data between two groups, while for qualitative data Chi-square/Fisher test was used.

Results

We invited 51 women to participate in this study where cervical scrapes were collected just before the colposcopic guided biopsy. The demographic profile for all women is shown in Table 1.

PAX 1 Methylation Levels According to Histopathology

Cervical scrapes taken during colposcopy were analyzed for PAX1 gene methylation levels. Due to problems like sample contamination and DNA degradation, results of only 48 samples could be obtained. Of these, 14 samples were of benign lesions (Group I), 17 were CIN 2/3(Group 2) and 17 were invasive cancer (Group 3). The mean methylation level in benign lesions was lowest, i.e., 9.58% (SD ± 2.37%), while the mean methylation level in CIN 2/3 was 18.21% (SD ± 2.67%) and mean methylation level in invasive cancer was highest 24.34% (SD ± 4.09%), with p-value of < 0.001. (Fig. 1).

Receiver Operating Characteristic (ROC) Curve Analysis of PAX1 Methylation for Invasive Cancer Versus CIN 2/3

The ROC curve for analysis of PAX 1 methylation percentage in invasive cancer versus CIN 2/3 showed the area under the curve was 0.865 with 95% CI (0.704 to 0.957) with P-value of < 0.0001. Best cutoff point was 21.8% at which the sensitivity was 76.67% and specificity was 100% (P < 0.001). All cases of invasive cancer had PAX 1 methylation levels above the cutoff value of 21.8%. (Fig. 2).

Frequency of PAX 1 Methylation in Invasive Cancer, Pre-Invasive Cancer and Benign Cervices

High frequency of PAX 1 methylation percentage was observed with increasing severity of neoplasia. The percentage PAX 1 methylation for cancer group and CIN 2/3 was significantly higher. At a cutoff of 21.8% the PAX 1 methylation frequency for benign cervices was calculated to be 0%; for CIN 2/3 it was 5.9% and for cancer cases it was 76.5% (Table 2).

Papanicolaou (Pap) Test

Pap test was performed in 40 women (excluding 11 women with obvious growth). Interpretation of Pap test was done using the 2001 Bethesda System. Positive test was seen in 21 (52.5%) out of the 40 women, i.e., 12 women were reported to have unsatisfactory pap test twice consecutively, LSIL was seen in two, HSIL in four and squamous cell carcinoma in three respectively (Fig. 3).

Table 1 Comparison of Histology and mean age of study participants

Variables	BIOPSY			Total	*P* value
	Group 1 = benign lesions	Group 2 = CIN 2/3	Group 3 = Cancer cervix		
Age	38.45 ± 8.49	43.1 ± 10.99	49.2 ± 11.19	43.58 ± 11.05	0.007**
Age at marriage	18.5 ± 2.14	17.45 ± 1.96	16.6 ± 1.5	17.52 ± 2.01	0.009**
Age at first intercourse	18.8 ± 2.21	17.5 ± 2.14	16.55 ± 1.43	17.62 ± 2.14	0.003**
Age at first child birth	21.2 ± 2.07	19.25 ± 2.02	18.4 ± 1.47	19.62 ± 2.19	< 0.001**
Age at menarche	14.45 ± 1	13.95 ± 1	13.75 ± 1.02	14.05 ± 1.03	0.085 +

