

Combined Cytological, Immunohistochemical, and Molecular Detection of Human Papillomavirus in Cervical Intraepithelial Lesions

Roaa Basim Najem¹, Marwa Jamal Hussain Al-Kinani^{2*}, Alaa Yacooab Rahee³

^{1,3} Department of Science, College of Basic Education, University of Sumer, Dhi Qar, Iraq

² College of Medicine, University of Sumer, Dhi Qar, Iraq

* Corresponding Author Email: marwa.jamal@gmail.com

Abstract

This study included 50 cervical specimens collected from women aged 25–60 years attending hospitals in Baghdad, Iraq. Normal cervical epithelium, cervical intraepithelial neoplasia CIN1 and CIN2 were included in this study. Specimens were evaluated using Pap smear cytology, p16 immunohistochemistry, and Real-Time PCR for detection of HPV-associated cervical abnormalities. Human papillomavirus (HPV) is strongly associated with cervical carcinogenesis through the action of viral oncogenes E6 and E7. The results showed that cervical lesions were most common among women aged 30–49 years. Histopathological examination revealed that CIN1 represented 60% of cases, while CIN2 accounted for 30%, and normal cervical tissue represented 10%. Cytological findings demonstrated that LSIL was the most frequent diagnosis (50%), followed by HSIL (24%). Positive p16 expression increased with lesion severity, while Real-Time PCR demonstrated high sensitivity for detecting high-risk HPV DNA. Combined diagnostic methods improved early detection and diagnostic accuracy of HPV-associated cervical lesions.

Keywords: Human papillomavirus, Pap smear, p16, PCR, cervical cancer, immunohistochemistry.

الكشف المشترك الخلوي والمناعي النسيجي والجزئي عن فيروس الورم الحليمي البشري في الآفات داخل الظهارة العنقية

روى باسم نجم¹، مروة جمال حسين الكنانى² آلاء يعقوب راهي³

^{1,3} قسم العلوم، كلية التربية الأساسية، جامعة سومر، ذي قار، العراق

² كلية الطب، جامعة سومر، ذي قار، العراق

الخلاصة

شملت هذه الدراسة 50 عينة من أنسجة عنق الرحم جُمعت من نساء تراوحت أعمارهن بين 25–60 سنة من مراجعات مستشفيات في مدينة بغداد، العراق. تضمنت العينات نسيج عنق رحم طبيعي وآفات التكتس داخل الظهارة العنقية من الدرجتين الأولى والثانية (CIN1 وCIN2). تم تقييم العينات بواسطة فحوصات مختلفة منها الفحص الخلوي لمسحة بابانيكولاو، والكشف المناعي النسيجي لمعلم p16، وتقنية Real-Time PCR للكشف عن التغيرات المرتبطة بفيروس الورم الحليمي البشري (HPV). يُعد فيروس HPV من أهم العوامل المرتبطة بسرطان عنق الرحم من خلال تأثير الجينات الورمية E6 وE7. أظهرت النتائج أن الآفات العنقية كانت أكثر شيوعاً لدى النساء بعمر 30–49 سنة. كما بين الفحص النسيجي أن CIN1 تضمنت نسبة 60% من الحالات وCIN2 نسبة 30%، بينما مثلت الأنسجة الطبيعية 10%. وأظهر الفحص الخلوي أن LSIL كان الأكثر شيوعاً بنسبة 50% يليه HSIL بنسبة 24%. وازدادت إيجابية p16 مع شدة الإصابة، بينما أظهرت تقنية Real-Time PCR حساسية عالية في الكشف عن HPV عالي الخطورة.

1. Introduction

Human papillomavirus (HPV) is a small non-enveloped double-stranded DNA virus belonging to the Papillomaviridae family. HPV infects stratified squamous epithelial tissues of the skin and mucous membranes and is considered the most common sexually transmitted viral infection worldwide [1]. Persistent infection with oncogenic HPV types is strongly associated with the development of cervical cancer, which remains one of the leading causes of cancer-related mortality among women, particularly in developing countries [2]. More

than 200 HPV genotypes have been identified, of which approximately 14 are classified as high-risk oncogenic types. Among these, HPV 16 and HPV 18 are responsible for nearly 70% of cervical cancer cases globally [3]. In addition to cervical carcinoma, HPV infection has also been linked to malignancies of the vulva, vagina, anus, penis, and oropharynx [4].

