# Detection of Human *Papillomaviruses* (HPVs) by Immunohistochemistry (P16^ink4a) In Verrucous Skin Lesions

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*Authors' contributions*

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

*Original Research Article*

## ABSTRACT

Even though the p16 marker allows us to distinguish between benign and malignant verrucous skin lesions, we are unable to determine the patient's Human *Papillomaviruses* (HPVs) status despite the existence of recognized histopathological features that indicate its presence like koilocytic change, since a proper correlation couldn't be made between this feature and p16 expression. Even though PV type 16 and 18 are a risk factor for developments of anogenital skin lesion diffuse p16 expression cannot always be attributed to HPVs as there may be several other risk factors causing skin lesions, unlike in cervical lesions such as squamous cell carcinomas, many studies have established the role of oncogenic HPVs with its carcinogenesis. This marker cannot be used as a surrogate for detection of HPV infection. The present study was the expression of p16INK4A in histological sections of verrucous skin lesions. To compare the expression of p16INK4a in benign, premalignant and malignant lesions involving the skin and comparing the pattern of expression of p16INK4a in skin lesions by immunohistochemistry and correlating the results with certain histological parameters that might indicate HPV infection.

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# 1. INTRODUCTION

HPVs are a group of more than 200 DNA viruses that infect human epithelial cells. It commonly causes skin/mucosal squamous intraepithelial lesion-low grade. HPV infection involves the genital area and perianal skin mostly. Beyond this, more common are warts with an array of lesions as Common skin warts, verruca vulgaris, Plantar warts, verruca plantaris and verruca plana. It shows a propensity to invade actively differentiating epithelial squamous cells and almost any part or location of human skin can be infected [1].

Over 200 HPV types have been described based on their DNA sequences similarity, distributed over 5 genera (Alpha, Beta, Gamma, Mu and Nu *Papillomaviruses*. Each genus is further divided into species containing one or more PV genotypes)Presently, Over 200 HPV types have been described based on their DNA sequences similarity, distributed over 5 genera (Alpha, Beta, Gamma, Mu and Nu *Papillomaviruses*. Each genus is further divided into species containing one or more PV genotypes) [2]. Oncogenic HPVs are very common causes of cervical cancer (especially HPV16 and 18 that cause 70% of cervical cancer), the second most common cancer in females all over the world. Risk of acquiring HPV infection is high, especially for young and sexually active people, so more common in cervical than skin lesions [3]. In India, poor hygiene, malnutrition, oral contraceptives and lack of awareness are significant risk factors for HPV infection [4]. HPVs are can cause other types of cancers such as anus, vulva, vagina, penis and head and neck cancers [5]. HPVs can be HPVs can be divided into cutaneous and mucosal types. HPVs are divided into low risk and high risk groups based on oncogenicity Based on oncogenicity [6-7]. A large epidemiologic study has identified 15 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58,59, 68, 73, and 82), 3 probable high-risk HPV types (26, 53, and 66), and 12 LR HPV types (6, 11, 40, 42, 43, 44, 54,61, 70, 72, 81 [8]. Role of p16 Genetic susceptibility is a multistep, complex process that includes initiation, promotion and progression. The most important and decisive event of the chemical carcinogenesis is between presumed carcinogens and macromolecules such as DNA, proteins and lipids [9]. These genetic damages result in tumors by disrupting the normal regulatory pathways that control basic cellular functions.

Recently, it is found that malignancy arises from accumulation of mutation in two major categories of genes, which are proto-oncogenes and tumor suppressor genes [10]. Tumor suppressor genes stop the growth of tumors by arresting cells in cell cycles [11]. The expressed product suppresses the expression or function of the other genes involved with cell growth and proliferation [12]. P16 was discovered in 1993 by Manuel Serrano, Gregory J. Hanson, and David Beach and was characterized as a protein functioning as cyclin dependent kinase inhibitor. It is a tumor suppressor protein encoded by CDKN2A gene [13-14]. It plays an important role in cell cycle regulation by decelerating cell progression from G1 phase to S phase acting as a tumor suppressor notably in melanomas, oral squamous cell carcinomas, cervical cancer and esophageal carcinomas.

p16 has an open reading frame of 148 amino acids encoding a molecular weight of 16,533 Da comprising of 4 ankyrin repeats, and hence the name is derived from its molecular weight with role in inhibiting CDK4 [15]. The other names are p16INK4a, CDKN2A, CDKN2, Multiple Tumor Suppressor 1, TP16, ARF, and MLM. This study was carried out mainly to see if histopathology and immunohistochemistry with p16INK4a was an effective surrogate over the more expensive and tedious DNA amplification techniques for detection of HPV in verrucous skin lesions.

