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LINE-1 ORF1p is a Promising Biomarker in Cervical Intraepithelial Neoplasia Degree Assessment

Réka Karkas, MD,† Khaldoon Sadiq Ahmed Abdullah, MSc,*† László Kaizer, MD, FRCPath,‡ Ádám Ürmös, BA,§ May Raya, MSc,*† Lilla Tiszlavicz, MD,‖ Tibor Pankotai, PhD,‡§¶ István Nagy, PhD,#** Lajos Mátés, PhD,* and Farkas Sükösd, MD, PhD‡*

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Abstract: Cervical intraepithelial neoplasia (CIN) represents a spectrum of preinvasive squamous lesions within the cervical epithelium, whose identification is a diagnostic challenge due to subtle histomorphological differences among its categories. This study explores ORF1p, a nucleic acid-binding protein derived from long interspersed nuclear element-1 (LINE-1), as a potential biomarker for enhancing CIN diagnosis. A comprehensive analysis of 143 cervical specimens, encompassing CIN I (n = 20), CIN II (n = 46), CIN III (n = 14), invasive cancer (n = 32), and nondysplastic cases (normal cervical epithelia (n = 24) and atrophy (n = 7) were conducted. ORF1p, Ki67, and p16 expressions were evaluated using immunohistochemistry. ORF1p immunopositivity was detected in the vast majority [110/112 (98.2%)] of dysplastic and neoplastic (CIN and invasive cancer) specimens, whereas 19/24 (79.2%) of normal cervical specimens lacked ORF1p expression. The observed pattern of ORF1p expression showed a progressively increasing extent and intensity with advancing CIN grades. CIN I exhibited mild ORF1p expression in the lower one or two-thirds of the cervical epithelium [14/16 (87.5%)], whereas CIN II demonstrated moderate to strong ORF1p expression spanning the lower two-thirds [29/46 (63.0%)]. Pronounced transepithelial ORF1p immunopositivity characterized CIN III cases [13/14 (92.8%)] and cervical cancer [30/32 (93.8%)]. These findings propose ORF1p as a valuable indicator even for detecting CIN I, effectively discerning them from normal cervical tissue ($p < 0.0001$). Our findings underscore the potential of ORF1p as an early diagnostic marker for cervical neoplasia.

Key Words: cervical intraepithelial neoplasia, diagnostic biomarker, ORF1p, LINE-1, carcinogenesis

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From the *Laboratory of Cancer Genome Research, Institute of Genetics, HUN-REN Biological Research Centre, Szeged, Hungary; †Doctoral School of Multidisciplinary Medical Sciences, University of Szeged, Albert Szent-Györgyi Medical School, Szeged, Hungary; ‡Department of Pathology, Albert Szent-Györgyi Health Centre, University of Szeged, Szeged, Hungary; §Genome Integrity and DNA Repair Core Group, Hungarian Centre of Excellence for Molecular Medicine, Szeged, Hungary; ‖Department of Pediatrics and Pediatric Health Centre, Albert Szent-Györgyi Health Centre, University of Szeged, Szeged, Hungary; ¶Competence Centre of the Life Sciences Cluster of the Centre of Excellence for Interdisciplinary Research, Development and Innovation, University of Szeged, Hungary; #Seqomics Biotechnology Ltd, Mórahalom, Hungary; and **Sequencing Platform, Institute of Biochemistry, HUN-REN Biological Research Centre, Szeged, Hungary.

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R.K. and K.S.A.A. are joint first authors

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Address correspondence to Farkas Sükösd, MD, PhD, Department of Pathology, University of Szeged, Állomás Street 1, Szeged H-6721, Hungary. E-mail: sukosd.farkas@med.u-szeged.hu, and Lajos Mátés, PhD, Laboratory of Cancer Genome Research, Institute of Genetics, HUN-REN Biological Research Centre, Temesvári Blvd. 62, Szeged H-6726, Hungary. E-mail: mates.lajos@brc.hu.