HPV infection and its related malignancy are primarily mediated by the viral oncoproteins E6 and E7, which interfere with tumor suppressor pathways, such as p53 and retinoblastoma protein. These molecular alterations result in genomic instability, dysregulation of the cell cycle, and progressive neoplastic transformation [5].

Despite the high prevalence of HPV infection, most cases are transient and cleared spontaneously by the host immune response within one to two years. However, persistent infection with high-risk HPV types may result in cervical intraepithelial neoplasia (CIN), which can progress from low-grade lesions to invasive carcinoma if left untreated [6].

Early detection of HPV-associated cervical lesions is therefore essential for reducing morbidity and mortality. Several diagnostic approaches are currently available, including cervical cytology using the Papanicolaou (Pap) smear test, histopathological evaluation with p16 immunohistochemistry, and molecular detection using polymerase chain reaction (PCR)-based techniques. Each method has a unique diagnostic role and contributes significantly to screening, diagnosis, and patient management [7]. This study aimed to investigate the role of cytological, immunohistochemical, and molecular diagnostic techniques, including Pap smear, p16 immunostaining, and Real-Time PCR, in the early detection and evaluation of HPV-associated cervical epithelial lesions.

2. Subjects and Methods

2.1 Study Design and Sample Collection

This study included 50 women attending several hospitals in Baghdad City, Iraq. Cervical samples were collected from women undergoing cervical screening. Inclusion criteria included women aged 25–60 years diagnosed with normal cervix, CIN I, or CIN II lesions. Pregnant women and those with inadequate biopsy specimens were excluded. Pap smear and cervical biopsy specimens were obtained for cytological, histopathological, immunohistochemical, and molecular examinations. Patient information was obtained from all participants prior to sample collection.

2.2 Cytological Examination

Pap smear slides were prepared using routine technique and stained by Papanicolaou stain. Cytological results were classified according to the Bethesda System.

2.3 Histopathological Examination

Cervical specimens were fixed in 10% buffered formalin, processed routinely, embedded in paraffin, and stained with hematoxylin and eosin stain. Histopathological grading was classified into CIN1 and CIN2. Slides were evaluated by an experienced pathologist for definitive diagnosis.

2.4 Immunohistochemistry

Immunohistochemical (IHC) staining of p16 marker was evaluated according to the recommended criteria for block-type staining. IHC staining was performed using a monoclonal antibody against p16 protein. Strong diffuse nuclear and cytoplasmic staining in a significant proportion of epithelial cells was considered positive. The p16 immunostaining

score was evaluated semi-quantitatively according to staining intensity and extent of epithelial involvement.

2.5 Real-Time PCR for HPV Detection

DNA was extracted from cervical specimens using a commercial genomic DNA extraction kit according to the manufacturer's instructions. The concentration and purity of the extracted DNA were assessed using a NanoDrop spectrophotometer. For molecular detection of high-risk HPV DNA, Rapid Real-Time PCR was performed using specific primers targeting oncogenic viral genotypes. Amplification reactions were carried out using a PCR system with a final reaction volume of 25 μ L containing master mix, forward and reverse primers, and extracted DNA template. The thermal cycling conditions included initial denaturation at 95°C for 5 minutes, followed by 40 amplification cycles consisting of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 30 seconds. Positive and negative controls were included in each run to ensure assay validity. Samples with amplification curves crossing the threshold cycle (Ct) value were considered positive for HPV DNA.

2.6 Statistical Analysis

The collected data were statistically analyzed using the Statistical Package for Social Sciences (SPSS) software version 26. Descriptive statistical analysis was performed to calculate frequencies, percentages, means, and standard deviations. The association between categorical variables was evaluated using the Chi-square (χ^2) test. A p-value of less than 0.05 was considered statistically significant.