# 2. MATERIALS AND METHODS

## 2.1 Study Design

The present cross-sectional study was a retrospective and prospective study conducted in the Department of Pathology during the period of December 2017-September 2019.

A total number of 50 cases of formalin fixed, paraffin embedded tissue and punch biopsy samples from lesions involving the skin were collected. 10 out of which were controls. STUDY POPULATION: They were divided into control group and study group.

The control group consisted of 10 formalin fixed paraffin embedded samples – 3 diagnosed squamous cell carcinoma of cervix samples and 7 normal skin samples with age group ranging from 22 years to 70 years.

The study group consisted of 40 formalin fixed paraffin embedded samples further sub-grouped as:

Group 1: 22 samples Benign verrucous skin lesions.

Group 2: The research group consisted of 40 formalin-fixed paraffin-embedded samples that were further divided into the following categories: 22 samples in Group 1 benign. Verrucous skin lesions 8 samples in Group 2 Premalignant verrucous skin lesions 10 samples in Group 3 Malignant verrucous skin lesions. Relative clinical history was obtained.

## 2.2 Inclusion Criteria

Control and study groups as mentioned above.
Skin and punch biopsy specimens with a verrucous appearance.
Skin and punch biopsy specimens with HPV infection as a risk factor.
Skin and punch biopsy specimens with at least1 histological feature suggestive of HPV involvement.

## 2.3 Exclusion Criteria

Skin and punch biopsy specimens which are not received in formalin.

Skin samples that have no HPV infection as a risk factor. Biopsy specimen from patients who underwent chemotherapy or radiotherapy as it will modify the morphology and antigen expression.

## 3. RESULTS

The present study was designed to determine the expression of p16 in benign, premalignant and malignant verrucous skin lesions. The control group (C) consisted of 10 formalin fixed paraffin embedded tissue samples which included 3 squamous cell carcinoma of cervix samples and 7 skin samples with normal histology. The study group included histologically diagnosed formalin fixed, paraffin embedded tissue samples of skin and punch biopsy specimens grouped into: Group I (SI) – Benign skin lesions; Group II(SII) – Premalignant skin lesions and Group III(SIII) – Malignant skin lesions.

## 4. DISCUSSION

The gene expression of p16INK4A is prominent in premalignant and malignant lesions where it acts as a cell cycle inhibitor by binding to CDK4/6 and prevents its interaction with cyclin D and thereby arresting cell proliferation and arresting carcinogenesis.

In my current study, expression of p16 in verrucous premalignant and malignant lesions of skin evaluated, showed high expression but none in the benign verrucous lesions.



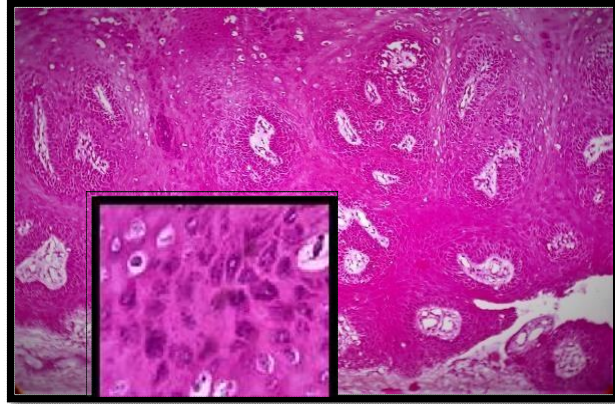**Fig. 1. Positive control SCC of Cervix - Diffuse p16 pattern(X100) benign verrucous lesions**