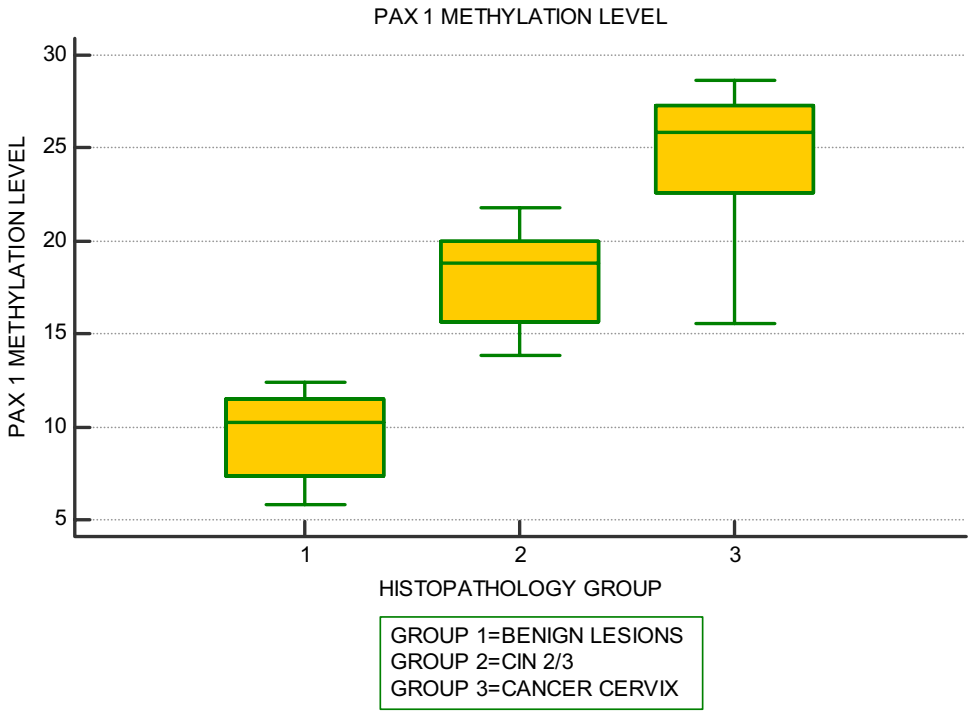

Fig. 1 PAX 1 methylation percentage in benign lesions, CIN 2/3 and cancer. Distribution of PAX1 methylation percentage in women having benign lesions, CIN 2/3 and cancer. Boxes, horizontal line and whiskers represent 25–75% quartile, median value and range, respectively. The median value for Group 1 (benign lesions) was 10.19, range 5.8–12.38; for Group 2 (CIN 2/3) the median value was 18.8 with a range of 13.8–21.8, while the median value for Group 3 (cancer) was 25.86, with the range of 15.6–28.62

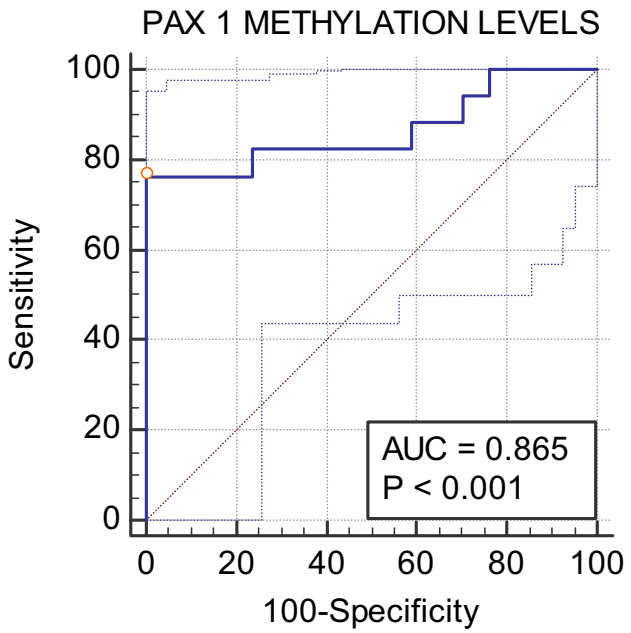

Fig. 2 Receiver operating characteristic (ROC) curve analysis of PAX1 methylation for cancer versus CIN 2/3

PAX-1 Methylation Status, Histopathology and Cytology

Although statistically insignificant due to small data, correlation between the three parameters showed that while Pap test did not identify the high-grade lesions, performing PAX 1 methylation analysis could help identify patients who have a high propensity for progressing to cancer. Out of 8 women with normal Pap test, 4 women with CIN 2/3 on biopsy were identified as having high-grade lesions by PAX1 methylation analysis. Similarly, out of 12 women with unsatisfactory pap report, 9 women had CIN 2/3, while one woman was diagnosed with invasive cervical cancer on biopsy. She was again picked up as high-grade lesion using the PAX 1 level cutoff**.** (Table 3).

Table 2 Frequency of methylation of PAX 1 (at cutoff of 21.8%) and histopathology

Histopathology	PAX 1 Methylation	p-value
Benign cervices	0/14 (0%)	
CIN 2/3	1/17 (5.9%)	0.0003
Cancer	13/17 (76.5%)	0.02

Discussion

Cervical cancer is caused by HPV and it's not just the persistent infection but the slow accumulation of various genetic and epigenetic changes over several years which actually causes cancer. This time period provides the clinicians with the golden opportunity to detect it early and prevent further morbidity and mortality. Cytology has been used over the past century as the main screening tool but with drawback of low sensitivity and false positive rate of 20%. (19) Even

