Article published online: 2020-12-21

ORIGINAL ARTICLE Gynaecologic Cancers

Expression of p16^{INK4a} and human papillomavirus 16 with associated risk factors in cervical premalignant and malignant lesions

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Abstract

Introduction: Human papilloma virus (HPV) which is causative factor for cervical cancer may interact with p16 leading to malignant transformation of cervical epithelial cells. The present study was conducted to assess the immunoexpression of p16 INK4a in premalignant and malignant lesions of cervix and to correlate it with HPV 16 expression. It was also intended to study the various risk factors which may be associated with cervical cancer in this north Himalayan region of India. **Material and Methods:** The study included 50 cases of premalignant and malignant cervical lesions and 50 controls diagnosed on histopathology over a period of one year. All the relevant clinical details were noted and both cases and controls were subjected to HPV 16 and p16 INK4a immunohistochemical staining. **Results:** 67% of subjects (including cases and controls) and 94% of the cases were positive for HPV 16 expression. p16 INK4a expression was negative in all the controls, positive in 96% of invasive cancer, 66.6% in HSIL and 37.5% in LSIL. **Conclusion:** Cervical cancer is associated with low socio economic status, illiteracy, smoking, early age of marriage and conception in north Himalayan region of India. HPV 16 infection is positive in both cases and controls indicating high prevalence of HPV 16 in this region. Neoplastic transformation by HPV is identified by over expression of p16 INK4a in premalignant and malignant cases. The immunopositivity of p16 INK4a increases with the severity of cervical lesions and thus may play an important role in stratification of premalignant and malignant lesions.

Key words: Cervical cancer, human papillomavirus 16, p16^{INK4a}, premalignant lesions

Introduction

Cervical cancer is the fourth most common cancer in females worldwide, with 85% cases occurring in developing countries. It is the second most common cancer in India with the age-standardized rate of 22 per 100,000 females and mortality rate of 20.7%.[1] The premalignant lesions of the cervix which include low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) have variable rate of transformation to invasive cancer. It is estimated that 1%–2% of women have HSIL or cervical intraepithelial neoplasm 2+ (CIN2+) each year and progression rate to CIN3 and invasive carcinoma is approximately 20% and 5%, respectively.[2,3] Human papillomavirus (HPV), which is well-known causative factor for cervical cancer, mediates oncogenesis through E6 and E7 proteins, which may in turn interact with tumor-suppressor gene p53 and cell cycle regulatory protein p16, leading to malignant transformation of cervical or oral epithelial cells.[4,5] In addition, various risk factors including socioeconomic status and sexual and reproductive factors may also be associated with cervical cancer which may vary in different geographical regions.[6] The present study was therefore conducted to assess the immunoexpression of p16^{INK4a} in premalignant and malignant lesions of the cervix and to correlate it with HPV 16 expression. In addition, it was also intended to study the various risk factors which may be associated with cervical cancer in this North Himalayan Region of India.

Materials and Methods

The prospective study was conducted in the Department of Pathology in the institute situated in the North Himalayan Region of India, which included cases of premalignant and malignant lesions of the cervix iagnosed on histopathology over a period of 1 year.[7] All the relevant clinical details along with socioeconomic status, dietary habits, smoking status, and sexual and obstetrical history were noted for every case after written informed consent. The study also included age-matched controls who were diagnosed with chronic cervicitis on histopathology in this period. Immunohistochemical staining on paraffin blocks for all cases and controls was done for HPV 16 (Biocare Medical, California, USA) and p16^{INK4a} (BioGenex, California, USA) as per instructions given by manufacturers using automated immunohistochemical stainer. HPV 16-immunostained slides were reported as negative or positive depending on the percentage of cells showing immunopositivity (<5% cells showing immunostaining – HPV negative, staining of 5%–100% cells – HPV positive). p16^{INK4a} grading was done depending on the staining pattern and percentage of involved cells as follows. Grade 0: no staining, Grade 1: focal staining of metaplastic or endocervical cells in <5% of cells, Grade 2: diffuse staining of cells involving basal and parabasal layers in premalignant lesions and 5%–50% staining of cells in malignant lesions, Grade 3: diffuse staining of full thickness of squamous epithelium in premalignant lesions and >50% cells staining in malignant lesions. Grade 0 and Grade 1 were considered negative for p16^{INK4a} staining, whereas Grade 2 and Grade 3 were considered positive for p16. Statistical analysis was done using SPSS version 20 (IBM, Armonk, NY, USA) and comparison of results was done using Fischer's exact test and Student's *t*-test. $P < 0.01$ was considered statistically significant.