**Fig. 2. Verruca vulgaris with foci of koilocytic change(X100). Inset - Koilocytic change & Keratohyaline granules(X400)**
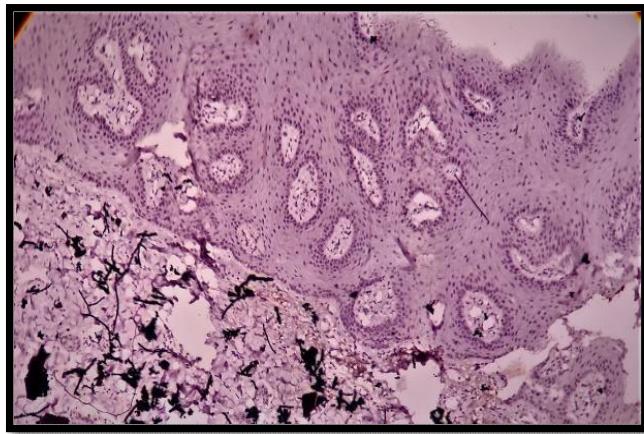


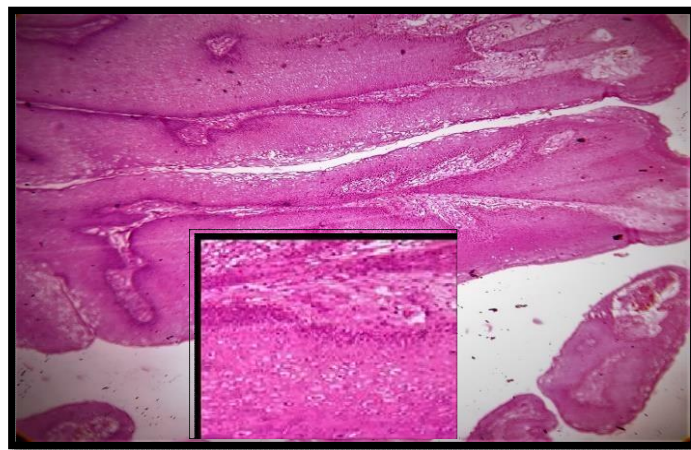**Fig. 3. Verruca vulgaris - Negative p16 staining(X100)**



**Fig. 4. Cornucutaneum with hyperkeratosis, papillomatosis & acanthosis(X40). Inset - Many koilocytes(X100)**
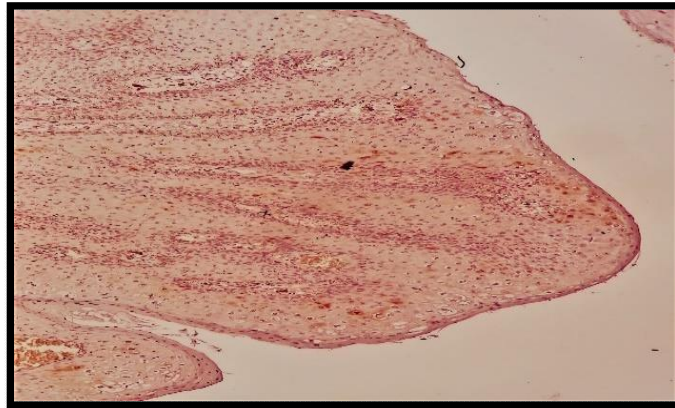
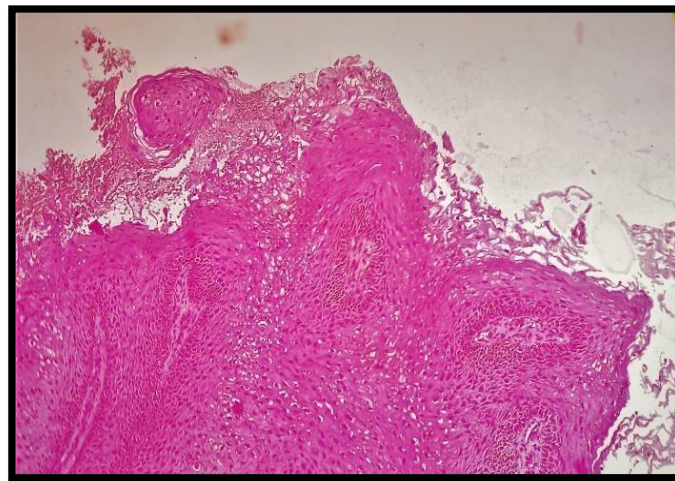**Fig. 5. Cornu cutaneum - equivocal p16 staining(X100)**
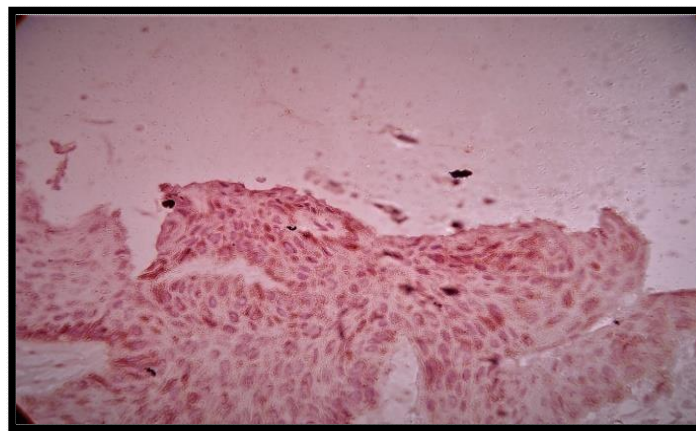