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Cervical cancer is a significant global health burden and a leading cause of cancer-related morbidity and mortality among women.[1] It is the fourth most common cancer in women worldwide,[2] primarily caused by persistent infection with high-risk human papillomavirus (HPV).[3,4] Despite advances in prevention and early detection, cervical cancer continues to pose a substantial threat, particularly in low and middle-income countries with limited access to screening programs and HPV vaccination.[5] Understanding the importance of early detection and timely intervention as key preventive measures can significantly reduce the burden of this disease and improve women's health outcomes worldwide.[6]

Preinvasive squamous lesions of the cervix, collectively known as cervical intraepithelial neoplasia (CIN), encompass a range of cellular changes that occur within the cervical epithelium. CIN is commonly classified into different grades based on the severity of the observed cellular abnormalities. The CIN I is a low-grade squamous lesion (LSIL) mostly caused by low-risk HPV types that regress spontaneously in more than three-quarters of cases. By contrast, high-grade squamous cell lesions (HSILs), such as

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CIN II and CIN III, have a significantly higher risk of progression to invasive cervical cancer. This difference highlights the clinical significance of accurate diagnosis.[6]

The diagnosis of CIN primarily relies on histological evaluation of cervical biopsy specimens. However, interobserver variability and subjectivity in interpreting histopathological features of CIN lesions can impact diagnostic accuracy and reproducibility.[7–10] Therefore, there is a growing need for objective and reliable biomarkers that can complement traditional diagnostic approaches and improve the accuracy of CIN diagnosis. The most commonly used immunohistochemical markers are Ki67 and p16.[11] Ki67 is a marker of cellular proliferation dominantly present at sites of ribosomal RNA transcription.[12] In the context of cervical lesions, a high Ki67 labeling index (with more than 30% of the cells exhibiting nuclear immunopositivity) is associated with more aggressive or advanced lesions, such as high-grade CIN or cervical cancer.[13] The p16, also known as p16INK4a, is a tumor suppressor protein that acts as a negative regulator of the cell cycle, inhibiting the phosphorylation of retinoblastoma (Rb) protein and preventing cell proliferation. In high-risk HPV-associated lesions, the viral oncoproteins E6 and E7 disrupt the normal function of p53 and Rb, respectively, leading to increased p16 expression as a cellular response to this dysregulation.[14] Consequently, cytoplasmic p16 overexpression has been recognized as a surrogate marker for high-risk HPV infection and a reliable indicator of HPV-associated neoplasia.[15,16] In the cases of HSIL and carcinoma in situ, HPV genotypes 16, 31, 33, and 35 are primarily involved. Consequently, these cases exhibit p16 immunopositivity.[4] Conversely, CIN I lesions are associated with a more diverse range of over 20 different HPV genotypes and are not expected to express p16. However, in 25% to 30% of LSIL cases, high-risk HPV infection can be identified, rendering their positive p16 status an insufficient classifier of the degree of CIN.[17,18] This incorrect application of p16 IHC can overdiagnose HSIL when performed on unequivocal low-grade lesions or when it upgrades questionable lesions based on non-block p16 staining patterns.[19] According to the follow-up studies, p16 immunopositivity does not correlate with LSIL to HSIL progression either,[17] so it is only the negative p16 staining result that has diagnostic value, suggesting a case of LSIL.

Long Interspersed Nuclear Elements 1 (LINE-1) are autonomous retrotransposons accounting for 17% of the genome, where their copy number reaches 500,000.[20] LINE-1 retrotransposons contribute to evolution and genomic diversity,[21–23] although they might have harmful genotoxic effects as well. LINE-1 elements can insert themselves into new genomic loci with a "copy-and-paste" mechanism by which they potentially disrupt the function of genes, contributing to the development of a variety of genetic disorders, including cancer.[24] The seeming conflict between their positive contribution to human evolution and their disease-causing genotoxicity is clarified by the fact that, under nonpathological conditions, their well-controlled activity is restricted to germ cells[25] and early embryonic development.[26] De novo retrotransposition events in the germline contribute to heritable genetic changes and the rate of new germline events was estimated as ~1 insertion for every 200 births based on genome comparisons.[27] The vast majority of these events have no discernible effect, but a very small proportion can cause familial inherited diseases.[24] By contrast, in differentiated tissues under nonpathological conditions, only sporadic and weak LINE-1 activity is observed in some epithelial cells and neurons.[28] However, when LINE-1 activity is initiated in somatic tissues, it leads to the development of cancers through the formation of cancer driver mutations.[24,29,30] In differentiated tissues, numerous defense mechanisms tightly suppress LINE-1 retrotransposition to maintain the integrity of the somatic genome.[31,32] However, under pathological conditions, these protective systems may decline, allowing the activation of LINE-1 elements, which in turn contribute to tumor development.