Results

The study included a total 50 cases with premalignant lesions (LSIL and HSIL) comprising 19 cases and malignant lesions comprising 31 cases. The mean age of total cases was 48.28 ± 8.9 years with a range of 30–61 years and the maximum number of cases was in the age group of 51–60 years (36% of total cases). The mean age of cases with premalignant lesions was 44 ± 8.61 years and with

Access this article online
Quick Response Code:
Website: www.sajc.org
DOI: 10.4103/sajc.sajc_118_17

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How to cite this article: Pandey A, Chandra S, Nautiyal R, Shrivastav V. Expression of p16^{INK4a} and human papillomavirus 16 with associated risk factors in cervical premalignant and malignant lesions. South Asian J Cancer 2018;7:236-9.

malignant lesions was 50.19 ± 8.47 years, with a maximum number of cases in age group of 41–50 and 51–60 years, respectively. Table 1 shows the various sociodemographic and reproductive characteristics of the cases and controls in the study. It showed that there was significantly higher rate of illiteracy, smoking, and low socioeconomic status in cases in comparison to controls. Table 2 shows the clinical complaints of the cases showing that vaginal discharge was the most common complaint. Table 3 shows the mmunohistochemical expression of HPV 16 and $p16^{INK4a}$ in cases and controls. It shows that $p16^{INK4a}$ expression was negative in all the controls while 76% of cases showed positivity for $p16^{INK4a}$. Table 4 shows the immunoexpression of $p16^{INK4a}$ and HPV 16 in different histopathological types of premalignant and malignant lesions [Figures 1-3]. All the cases of invasive carcinoma were positive for HPV 16 except for one case of moderately differentiated squamous cell carcinoma (SCC). The same case was also negative for $p16^{INK4a}$ grading.

Discussion

Cervical cancer is common cancer worldwide, with 85% of cases occurring in developing countries. In India, it is the second most common cancer in females after breast cancer, with a high mortality rate of 20.7%.[1] In the present study, the maximum number of cases of invasive carcinoma belonged to the age group of 51–60 years while premalignant cases were maximum at decade earlier. This age of presentation is comparatively higher than other studies from the Indian Subcontinent which observed maximum number of malignant cases in 40–54 years of age group.[6,8] The probable reason may be that females present late to the hospital from remote hilly regions of this North Himalayan state with higher stage of carcinoma cervix for diagnosis and treatment. Although HPV infection is a well-established causative factor for cervical cancer, various other sociodemographic factors may also be associated which may vary in different geographical regions depending on social and cultural trends.[9-11] It was observed in the present study that premalignant and malignant cervical lesions were associated with significantly higher rate of illiteracy (84%), smoking (74%), and low socioeconomic status (62%) in comparison to controls. Low socioeconomic status and low literacy rate may be related to poor hygiene, leading to persistent genitourinary tract infections along with limited knowledge and awareness of cancer preventive measures further associated with increased risk of cervical cancer.[6,12] Although smoking habit (active and passive) was significantly higher in cases in the present study, another study from a neighboring state in India did not find any significant association of cervical cancer with smoking.[8] A recent study from China observed that risk of HPV infection from smoking was fivefold higher and thus, concluding that it is an important risk factor for HPV infection.[13] Further, it was also observed in our study that age of marriage and first conception was statistically significantly lower in cases in comparison to controls ($P < 0.05$) and thus, suggesting it to be important risk factor for cervical malignancy. Studies have concluded that lower age of sexual experience and menarche are associated with early exposure of HPV infection and increased risk