**Fig. 6. Condyloma acuminatum (X100)**



**Fig. 7. Condyloma acuminatum - Area with Focal p16 staining(X400) Color Charts of Premalignant lesion**
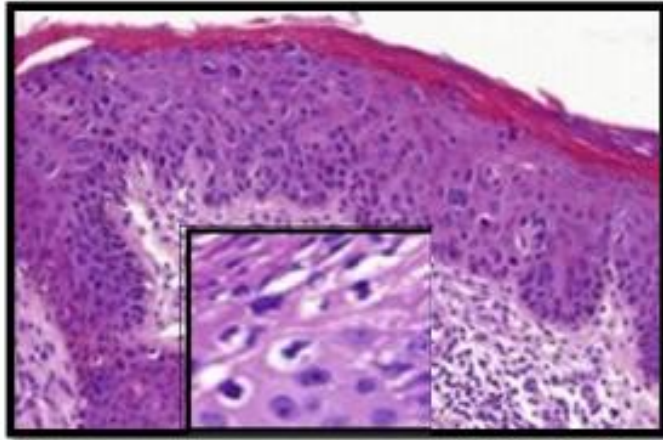
**Fig. 8. Bowen's disease with dysplasia in any layer (X100). Inset - foci of koilocytes (X400)**



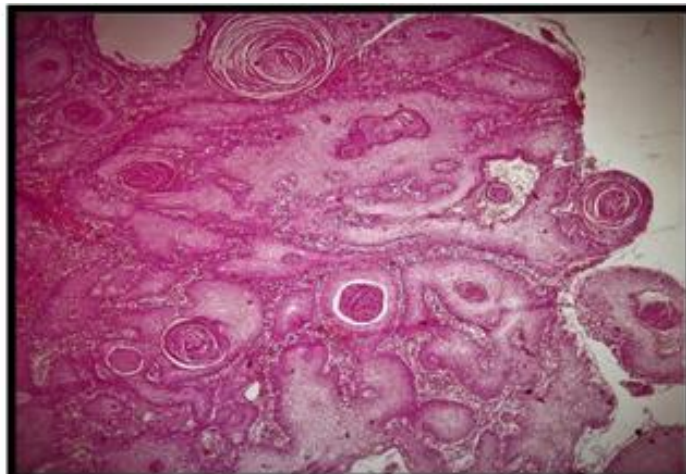**Fig. 9. Bowen's disease - diffuse nuclear & cytoplasmic staining with p16**