Active LINE-1 loci produce transcripts encoding 2 proteins, ORF1p and ORF2p. ORF1p acts as a high-affinity RNA binding protein and nucleic acid chaperone, playing a critical role in facilitating retrotransposition by binding to LINE-1 RNA and forming stable ribonucleoprotein particles.[33] ORF2p is responsible for reverse transcription and integration of LINE-1 elements.[34]

The emerging potential of ORF1p as a biomarker has been recognized in different types of cancer and their precursor lesions.[35] ORF1p expression serves as a characteristic feature in numerous cancer types, such as breast, ovarian, bladder, esophageal, and pancreatic cancer, among many others.[36–41] However, its association with CIN and cervical cancer has not been investigated.

This study aims to explore the expression of ORF1p in preneoplastic and neoplastic cervical specimens and evaluates its potential as a diagnostic biomarker for CIN. By investigating the diagnostic value of ORF1p, we aim to enhance the accuracy and objectivity of CIN diagnosis. Identifying a reliable biomarker like ORF1p could aid in risk stratification and reduce interobserver variability in CIN diagnosis.

MATERIALS AND METHODS

Tissue Specimens

Archived pathological samples collected for diagnostic purposes were used for TMA assembly. The study was performed on 112 selected formalin-fixed paraffin-embedded (FFPE) specimens of CIN I (n = 20), CIN II (n = 46), CIN III (n = 14), and cervical cancer (n = 32). The control group comprised 31 cases of nondysplastic cervical tissue obtained from patients who underwent total hysterectomy for uterine leiomyomas and uterine prolapsus. These specimens were further categorized into the following groups: normal (n = 24) and atrophic epithelium (n = 7). Cores were chosen from each case, and their representativeness was reviewed after H&E staining. For tumor specimens, the following parameters were investigated: tumor grade, primary tumor size, lymph

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node, and metastasis status (pTNM). All the patient data acquired in this study were anonymized and did not impact diagnosis or treatment. The age range of the patient cohort encompassed a spectrum of 22 to 83 years, with a mean age of 52 years, selected from the period between 2018 and 2022 at the Clinical Centre of Albert Szent-Györgyi Medical School at the University of Szeged, Hungary. (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/IJGP/A176) The research protocol was reviewed and approved by the Institutional Committee of Science and Research Ethics of the Medical Research Council, Budapest, Hungary (Reference number: BM/3049/2023).

Immunohistochemistry

Immunohistochemical staining was run on the Bond Max Autostainer (Leica Biosystems, Wetzlar, Germany) with Bond Polymer Detection System (Vision BioSystems, Newcastle upon Tyne, UK). Monoclonal mouse anti-p16 antibody (clone MX007, MAD-000690QD-12, Master Diagnostica S.L, Granada, Spain), monoclonal anti-Ki67 (clone SP6, #10080, Histopathology Ltd., Pécs, Hungary), and monoclonal anti-LINE-1 ORF1p antibody (clone 4H1, MABC1152, Millipore Darmstadt, Germany) were used. Negative controls were performed on all sections using an equivalent concentration of a subclass-matched IgG_{1K}. The IHC sections were scored by 2 experienced pathologists (L.K. and F.S.) independently to ensure interobserver agreement. The observers were blinded to any clinical parameter. A consensus outcome was reached in case of discordance.

To evaluate ORF1p immunostaining results, a modified version of the immunoreactive scale (IRS) by Remmele and Stegner was used.[42] The samples underwent scoring based on both staining intensity and the extent of epithelial involvement. The cytoplasmic staining intensity was assessed using a semi-quantitative scale: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The extent of positively stained epithelium was scored as follows: 0 (absent), 1 (involving the basal one-third), 2 (involving the basal two-thirds), and 3 (involving the entire epithelial thickness). To determine the ORF1p immunoreaction score, the intensity and extent scores were multiplied (Table 1). The ORF1p staining was graded negative in the presence of less than 10% weakly reactive epithelial cells.[43]

The degree of p16 expression was quantified based on the percentage of p16-positive cells. In a semi-quantitative scale, a 0 designation was assigned when the proportion of positive cells fell below 1%. Grades 1 and 2 were allocated to cases with clustered positive cells and percentages of positive cells ranging from 1% to 5% and 5% to 25%, respectively. Grade 3 was designated in instances where widespread positive cells constituted more than 25% of the total.[13,44]

To assess the degree of Ki67 expression, the nuclei of 200 epithelial cells spanning the entire epithelial layer were examined in each specimen. The Ki67 index was established as the percentage of Ki67 positive cells. Grades 1, 2, and 3 were applied to categorize the extent of expression. Grade 1, 2, and 3 were given when the Ki67 index was below 5%, 5% to 30%, and greater than 30%, respectively.[13,45]