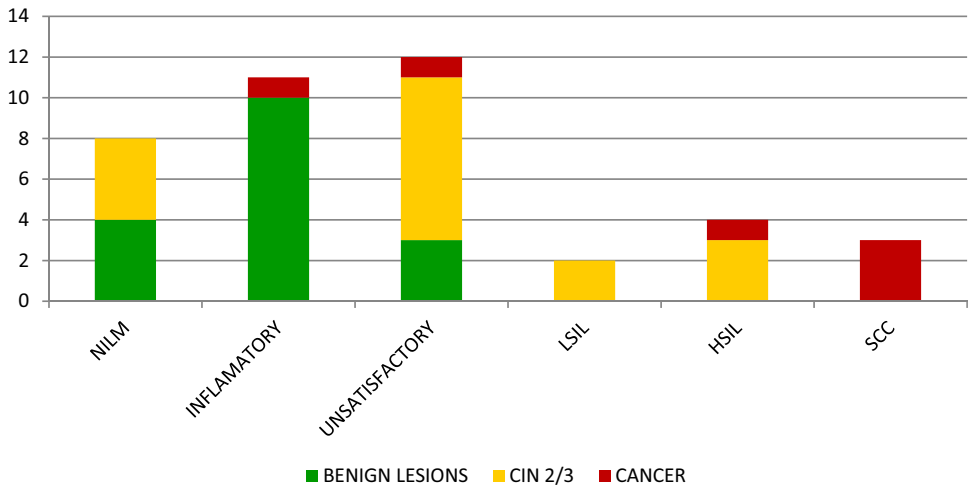

Fig. 3 Distribution of Pap Smear Reports According to Histopathology

Table 3 Comparison of PAX-1 methylation percentage, histopathology and cytology

Pap Test	Chronic Cervicitis	CIN 2/3	Cancer cervix
NILM($n=8$)	8.6%($n=4$)	17.16%($n=4$)	
Inflammatory($n=11$)	10.35%($n=10$)		25.6%($n=1$)
Unsatisfactory($n=12$)	8.43%($n=5$)	18.09%($n=6$)	28.5%($n=1$)
LSIL($n=2$)		21.8%($n=2$)	
HSIL($n=4$)		19.08%($n=3$)	21.6%($n=1$)
SCC($n=3$)			25.5%($n=3$)
NILM($n=8$)	8.6%($n=4$)	17.16%($n=4$)	

NILM Negative for Intraepithelial Lesion or Malignancy, *LSIL* Low-Grade Squamous Intraepithelial Lesion, *HSIL* High-Grade Squamous Intraepithelial Lesion, *SCC* Squamous Cell Carcinoma

though HPV testing has now become the standard test for diagnosis, other low risk strains may not be picked up and prevent early detection and addition of another molecular test may prevent such lapses. The use of genome wide approach including next-generation sequencing and microarray has allowed the identification of newer diagnostic parameters for the early recognition of cervical cancer. Studies have now shown that altered DNA methylation especially site specific hypermethylation of PAX 1 gene has a promising role in detection of pre-invasive and invasive cervical cancer. The various studies conducted worldwide have validated the potential of PAX 1 methylation as the diagnostic biomarker in cervical cancer molecular screening. Taiwanese FDA has approved the use of PAX 1 methylation as an adjunct to cytology in cervical cancer screening in 2016.

This pilot study was aimed to see whether PAX1 methylation helped detect the pre-invasive and invasive disease in the Indian population. The present study showed that the mean PAX 1 methylation in benign lesions was lowest, i.e., 9.58% (SD ± 2.37%), while the mean methylation in CIN 2/3 was 18.21% (SD ± 2.67%) and mean methylation in invasive cancer was highest at 24.34% (SD ± 4.09%). These findings are in accordance with results obtained in a study conducted by Xu Jun et al. which showed that the mean methylation status for normal, CIN 2/3 and cancer was 3.3%(SD ± 0.41%), 13.0%(SD ± 2.2%) and 26.3%(SD ± 3.5%), respectively. [11]

In another study by Lai HC et al., the authors concluded that quantitative measurement of PAX1 hyper-methylation in cervical scrapings is highly sensitive for detection of cervical cancer [13], while study conducted by Xu et al. showed that methylated PAX-1 demonstrated greater ability to detect cervical cancer with the sensitivity and specificity at the cutoff point being 79.1% and 89.3%, respectively. [14]

The recent meta-analysis conducted by Kelly H et al. showed that women with CIN 2/3 had remarkably high DNA PAX 1 methylation than CIN 1. The study also found that DNA methylation has higher specificity than cytology ASCUS+ and higher sensitivity than HPV 16/18 genotyping when used as triage test [15]. In the present study, although the sample size was small, it was found that women with normal pap smear who had high-grade lesion on biopsy corresponded with higher PAX 1 methylation levels in cervical scrapes. Hence, PAX -1 methylation can be used as a useful adjunct to cytology to increase the accuracy. In the present times, it is not possible to do it at all levels and is considered useful for research settings than clinical usefulness.

This study can be considered as pioneering study in Indian context, as not many studies have been conducted in India and is still in research phase. Long-term study at different centers is needed to prove its efficacy. In another meta-analysis Fang et al. concluded that combined testing for HPV and PAX 1 methylation was an effective triage tool in cervical cancer screening program.