**Fig. 10. Well differentiated SCC of skin - Nests of squamous epithelium and extensive keratinization (X40)**
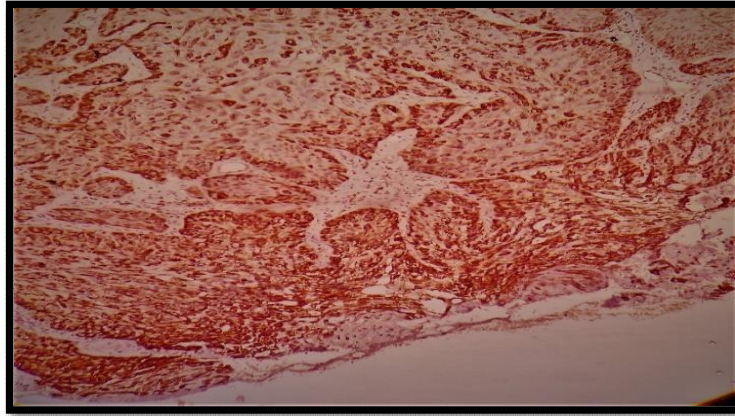
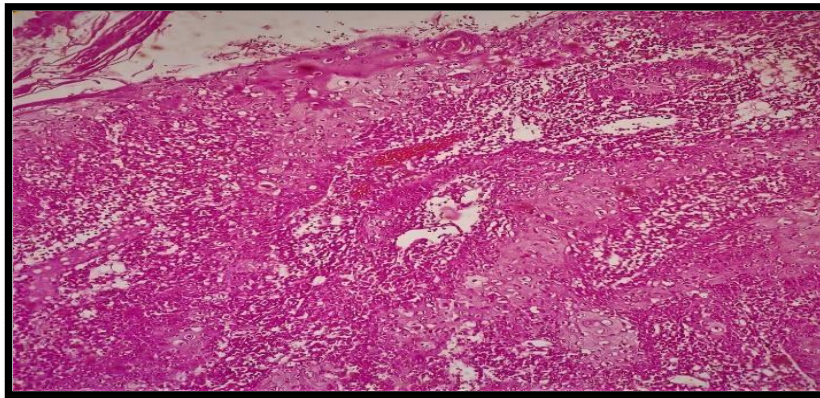**Fig. 11. Well differentiated SCC of skin - diffuse nuclear and cytoplasmic staining with p16(X100)**



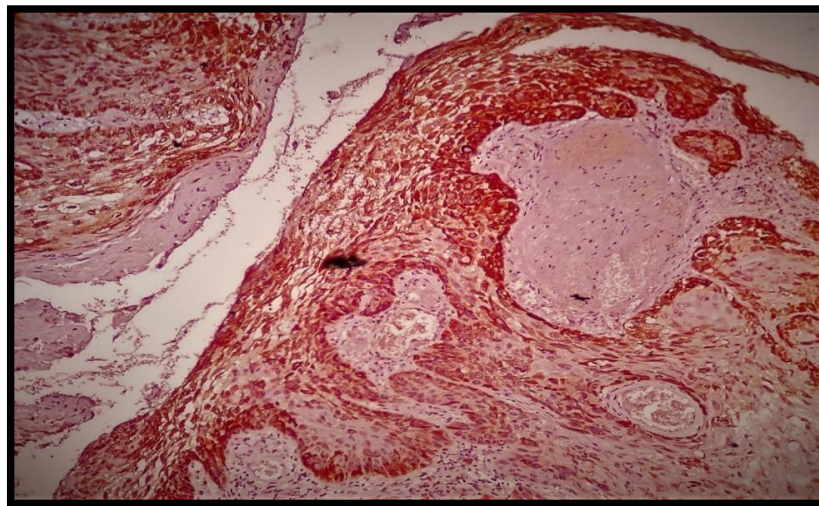**Fig. 12. Moderately differentiated SCC(X100)**



**Fig. 13. Moderately differentiated SCC of skin - Diffuse p16 staining(X100)**
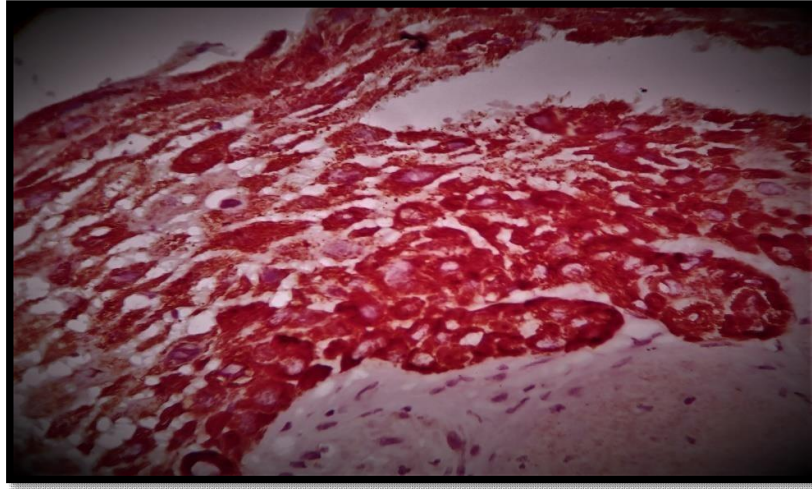
**Fig. 14. Moderately differentiated SCC of skin - diffuse nuclear & cytoplasmic staining with p16(X400)**

The other previous studies which are either in support or not in support of the results I have obtained are:

Dunwell P et al. [16]. According to this study carried out in the Afro-carribean population, most skin lesions belong to the benign category. The commonest skin diseases seen were acne vulgaris made up to 29.2%, seborrhea eczema made up 22.0%, pigmentary disorders summed up to 16.6%, and finally atopic eczema around 6.1%. Other lesions also included viral warts. This is in support of the present study where most of the skin lesions selected turned out to be benign or even inflammatory lesions. Benign lesions made up about 55% of all the lesions in my study.