TABLE 1. Modified version of the immunoreactive scale (IRS) scoring system[42] for the evaluation of ORF1p immunohistochemistry in cervical specimens. (A) The measured parameters and their scaling (B) The final immunoreaction scores and their classification

A

Positively stained epithelial thickness (PP)	PP score	Intensity of staining (SI)	SI score
No staining	0	No color reaction	0
Basal one-third	1	Mild reaction	1
Basal two-thirds	2	Moderate reaction	2
Transepithelial	3	Strong reaction	3

B

IRS Scores (PP × SI)	IRS Classification
0	Negative
1–2	Positive, mild immunoreaction
3–4	Positive, moderate immunoreaction
6–9	Positive, strong immunoreaction

Statistical Analysis

Staining score data handling and analysis were carried out in the R statistical programming environment.[46] Comparisons across entities were made using Pearson χ^2 and Fisher exact tests. The associations were considered statistically significant for $P < 0.05$ in all analyzed cases.

RESULTS

We investigated the LINE-1 ORF1p immunopositivity of 112 formalin-fixed paraffin-embedded (FFPE) specimens of CIN I (n = 20), CIN II (n = 46), CIN III (n = 14), and cervical cancer (n = 32), and a control group of 31 nondysplastic cervical tissue samples. The ORF1p exhibited increasing expression with increasing grades of CIN towards cancer, while it was barely or undetectable in normal cervical epithelium (Fig. 1). We also performed immunostaining for the protein markers Ki67 and p16 in our entire sample panel. The Ki67 expression deviates from the normal pattern in the CIN II stage only (Fig. 1). Similarly, abnormal expression of the p16 marker is usually not observed before CIN II stage (Fig. 1). In contrast, the expression of ORF1p typically shows an abnormal pattern already from CIN I stage onwards, with a characteristic increase in immunopositivity with increasing CIN grading (Fig. 1). Representative images of CIN I stage at higher magnification clearly show patterns of Ki67 and p16 immunostaining typical of normal cervical epithelium, while ORF1p overexpression is easily discernible from normal staining patterns (Fig. 2).

The ORF1p positivity often does not extend to the full thickness of the cervical epithelia. In some cases, only the lower one-third, in other cases, the lower two-thirds,

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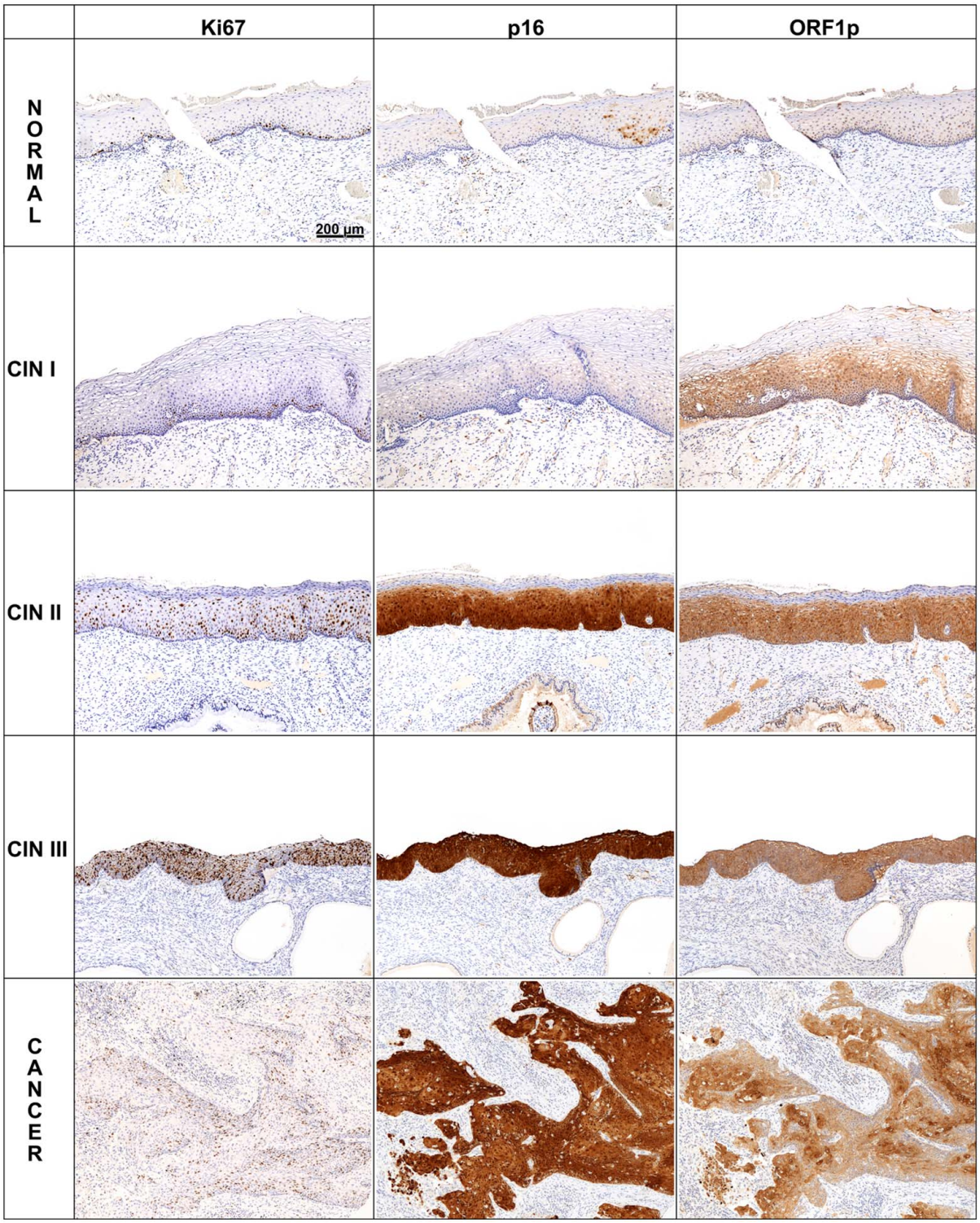