Conclusions

To summarize, in this biopsy affirmed pilot study, the PAX 1 gene methylation status obtained from the cervical scrapes demonstrates significantly higher status in women with pre-malignant and malignant lesions. PAX 1 methylation status may accurately identify women with persistent and transforming HPV infection and identify women with precancerous lesions that are going to progress to cancer, with higher precision. However, studies with larger sample size and with HR HPV genotyping correlation need to be conducted before PAX 1 methylation test can be considered as a triage test.

Acknowledgements This study was funded by Indian Council of Medical Research

Funding The study was funded by Indian Council of Medical Research.

Declarations

Conflict of interest No conflict of interest among the authors.

Ethical approval Study has been approved by institutional ethics board and all procedures performed in study involving human participants were in accordance with the ethical standards of the institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all the participants included in the study.

Consent for publication The work described has not been published before and is not under consideration for publication anywhere else. The publication has been approved by all co-authors at the institute where the work has been carried out.

References

1. World Health Organization (WHO). Cervical Cancer Estimated Incidence, Mortality and Prevalence Worldwide in 2018. https://www.uicc.org/new-global-cancer-data-globocan-2018. Accessed on 25th jan. 2022.
2. Snijders PJ, Steenbergen RD, Heideman DA, Meijer CJ. HPV-mediated cervical carcinogenesis: concepts and clinical implications. J Pathol. 2006;208:152–64.
3. McCredie MR, et al. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. Lancet Oncol. 2008;9:425–34.
4. Vink MA, Bogaards JA, van Kemenade FJ, de Melker HE, Meijer CJ, Berkhof J. Clinical progression of high-grade cervical intraepithelial neoplasia: estimating the time to preclinical cervical cancer from doubly censored national registry data. Am J Epidemiol. 2013;178:1161–9.
5. Steenburgen RD, Snijders PJ, Heideman DA, Meijer CJ. Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. Nat Rev Cancer. 2014;14(6):395–405.
6. Shen H, Laird PW. Interplay between the cancer genome and epigenome. Cell. 2013;153(1):38–55.
7. Lai HC, Lin YW, Huang TH, Yan P, Huang RL, Wang HC, et al. Identification of novel DNA methylation markers in cervical cancer. Int J Cancer. 2008;123:161–7.
8. Eijsink JJ, Yang N, Lendvai A, Klip HG, Volders HH, Buikema HJ, et al. Detection of cervical neoplasia by DNA methylation analysis in cervico-vaginal lavages, a feasibility study. Gynecol Oncol. 2011;120(2):280–3.
9. Overmeer RM, Louwers JA, Meijer CJ, van Kemenade FJ, Hesselink AT, Daalmeijer NF, et al. Combined CADM1 and MAL promoter methylation analysis to detect (pre-) malignant cervical lesions in high-risk HPV-positive women. Int J Cancer. 2011;129(9):2218–25.
10. Su PH, Lai HC, Huang RL, et al. Paired box-1 (Pax1) activates multiple phosphatases and inhibits kinase cascades in cervical cancer. Sci Rep. 2019;9(1):9195.
11. Xu J, Xu L, Yang B, Wang L, Lin X, Tu H. Assessing methylation status of PAX1 in cervical scrapings, as a novel diagnostic and predictive biomarker, was closely related to screen cervical cancer. Int J Clin Exp Pathol. 2015;8(2):1674–81.
12. Bowring J, Strander B, Young M, Evans H, Walker P. The swede score: evaluation of a scoring system designed to improve the predictive value of colposcopy. J Low Genit Tract Dis. 2010;14(4):301–5.
13. Huang TH, Lai HC, Liu HW, Lin CJ, Wang KH, Ding DC, et al. Quantitative analysis of methylation status of the PAX1 gene for detection of cervical cancer. Int J Gynecol Cancer. 2010;20(4):513–9.
14. Xu L, Xu J, Hu Z, et al. Quantitative DNA methylation analysis of paired box gene 1 and LIM homeobox transcription factor 1 α genes in cervical cancer. Oncol Lett. 2018;15(4):4477–84.
15. Kelly H, Benavente Y, Pavon MA, De Sanjose S, Mayaud P, Lorincz AT. Performance of DNA methylation assays for detection of high-grade cervical intraepithelial neoplasia (Cin2+): a systematic review and meta-analysis. Br J Cancer. 2019;121(11):954–65.

Authors and Affiliations

Bhavya[1] · Shalini Rajaram[2] · Bindiya Gupta[1] · B. D. Banerjee[3] · Vinod Kumar Arora[4] · Gaurav Thakur[3] · Sandhya Jain[1]

[1] Department of Obstetrics & Gynaecology, UCMS & GTB Hospital, A-318, Mangal Apartments, Vasundhara Enclave, Delhi, India

[2] Division of Gynecologic Oncology, AIIMS, Rishikesh, India

[3] Department of Biochemistry, UCMS & GTB Hospital, Delhi, India

[4] Department of Pathology, UCMS & GTB Hospital, Delhi, India