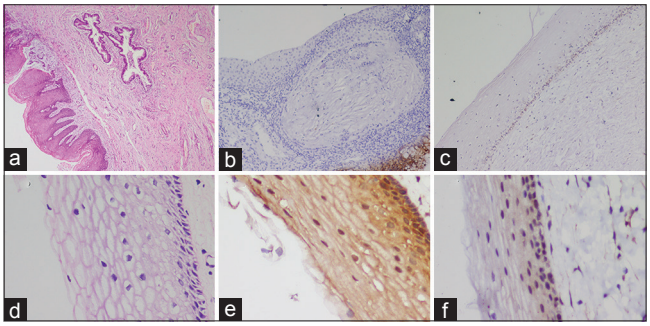

Figure 1: (a) Histopathological section showing chronic cervicitis (H and E, ×4), (b) negative immunohistochemical expression of human papillomavirus in chronic cervicitis (HPV, ×10), (c) Grade 0 (negative) immunohistochemical expression of $p16^{INK4a}$ in chronic cervicitis (p16, ×4), (d) koilocytosis in case of low-grade squamous intraepithelial lesion (H and E, ×40), (e) positive immunohistochemical expression of human papillomavirus in low-grade squamous intraepithelial lesion (HPV, ×10), (f) Grade 1 (negative) immunohistochemical expression of $p16^{INK4a}$ in low-grade squamous intraepithelial lesion (p16, ×4)

Table 1: Sociodemographic and reproductive characteristics of the cases and controls

Characteristics	Cases, *n* (percentage of total cases)	Controls, *n* (percentage of total controls)	*P*
Education status			
Illiterate	42 (84)	5 (10)	0.05
Smoking status			
Active smokers	20 (40)	1 (2)	0.001
Passive smokers	17 (34)	3 (6)	
Nonsmokers	13 (26)	46 (92)	
Socioeconomic status			
Low socioeconomic	31 (62)	14 (28)	0.05
Middle socioeconomic	19 (38)	36 (72)	
Age of marriage			
Years (mean±SD)	18.64±3	22.18±3.5	0.001
Age of first conception			
Years (mean±SD)	20.18±4.4	24±3.8	0.006
Parity			
Multiparous	16 (32)	23 (46)	0.07
Contraceptive method used			
Nil	28 (56)	11 (22)	0.01
Oral pills, barrier method, Cu T	22 (44)	39 (78)	
Dietary habits			
Vegetarian	18 (36)	22 (44)	0.9
Mixed (vegetarian and nonvegetarian)	32 (64)	28 (56)	

SD=Standard deviation

Table 2: Clinical complaints of the cases

Clinical complaints	Number of cases (%)
Vaginal discharge	31 (62)
Itching	20 (40)
Irregular vaginal bleeding	20 (40)
Dyspareunia	9 (18)
Menorrhagia	2 (4)
Postcoital bleeding	2 (4)

Table 3: Immunohistochemical expression of human papillomavirus 16 and $p16^{INK4a}$ in cases and controls

	p16 positive	p16 negative	HPV 16 positive	HPV 16 negative
Cases (%)	38 (76)	12 (24)	47 (94)	2 (4)
Controls (%)	0	50 (100)	20 (40)	30 (60)

HPV=Human papillomavirus

Table 4: Immunoexpression of p16^{INK4a} and human papillomavirus 16 in cervical premalignant and malignant lesions

Histopathological diagnosis	Number of cases	p16 negative (Grade 0,1)	p16 positive (Grade 2,3)	HPV 16 negative	HPV 16 positive
LSIL	16	10	6	2	14
HSIL	3	1	2	0	2
WD SCC	1	-	1	-	1
MD SCC	22	1	21	1	21
PD SCC	3	-	3	-	3
Basaloid SCC	1	-	1	-	1
Adenocarcinoma	4	-	4	-	4
Total	50	12	38	3	47