FIGURE 1. Immunostaining for ORF1p, p16 and Ki67 across differently graded lesions of cervical epithelium progressing towards cancer. Representative sections of FFPE specimens from normal cervical epithelium, cervical epithelial hyperplasia, cervical epithelial neoplasia (CIN I, CIN II, and CIN III) and cancer are presented. Scale bar = 200 µm.

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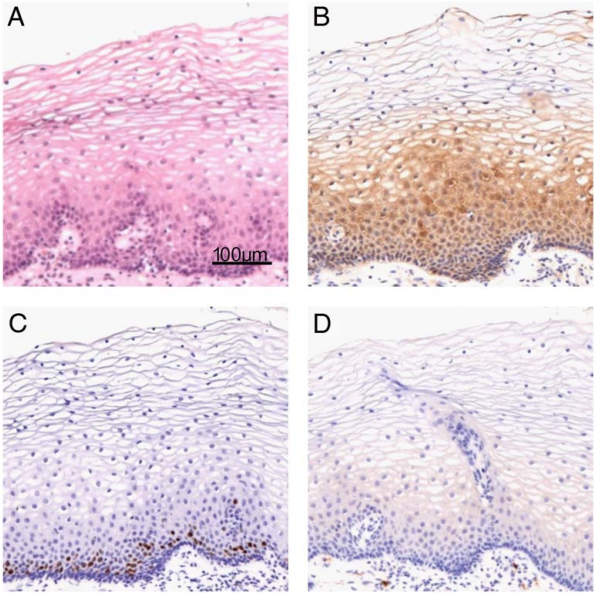

FIGURE 2. Immunostaining for ORF1p, p16, and Ki67 in FFPE specimens of cervical intraepithelial neoplasia grade I (CIN I). Hematoxylin & Eosin staining (A) with distinct upregulation of ORF1p in the basal two-thirds of the epithelial layer (B), accompanied by a negative p16 staining (C), and basal Ki67 expression pattern (D). Scale bar = 100 μm.