3. Results

In this study, 50 females were enrolled, aged between 25 and 60 years. Pregnant women were excluded to eliminate the effect of hormones on the morphology and immunohistochemical expression of cervical epithelial cells. Cervical intraepithelial lesions were most prevalent in women aged 30–49 years, the age bracket at greatest risk of HPV-associated cervical lesions.

Table 1- Age Distribution of Study Population

Age Group (Years)	Number of Cases	Percentage (%)
25–29	8	16%
30–39	19	38%
40–49	15	30%
50–60	8	16%
Total	50	100%

As shown in Table 1, the highest proportion of women was noted in the 30–39 years age group (38%), followed by the 40–49 years age group (30%). Women aged 25–29 years and 50–60 years each represented 16% of the study population, indicating that CIN1 and CIN2 lesions were more frequently detected among women aged 30–39 years.

Table 2- Histopathological Diagnosis Distribution

Diagnosis	Number of Cases	Percentage (%)
Normal cervix	5	10%
CIN I	30	60%
CIN II	15	30%
Total	50	100%

Table 2- presents the histopathological distribution of cervical lesions among the studied cases. CIN I was the most frequently observed lesion, representing 60% of cases, followed by CIN II (30%), while normal cervical tissue accounted for 10%. These results demonstrate a predominance of low-grade cervical intraepithelial lesions in the study population.

Table 3- Pap Smear Cytological Findings

Cytological Diagnosis	Number of Cases	Percentage (%)
Negative	5	10%
ASC-US	8	16%
LSIL	25	50%
HSIL	12	24%
Total	50	100%

Table 3 illustrates the cytological findings of the examined cervical smears according to the Bethesda System. LSIL represented the most frequent cytological diagnosis, accounting for 50% of the examined cases, followed by HSIL (24%). ASC-US was identified in 16% of cases, while negative cytological findings were observed in 10% of the study population. These findings indicate a predominance of low-grade squamous intraepithelial lesions among the studied women.

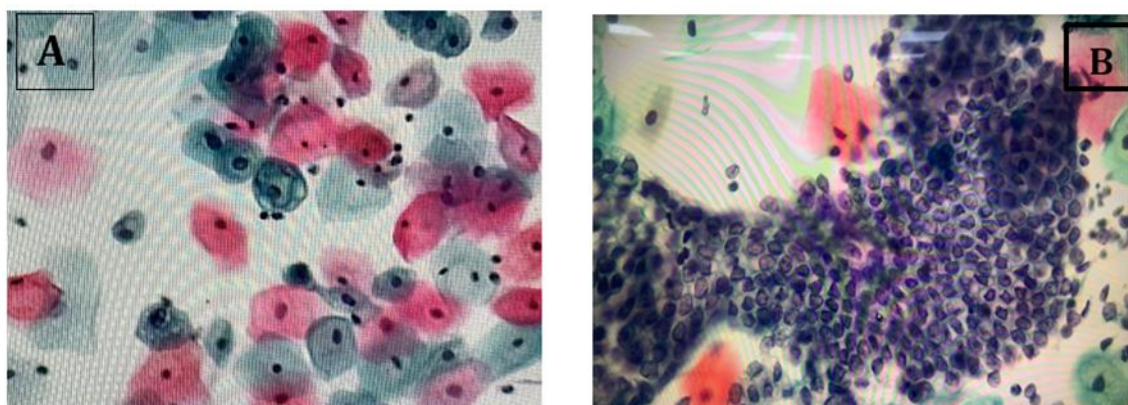


Figure -1 Pap smear cytology images (Pap stain, 400×). (A) Low-grade squamous intraepithelial lesion (LSIL/CIN I) showing cells with koilocytotic atypia characterized by perinuclear halo, nuclear enlargement, and mild hyperchromasia. (B) Cells showing HSIL with dyskeratosis.