Kilkenny M et al. [17]. A series of epidemiological studies were carried out for warts and the author concluded that by cross-sectional studies done in schools showed incidence in children to vary from 2 to 20% besides that, handlers of meat, domestic fowls and fish have a greater risk than other workers. He went on to say that children and young adults(late teens to early twenties to thirties) are the groups most affected, even though warts are very common skin lesions caused by HPV affecting most people at some point in life. This is in correlation with my study wherein more than two-thirds of the benign skin lesions are composed of warts including verruca vulgaris, plantar warts, cornu cutaneous, and condyloma. But the mean age from my study for benign skin lesions turned out to be 43 years on average (Middle adulthood: 41 – 64 years),

minimum age being 8 years and maximum being 67 years. This is not in coherence with my study.

Kyriako's K et al. [18]. A similar study was done wherein a small observational studies carried out suggested that 5 –30% of children and young adults have warts, again it is not in support of the present study.

Seung-Hwan Choi et al. [19]. This study was conducted on the Korean population in Busan city with a total of 1292 cases. The average premalignant lesion of skin yearly incidence was 1.8%, and it constantly increased from 0.7% to 4.3% over the period. The most common cutaneous premalignant lesion was Actinic Keratosis (75.9%), followed by Bowen's Disease (24.2%). Mean age of onset was 68.8 years (men, 70.9 years; women, 65.6 years), and the male: female ratio of patients was 1:1.5. The results from the present study showed that premalignant lesions make up 20% of the lesions out of which Bowen's disease makes up 37.5% of the premalignant lesions making it the more common among the list of premalignant lesions in my study, therefore there is a close correlation to the above study. The mean age of onset for the premalignant group of skin lesions is 58.8 years and the mean age of onset for Bowen's disease turned out to be 68.7 years which is in support of the present study. But the male: female ratio in the premalignant lesions of the present study unfortunately was also not correlating with the above study. It turned out to be 7:1. Probably a larger sample would have negated the error in ratios.

Jianjun Liu et al. [20]. In this study, 15,384 college students in three colleges in Beijing city were examined for the presence of cutaneous warts, and a follow up was done 2 to 3 years later. A total of 215 students were identified with warts. More in males than in female students (2.0% vs. 0.9%, P < 0.0001). The present study showed a higher prevalence in males (82%) than females (18%), hence the above study is in favor of the present study [20].

Vergilis-Kalner IJ et al. [21]. Information on the distribution of Keratoacanthomas vary amongst the sexes. Estimates of the sex ratio range from a similar incidence in both sexes to a three times more risk for KA among males. In the present study all Keratoacanthoma cases were from males, so as per the above literature sex ratio varies and hence a proper correlation cannot be made.

Schwartz RA et al. [22]. Solitary type of KA shows a peak between the ages of 50 and 69 according to a previous literature. And it rarely occurs under 20 years of age. This study is not in support of the present study where the mean age was 42 years.

Marián Švajdler et al. [23]. This study describes about certain histopathological features that might guide us in the detection of HPV infection. Koilocyte-like changes and papillomatosis are two findings that are frequently seen in HPV associated lesions, especially genital warts or condylomas. Even though these features were found more in HPV-positive compared to HPV-negative BD specimens, the variations in proportions were not statistically significant; therefore it was concluded that these features can't be used as reliable markers for BD cases to detect HPV. This finding also was similar to the present study were in koilocytic change was not significant and that there was no correlation with p16 expression.

Emad Kaabipour et al. [24]. In this study p16 staining appeared to be variable in both keratoacanthomas and SCC, but more number of cases of SCC showed diffuse positivity. This finding is consistent with the present study since all keratoacathoma cases showed only equivocal staining pattern. Whereas SCC were mostly showing Diffuse positivity.