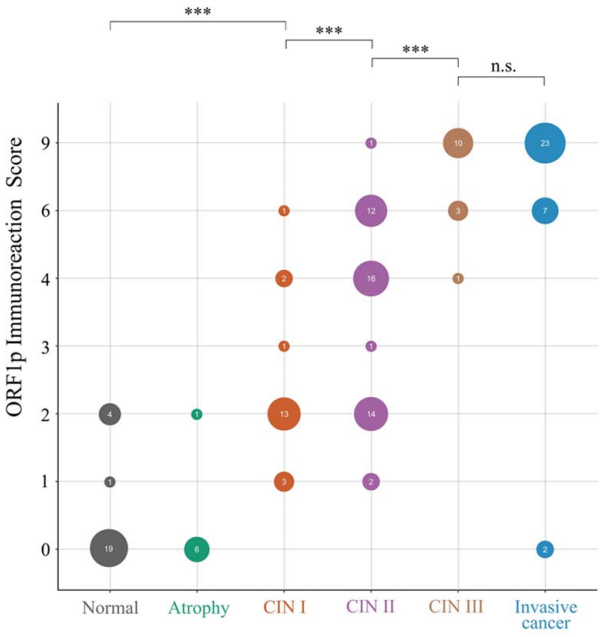

FIGURE 3. Bubble plot to visualize ORF1p immunoreaction scores across different cervical tissue specimens. Bubble sizes and the embedded numbers reflect the number of individuals with a particular immunoreaction score in each tissue sample group. Brackets connect the subjects of contingency analysis (Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177). Three stars indicate $P < 0.001$; n.s, nonsignificant.

and in further cases, the whole cross-section of the epithelium was positive. The extent of ORF1p positivity correlated with CIN grade (Fig. 1). In light of these observations, we have developed a scoring system, which is a modified version of the Immunoreactivity Scale (IRS) by Remmele and Stegner.[42] The final ORF1p immunoreaction score was determined by multiplying the numerical values of staining intensity and epithelial involvement (Table 1 and Materials and Methods section for details). Henceforth, this scoring scheme was used to evaluate the ORF1p immunostaining results of our entire sample panel.

Overall, ORF1p immunopositivity was detected in 98.2% of pre- and neoplastic cervical lesions (score 1–9), while only 20.8% of normal cervical specimens showed weak staining (score 1-2) (Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177). Among normal cervical epithelium samples, only 5 out of 24 displayed mild ORF1p immunoreaction at the basal one or two-thirds of the epithelial thickness (score 1–2), while the remaining 19 samples lacked ORF1p expression (score 0) (Fig. 3). Comparably, only 1 of the 7 atrophic cases exhibited mild ORF1p immunoreaction (score 2) (Fig. 3). In sharp contrast, ORF1p immunopositivity emerged in all CIN I cases, localized predominantly to the lower third of the cervical epithelium with a median value of score 2 (Fig. 3 and Table 2). Of the 20 cases of CIN I, 16 showed a mild immunoreaction (score 1–2), 3 showed a moderate immunoreaction (score 3–4), and 1 showed a strong immunoreaction (score 6) (Fig. 3). Likewise, ORF1p immunopositivity was also present in all CIN II cases, predominantly spanning the lower two-thirds of the cervical epithelium with an increased median score of 4 (Fig. 3 and Table 2). Of the 46 cases of CIN II, 16 showed a mild immunoreaction (score 1–2), 17 showed a moderate immunoreaction (score 3–4) and 13 showed a strong immunoreaction (score 6–9) (Fig. 3). Similarly to the CIN I and CIN II cases, ORF1p immunopositivity was present in all CIN III samples, showing a robust transepithelial appearance with a further increased median score of 9 (Fig. 3 and Table 2). Of the 14 cases of CIN III, only 1 showed a moderate immunoreaction (score 4) while the remaining 13 samples exhibited a strong immunoreaction (score 6–9) (Fig. 3). Samples of invasive cancer cases also exhibited strong ORF1p immunopositivity with a median score of 9 (Fig. 3 and Table 2). 30 of the 32 cancer cases showed strong immunoreaction (score 6–9) (Fig. 3). In the 2 cancer samples where ORF1p expression was absent (Fig. 3), it is conceivable that these tumors shut down LINE-1 expression concurrent with their accelerated growth.[47] The ORF1 immunoreaction scores did not correlate with tumor grade ($P = 0.4989$), and pTNM stage ($P = 0.6039$) (Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177).

Statistical analysis showed stronger immunoreaction for ORF1p in dysplastic lesions (CIN I-III and cancer) versus normal tissue ($P < 0.0001$). A significantly stronger immunoreaction for ORF1p was observed in CIN I versus normal cases ($P < 0.0001$) (Fig. 3. and Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.