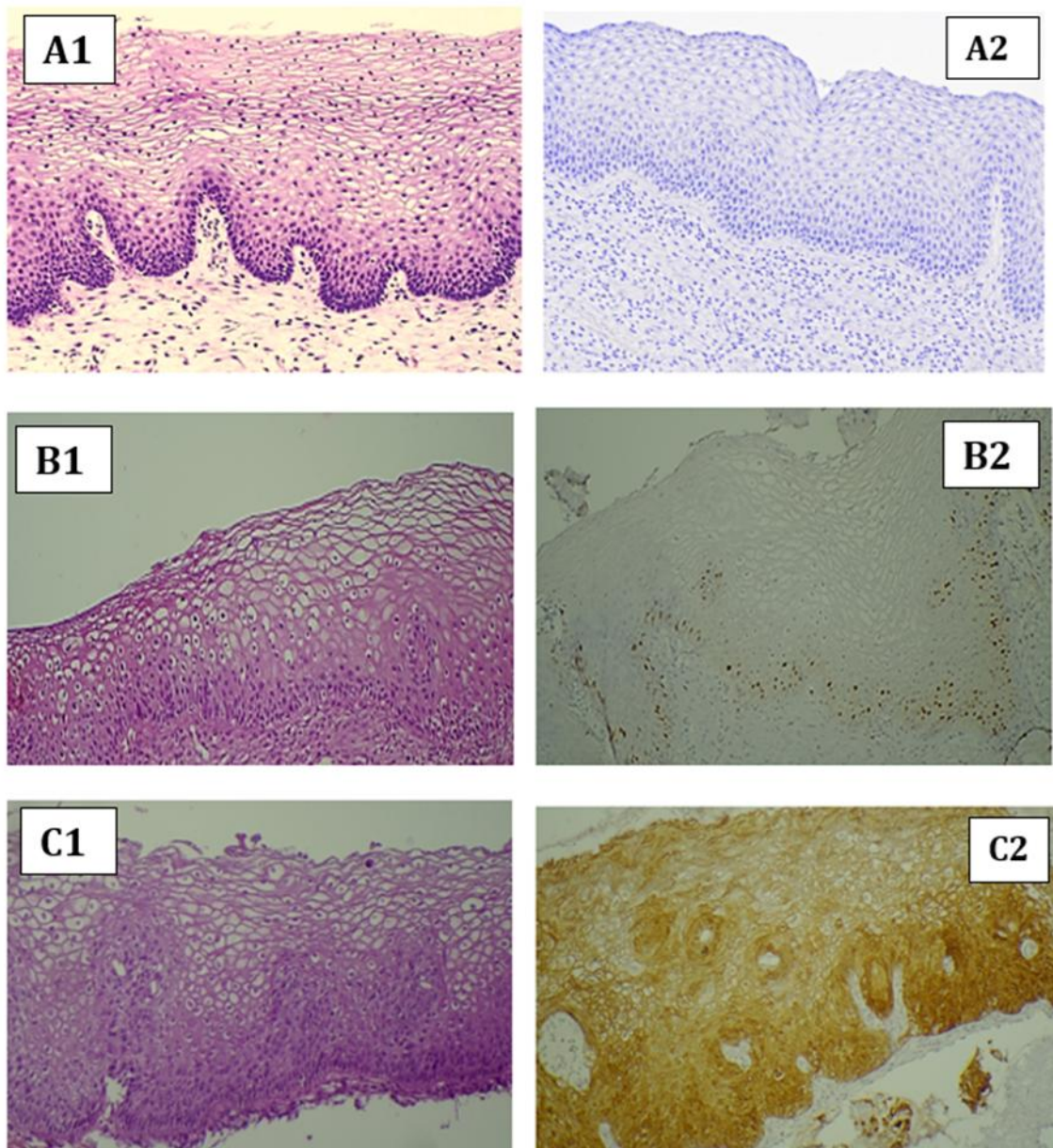


Figure -2 Histological sections of cervix. (A1) Normal squamous epithelium of the ectocervix with three layers, H&E stain, 100x. (A2) Negative p16 expression in normal cervix. (B1) CIN I/LSIL squamous epithelium with dysplastic changes in the lower third of the epidermis, H&E stain, 100x. (B2) CIN I cervical section showing p16-positive cell reaction in the lower third of epidermis, IHC staining, 100x. (C1) CIN II cervical section showing marked koilocytic change with squamous dysplasia confined to the lower third and focal extension to the middle third, H&E stain, 100x. (C2) CIN II cervical section showing positive p16 immunostaining in the middle third of epidermis, 100x.