LSIL=Low-grade squamous intraepithelial lesion, HSIL=High-grade squamous intraepithelial lesion, SCC=Squamous cell carcinoma, WD SCC=Well-differentiated SCC, MD SCC=Moderately differentiated SCC, PD SCC=Poorly differentiated SCC, HPV=Human papillomavirus

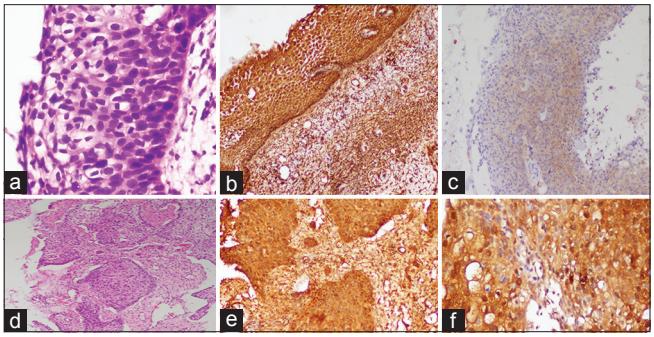

Figure 2: (a) Histopathological section showing high-grade squamous intraepithelial lesion (H and E, ×40), (b) positive immunohistochemical expression of human papillomavirus in high-grade squamous intraepithelial lesion (HPV, ×10), (c) Grade 2 (positive) immunohistochemical expression of p16^{INK4a} in high-grade squamous intraepithelial lesion (p16, ×10), (d) moderately differentiated squamous cell carcinoma (H and E, ×10), (e) positive immunohistochemical expression of human papillomavirus in moderately differentiated squamous cell carcinoma (HPV, ×10), (f) Grade 3 (positive) immunohistochemical expression of p16^{INK4a} in moderately differentiated squamous cell carcinoma (p16, ×40)

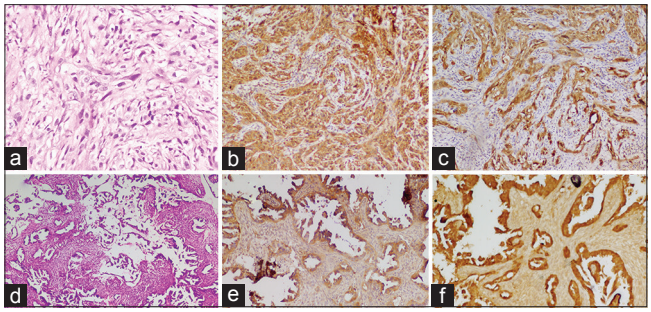

Figure 3: (a) Histopathological section showing sarcomatoid squamous cell carcinoma (H and E, ×10), (b) positive immunohistochemical expression of human papillomavirus in sarcomatoid squamous cell carcinoma (HPV, ×10), (c) Grade 3 (positive) immunohistochemical expression of p16^{INK4a} in sarcomatoid squamous cell carcinoma (p16, ×10), (d) papillary adenocarcinoma (H and E, ×10), (e) positive immunohistochemical expression of human papillomavirus in papillary adenocarcinoma (HPV, ×10), (f) Grade 3 (positive) immunohistochemical expression of p16^{INK4a} in papillary adenocarcinoma (p16, ×10)

of cervical cancer.[14] The common presenting complaints of females in the study were itching and vaginal discharge which may be due to genitourinary tract infection and further associated with HPV infection, leading to increased chances of cervical cancer. An interesting finding that was observed in the present study was that 67% of subjects (including cases and controls) and 94% of the premalignant and malignant cases were positive for HPV 16 expression. This indicates a high prevalence of HPV 16 infection in this hilly state of India and authors therefore suggest further larger studies to study the prevalence of high-risk HPV genotypes in general female population of this state. This would be in turn beneficial to formulate an effective cervical cancer screening program in this region as HPV prevalence has still not been studied here.