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TABLE 2. Overview of ORF1p immunoreaction scores in Cervical Tissue Specimens, CIN: Cervical Intrepithelial Neoplasia

			ORF1p immunoreaction scores			
	Normal	Atrophy	CIN1	CIN2	CIN3	Invasive cancer
Sample number	24	7	20	46	14	32
Median	0.000	0.000	2.000	4.000	9.000	9.000
Mean	0.375	0.286	2.300	3.870	8.000	7.781
SD	0.770	0.756	1.174	1.809	1.710	2.393

lww.com/IJGP/A177). In addition, the immunoreaction scores were significantly higher in HSIL than in the CIN I group ($P = 0.001$) (Fig. 3 and Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177). Furthermore, we found statistically significant differences in ORF1p immunoreaction scores between CIN I and CIN II ($P = 0.0075$) and between CIN II and CIN III lesions alike ($P < 0.0001$) (Fig. 3 and Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177). Across our entire sample cohort, no statistically significant correlation was found between age groups (<35, 35–45, $45<$) and ORF1p immunopositivity ($P = 0.095$)) (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/IJGP/A176 and Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177). Although, for cases of invasive cancer, we observed slightly elevated median immunoreaction scores in tumor samples from older patients (Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177).

Subsequently, we evaluated the results of the p16 and Ki67 immunostainings for the whole sample panel. To quantify the degree of p16 and Ki67 immunoreactions, we used 3-grade scales, as recommended in references.[13,44,45] As demonstrated by the statistical analyses, both p16 and Ki67 immunoreactions were stronger in the group of all neoplastic and dysplastic lesions (CIN I-III and cancer) versus the control group ($P < 0.001$) (Supplementary Tables 2-3, Supplemental Digital Content 1, http://links.lww.com/IJGP/A176 and Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177). Furthermore, the immunopositivity of these 2 markers graded higher in HSIL than in LSIL ($P < 0.009$) (Supplementary Tables 2-3, Supplemental Digital Content 1, http://links.lww.com/IJGP/A176 and Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177). However, the immunoreaction scores of p16 and Ki67 did not show a significant difference between normal cervical tissue and CIN I ($P = 0.429$ and $P = 0{,}528$) (Supplementary Tables 2-3, Supplemental Digital Content 1, http://links.lww.com/IJGP/A176 and Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177).

Next, we compared the results of ORF1p immunostaining with the results of p16 and Ki67 immunostainings across the whole sample panel. The median

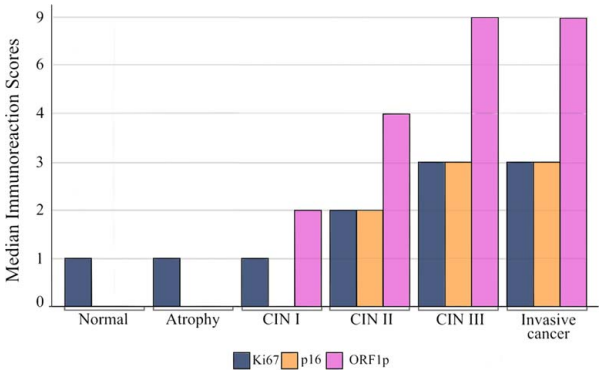

FIGURE 4. The median immunoreaction scores of ORF1p, p16, and Ki67 immunostainings in the entire palette of investigated cervical epithelial lesions. Data shown are derived from the analysis of our whole sample panel (Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177).

immunoreaction scores of the three markers revealed distinct patterns among the various sample groups (Fig. 4). The p16 and Ki67 immunoreaction scores showed a similar profile in the sense that they exhibited a progressive increase from CIN II stage onwards, but remained relatively low in normal, atrophic and CIN I cases (Supplementary Tables 2-3, Supplemental Digital Content 1, http://links.lww.com/IJGP/A176 and Fig. 4). Conversely, ORF1p scores demonstrated a gradual escalation, which was already detected between normal epithelium and CIN I stage samples, and this rising tendency was also observed in the comparison of CIN I and CIN II stages and CIN II and CIN III stages alike (Table 2 and Fig. 4). The immunoreaction scores showed no significant difference between invasive cancer and CIN III sample groups (Table 2 and Fig. 4). Consequently, ORF1p can be regarded as an early marker, serving as useful surrogate for p16 and Ki67, which are markers indicative of later events in the progression of cervical epithelial lesions.

DISCUSSION

Our study provides comprehensive insights into the differential expression patterns of ORF1p, p16, and Ki67 markers in cervical dysplasia and cancer. ORF1p emerges as a promising early marker, capable of distinguishing between various CIN grades by offering distinct staining patterns.