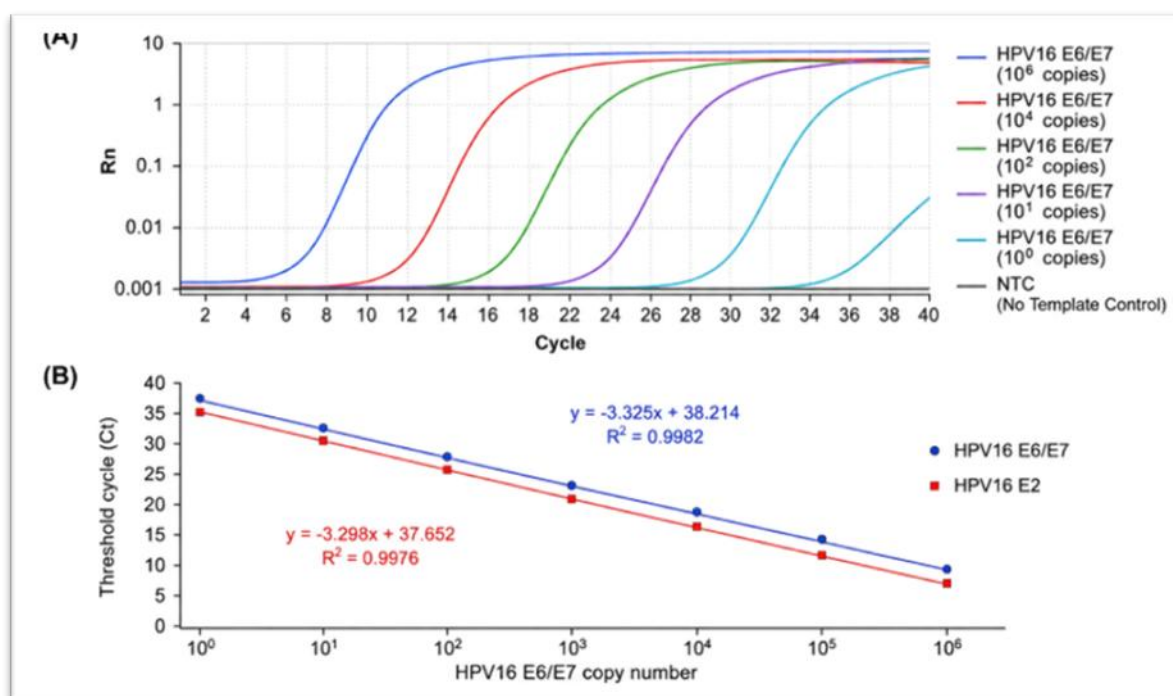


Figure -3 Detection of Human Papillomavirus (HPV16) by Real-Time PCR. (A) Amplification plots from serial dilutions of HPV16 DNA showing incremental increases in fluorescence intensity. (B) Standard curves showing Ct values graphed against the logarithm of HPV16 copy numbers, demonstrating high linearity and correlation coefficients (R^2).

4. Discussion

Infection with Human papillomavirus represents one of the leading causes of cervical epithelial abnormalities and cervical cancer worldwide. HPV16 and HPV18 genotypes play a chief role in the progression of both cervical intraepithelial neoplasia (CIN) and invasive cervical carcinoma [1, 8, 9].

Epidemiological results observed in the current study showed that the 30–49 years age group had the highest prevalence among the studied population, consistent with previous studies indicating that persistent HPV infection and cervical dysplasia are more frequently detected during the reproductive and perimenopausal age due to prolonged viral persistence and cumulative exposure to oncogenic HPV strains [10]. Moreover, the WHO has reported that reproductive and perimenopausal age represents the highest risk period for cervical lesions [11].

Histopathological examination established that CIN I lesions constituted the majority of diagnosed cases, followed by CIN II lesions. Similar findings were reported by Hosseini et al. [12], who observed that low-grade squamous intraepithelial lesions are the most frequently encountered abnormalities in cervical screening patients. The predominance of CIN I may reflect the transient nature of many HPV infections, as most low-grade lesions regress spontaneously through host immune responses [5].