An interesting finding that was observed in the present study was that 94% of cases were positive for HPV 16 and 76% of cases were p16^{INK4a} positive. In contrast, none of the control showed positivity for p16^{INK4a} although 40% of them were positive for HPV 16 [Table 4]. This suggests that HPV 16 infection gets clear in controls while cases in which HPV leads to neoplastic transformation identified by the overexpression of p16^{INK4a} show premalignant and malignant changes. Thus, p16^{INK4a} which is a cyclin-dependent kinase inhibitor may be used as a marker to identify the HPV-induced malignant transformation in cervical cancer. The E7 viral oncogenic protein overexpression due to HPV integration in host cell genome leads to binding of E7 with tumor-suppressor protein, retinoblastoma gene product which further causes enhanced expression of p16^{INK4a}.[15] Bergeron *et al*. have also concluded that p16^{INK4a} can serve as a useful biomarker in the diagnostic workup of patients with HPV infection and associated lesions of the female anogenital tract.[16] It was also observed in our study that p16^{INK4a} was positive in 96% of invasive cervical cancer, 66.6% in HSIL, and 37.5% in LSIL, while it was negative in all the nonneoplastic controls [Figures 1-3]. This suggests that p16^{INK4a} immunoexpression increases with the severity of cervical lesion and thus may be helpful in stratification of the cases according to severity. In addition, it may also be helpful in stratification of LSIL and nondysplastic cases showing equivocal histopathological features. Few recent studies have also observed that p16^{INK4a} immunopositivity was higher in HSIL and SCC in comparison to LSIL and nondysplastic lesions.[17,18] However, rates of immunopositivity are variable in different studies in various lesions. In the present study, invasive carcinoma showed the highest rate of immunopositivity followed by HSIL and LSIL. In contrast, Cheah *et al*. have shown highest rate of immunopositivity in HSIL (95.2%) followed by SCC (90.2%) and LSIL (3.7%).[18] This difference in expression may also depend on criteria which has been followed to define p16^{INK4a} immunohistochemical grading which has still not been standardized. Few studies include only the percentage of cells showing p16^{INK4a} immunopositivity for grading, while others follow more stringent criteria including both percentage and part of epithelial layers involved.[17,19] The present study has also included both the epithelial layers and percentage of cells involved as the criteria for p16^{INK4a} immunopositivity. Although adenocarcinoma included only 8% of total cases in our study, it did not show

any difference of expression in HPV 16 and $p16^{INK4a}$ in comparison to SCC, thus suggesting that HPV 16 is related to both the histopathological types and follows the same pathway of carcinogenesis [Figure 3]. The important limitation of the present study was that lesser number of cases included in the study, and therefore, authors suggest that larger study should be done in this region to clearly establish the association of HPV 16 and $p16^{INK4a}$ and explore the utility of $p16^{INK4a}$ as a biomarker in the routine diagnostic workup of cervical premalignant and malignant lesions.

Conclusion

The cervical premalignant and malignant lesions are associated with low socioeconomic status, illiteracy, smoking, early age of marriage, and conception in this North Himalayan Region of India. HPV 16 infection is positive in both cases and controls indicating high prevalence of HPV 16 in this region. This in turn may be beneficial to formulate an effective HPV-based cervical cancer screening program in this region. Neoplastic transformation by HPV is identified by over expression of $p16^{INK4a}$ in premalignant and malignant cases, which plays an important role in cervical carcinogenesis. The immunopositivity of $p16^{INK4a}$ increases with the severity of cervical lesions and thus may play an important role in the stratification of premalignant and malignant lesions of the cervix.

Further larger studies may be done in this region to assess the role of $p16^{INK4a}$ as a biomarker in routine diagnostic workup of cervical lesions.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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