Salama et al. [25]. There were 22 cases each of Bowen's disease and actinic keratosis. All the cases turned out to be negative with p16

expression, this finding was definitely against the results of the present study where there was a significant correlation between premalignant skin lesions like Bowen's with p16 expression showing diffuse pattern of staining. The author relates this issue with the fact that most of these lesions were not on UV sun-exposed areas of the skin.

Patel A et al. [26]. According to this study 70% of SCC in situ cases, 100% of invasive SCC showed full thickness diffuse pattern of staining. This was in agreement with our study especially for SCC insitu where 67% lesions showed diffuse block-type positivity.

Sauvarat et al. [27]. According to this study, 80.4% cases of Bowen's disease was positive for p16 and only 42.9% cases of Bowenoid papulosis showed positivity. This is in support of our study where mainly Bowen's disease cases were positive for p16 amongst the premalignant skin lesions.

Walts AE et al. [28]. This study deals with a variety of anal lesions which included mainly condyloma acuminatum and anal intraepithelial neoplasia (AIN) of varying degrees. A diffuse band-like pattern (>90% cells) of p16 staining was noticed in about 22% AIN I, 80% AIN II, and 88% AIN III cases. Not even 1 of the condylomas showed band-like p16 positive staining. Spotty (<10% cells) p16 expression was Observed in 8% of condylomas, 14% AIN I, 12% AIN II, and 13% AIN III cases. Therefore this study concluded that a band-like p16 pattern and Ki67 positivity in >50% of the squamous cells were strongly related to high-grade lesions. Whereas, absence of a p16 band-like staining along with Ki67 positivity in <50% of cells was mostly seen in benign lesions. The author also stated that band-like diffuse p16 staining also correlated strongly with the presence of oncogenic high-risk HPV. This finding was in support of our study, where less p16 expression was associated with benign lesions like condylomas in which 16% cases showed equivocal pattern of staining and higher grade lesions were associated with higher p16 expression.

Roel E Genders et al. [29-30]. In this study, 19% actinic keratosis, 92% Bowen's disease, 35% SCC and 12% benign keratotic lesions revealed more than 15% p16 -positive cells in the lesion. Strong p16-positivity was detected in 16% of actinic keratosis, 80% of Bowen's disease, 18%

of squamous cell carcinomas and 13% of benign keratotic lesions. This was in support of our study for higher grade lesions. But in my study all Bowen's disease cases showed diffuse positivity and only 70% SCC cases showed the same findings.

Sarier et al also showed that TUR may be the preferred option for the management of CA in the urinary bladder [31-32].

## 5. CONCLUSION

Expression of p16INK4A runs against the normal cycle of a cell. In case of carcinogenic pathophysiology, the expression of p16INK4a occurs in order to act as a cell cycle inhibitor by binding to CDK4/6, preventing its interaction with cyclin D, thereby arresting cell proliferation which in turn leads to a halt in carcinogenesis. In most premalignant and malignant lesions, p16INK4A is highly (Diffuse) expressed, thereby limiting the cell cycle  and putting a check on cells turning malignant and so p16 is found to be high in both verrucous premalignant and malignant lesions not in benign warts or inflammatory conditions. In malignancy, there is loss of p16 activity and hence there is a high turnover rate of cells without any checkpoint which is the cause for malignancy.

The current study supports this hypothesis and concludes it by saying whenever a skin biopsy is taken; expression of p16 can help in determining whether the sample is malignant or benign and can help with the prognosis of the condition. But true delineation cannot be made between premalignant and malignant skin lesions as stated earlier, both may show high p16 expression. Thus an inference can be made stating that p16 may be considered as a tumor marker and can be used in conjunction with routine hematoxylin & eosin slides to help in determining the prognosis of the patient.

With regards to verrucous skin lesions, even though we can make a distinction between benign and malignant with p16 marker, we cannot arrive at a conclusion with regards to the HPV status of the patient even with known histopathological features indicative of its presence, like koilocytic change, since a proper correlation couldn't be made between this feature and p16 expression. Even though HPV maybe a risk factor, especially in genital skin lesions, diffuse p16 expression cannot always be attributed to HPV as there may be several other risk factors causing skin lesions, unlike in cervical lesions such as squamous cell carcinomas, many studies have established the role of oncogenic HPV with its carcinogenesis. This marker cannot be used as a surrogate for detection of HPV infection.

## CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

## ETHICAL APPROVAL

Ethical clearance for the study was obtained from the Institutional Human Ethical Committee, SBMCH.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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