The Pap smear remains one of the most significant screening tools for cervical cancer owing to its wide availability, low cost, and minimally invasive nature. In the present study, LSIL was the most frequent cytological diagnosis, followed by HSIL. These results are comparable to those reported by Perkins et al. [6], who found that low-grade cytological abnormalities are commonly associated with HPV infection. Nevertheless, cytological

examination alone may have limited sensitivity, particularly in equivocal lesions such as ASC-US, due to observer variability and sampling limitations [13]. Therefore, adjunctive diagnostic methods are essential to improve diagnostic accuracy.

Immunohistochemical expression of p16 showed increased positivity in CIN lesions compared with normal cervical tissues, in agreement with many previous studies demonstrating that p16 overexpression is strongly associated with transforming high-risk HPV infection [14]. The biological mechanism underlying p16 overexpression involves HPV E7-mediated inactivation of retinoblastoma protein (pRb), resulting in compensatory accumulation of p16 protein within dysplastic epithelial cells [5, 14]. In the current study, p16 positivity increased with lesion severity, supporting its usefulness as a biomarker for distinguishing dysplastic lesions from benign reactive changes.

Molecular detection methods are currently considered the gold standard for detecting HPV infection risk, as they detect viral nucleic acid directly and enable accurate genotyping [15]. The results of Real-Time PCR in the present study confirmed high sensitivity and specificity for detecting HPV DNA in cervical samples, as demonstrated through amplification and standard curve analysis. These findings are consistent with numerous studies that confirm the role of molecular HPV testing in the early detection of cervical lesions and in reducing false screening results [16].

The combination of cytological, histopathological, immunohistochemical, and molecular diagnostic methods in the present study highlights the importance of a multimodal diagnostic strategy for HPV-associated cervical lesions. This integrated approach enhances diagnostic accuracy, facilitates early detection of high-risk lesions, and contributes to improved patient management and cervical cancer prevention [1, 7].

5. Limitations of the Study

The present study had several limitations, including the relatively small sample size, exclusion of CIN III lesions, lack of long-term patient follow-up, and restriction to a single geographic region. Further large-scale multicenter studies are recommended to validate these findings.

6. Conclusions

This study demonstrated that early detection and diagnosis of HPV-associated cervical lesions are significantly improved when Pap smears, p16 IHC, and Real-Time PCR are used in combination. The most frequent findings were CIN I and LSIL, and p16 expression increased proportionally with lesion severity. The excellent sensitivity of Real-Time PCR in identifying high-risk HPV further supports the utility of integrated diagnostic techniques in cervical cancer prevention.

Ethical Approval

Ethical approval was granted by the Institutional Review Board (IRB) of the University of Sumer. All procedures were carried out in full compliance with the ethical guidelines of the Declaration of Helsinki (2013 revision) and the institutional research committee.

Acknowledgment

The authors would like to express their sincere gratitude to the staff of the Department of Pathology and the laboratory team at Medical City Hospital, Baghdad, Iraq, for their invaluable technical assistance and cooperation during sample collection and processing.

Special thanks are extended to the University of Sumer for providing academic and institutional support. The authors also acknowledge the patients who participated in this study for their willingness to contribute to advancing cervical cancer diagnostics.

Conflict of Interest

The authors declare that they have no conflicts of interest.

References

- [1] World Health Organization, "Human papillomavirus (HPV) and cervical cancer," Geneva, Switzerland: World Health Organization, 2024.
- [2] Centers for Disease Control and Prevention, "Genital HPV Infection – Fact Sheet," Atlanta, GA, USA: Centers for Disease Control and Prevention, 2024.
- [3] M. Arbyn, E. Weiderpass, L. Bruni, S. de Sanjosé, M. Saraiya, J. Ferlay, and F. Bray, "Estimates of incidence and mortality of cervical cancer associated with human papillomavirus worldwide," *Lancet Glob. Health*, vol. 11, no. 2, pp. e191–e203, 2023.
- [4] P. E. Castle, M. H. Stoler, T. C. Wright, A. Sharma, T. L. Wright, and C. M. Behrens, "Performance of p16 immunohistochemistry in cervical cancer screening and diagnosis," *Mod. Pathol.*, vol. 36, no. 4, Art. no. 100091, 2023.
- [5] P. A. Cohen, A. Jhingran, A. Oaknin, and L. Denny, "Cervical cancer," *Lancet*, vol. 393, no. 10167, pp. 169–182, 2019.
- [6] R. B. Perkins, R. S. Guido, P. E. Castle, D. Chelmow, M. H. Einstein, F. Garcia, W. K. Huh, J. J. Kim, A. B. Moscicki, R. Nayar, N. Wentzensen, M. Schiffman, and D. Saslow, "Updated guidelines for management of cervical cancer screening abnormalities and precancerous lesions," *J. Low. Genit. Tract Dis.*, vol. 28, no. 1, pp. 1–24, 2024.
- [7] M. Schiffman, N. Hyun, T. Raine-Bennett, H. A. Katki, B. Fetterman, J. C. Gage, and N. Wentzensen, "Real-time PCR and HPV molecular testing in cervical cancer prevention," *Int. J. Cancer*, vol. 152, no. 7, pp. 1284–1295, 2023.
- [8] H. Sung, J. Ferlay, R. L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, and F. Bray, "Global cancer statistics 2024: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA Cancer J. Clin.*, vol. 74, no. 1, pp. 1–35, 2024.
- [9] American Society for Colposcopy and Cervical Pathology, "Risk-based management consensus guidelines for abnormal cervical cancer screening tests and cancer precursors," ASCCP Guidelines Committee, 2021.
- [10] World Medical Association, "Declaration of Helsinki: Ethical principles for medical research involving human subjects," *JAMA*, vol. 310, no. 20, pp. 2191–2194, 2013.
- [11] D. Mauricio, B. Zeybek, J. Tymon-Rosario, J. Harold, and A. D. Santin, "Immunotherapy in cervical cancer," *Curr. Oncol. Rep.*, vol. 23, no. 6, Art. no. 61, 2021, doi: 10.1007/s11912-021-01052-8.
- [12] M. S. Hosseini, M. Talayeh, N. Afshar Moghaddam, M. Arab, F. Farzaneh, and T. Ashrafganjoei, "Comparison of Ki67 index and p16 expression in different grades of cervical squamous intraepithelial lesions," *Caspian J. Intern. Med.*, vol. 14, no. 1, pp. 69–75, 2023, doi: 10.22088/cjim.14.1.69.
- [13] J. McIntyre, R. Marima, B. Alabi, G. Ramkilawon, and B. Mosoane, "Immunohistochemical Expression of Programmed Death-Ligand 1 Associated with Human Papillomavirus-Driven High-Grade Cervical Intraepithelial Neoplasia in South African Women," *Onco*, vol. 6, no. 1, Art. no. 14, 2026, doi: 10.3390/onco6010014.
- [14] S. N. Ansari, R. Kunwar, and S. A. Vaidya, "Immunohistochemical Expression of P16 in Cervical Lesions: An Observational Study," *JNMA J. Nepal Med. Assoc.*, vol. 63, no. 291, pp. 823–827, 2025, doi: 10.31729/jnma.v63i2091.9228.

- [15] A. Hamod, M. Grigore, I. A. Vasilache, R. G. Ursu, O. Mihaela, R. Popovici, A. M. Grigore, L. Lozneau, D. C. Andronic, and M. Ciocoiu, "Prospective evaluation of cytology, CINtec and PD-L1 for the detection of cervical intraepithelial neoplasia: A single-center study," *J. Clin. Med.*, vol. 15, no. 3, Art. no. 1171, 2026, doi: 10.3390/jcm15031171.
- [16] M. B. Chin, G. Liu, and N. Mugo, "A framework for cervical cancer elimination in low- and middle-income countries: A scoping review and roadmap for interventions and research priorities," *Front. Public Health*, vol. 9, Art. no. 670032, 2021, doi: 10.3389/fpubh.2021.670032.