In CIN I, we observed subtle ORF1p expression localized predominantly to the lower third of the cervical epithelium. CIN II displayed a moderate increase in ORF1p expression, spanning the lower one and two-thirds of the cervical epithelium. CIN grade 3 and invasive cancer demonstrated robust transepithelial ORF1p immunopositivity, while the normal cervical epithelium consistently lacked ORF1p staining or showed weak positivity (Fig. 1). Compared with the other markers, the greatest practical clinical value of ORF1p immunostaining will probably lie in

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its ability to reliably distinguish normal cervical epithelium from CIN I lesions (Figs. 1 and 2). This is evidenced by the mean ORF1p immunoreaction score of 0.37 for normal epithelium and 2.30 for CIN I lesions (Table 2). For CIN I detection, the calculated Positive Predictive Value (PPV) and Negative Predictive Value (NPV) scores (https://www.medcalc.org/calc/diagnostic_test.php) of ORF1p staining were 77% and 100%, respectively, when compared to non-dysplastic samples (Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177). The NPV is particularly favorable because all CIN I cases showed a positive ORF1p immunoreaction. PPV and NPV values were also calculated for other sample groups, which similarly support the usefulness of ORF1p immunostaining (Supplemental Digital Content 1.xls, Supplemental Digital Content 2, http://links.lww.com/IJGP/A177). The utilization of ORF1p as a potential biomarker for identifying CIN lesions bears similarity to the established paradigm wherein ORF1p expression has been exploited as a diagnostic indicator for HPV-independent differentiated vulvar intraepithelial neoplasia (dVIN), the precursor lesion of vulval squamous cell carcinoma.[43]

Besides, ORF1p immunostaining provides a clearer and more reliable distinction between higher CIN grades than the conventionally used p16 and Ki67 markers (Figs. 3 and 4). While p16 immunostaining has proven valuable in identifying high-risk HPV-associated lesions, its interpretation can sometimes be subjective and prone to interobserver variability.[48] This is also illustrated by the fact that the authors of Clark et al[19] found that 19% of the examined HSIL samples had to be reclassified as LSIL because positive p16 immunostainings were overvalued at the time of the original diagnosis. In combination with ORF1p immunostaining, the concomitant presence of a mild ORF1p immunoreaction characteristic of LSIL may help the investigator to avoid such an error due to the presence of positive p16 immunostaining. Our current study suggests that in HSIL cases, the moderate or strong ORF1p immunoreaction is much more typical.

Our present results strongly support that the retrotransposition activity of endogenous LINE-1 elements may be an important driver of cervical tumorigenesis from its very beginning, as evidenced by the generalized ORF1p immunopositivity already in CIN I stage (Figs. 1 and 3). Over the last decade, it has become clear that the appearance of LINE-1 proteins and the concomitant LINE-1 retrotransposition activity in somatic cells is not only a simple marker of tumorigenesis but is also a driver of that process, that is, a pathogenic factor. It was shown that LINE-1 activity is present in more than 50% of cancers[29,35] and is now recognized as a major hallmark of cancer accompanied by genomic instability and genetic heterogeneity.[49] In line with this, several cancer driver mutations were identified in different tumor types that were caused by novel somatic LINE-1 integration events.[29,30,41,50–53] Moreover, the targeted inhibition of the activity of LINE-1 elements showed a clinical benefit in a phase 2 clinical trial in the treatment of metastatic colorectal carcinoma.[54] Notably, in the cervical epithelium, there is a potential interplay between LINE-1 retrotransposition and HPV infection. HPV infection may lead to alterations in the epigenetic landscape of infected cells.[55] In turn, HPV-induced epigenetic modifications may contribute to the activation of LINE-1 retrotransposition.[32] This is underlined by the fact that LINE-1 elements were found to be differentially expressed among cervical cancer samples infected with different HPV serotypes.[56]

Invasive cervical cancer cases, similar to CIN III lesions, exhibited a strong ORF1p immunoreaction (median score 9) but with some degree of intra-tumor heterogeneity (Fig. 1, Fig. 3, and Table 2). Remarkably, the authors of Ardeljan et al proposed that LINE-1 retrotransposition may conflict with DNA replication in exceptionally fast-growing tumors or tumor subclones.[47] In other words, exceptionally fast-dividing cancer cells may benefit from the shutdown of the LINE-1 retrotransposition activity that promoted earlier stages of their transformation. This may explain both the intra-tumor heterogeneity of ORF1p immunoreaction and the presence of 2 cancer samples where ORF1p expression was absent (Figs. 1 and 3).

Rarely, we also saw sporadic and weak ORF1p immunopositivity in normal cervical epithelium (data not shown). This is validated by the recent demonstration of McKerrow et al that LINE-1 mRNA expression is sometimes detectable in normal epithelial cells across various tissue types.[28] This suggests that LINE-1 expression is not completely sealed in epithelial cells and leaky expression may occur. Epigenetic downregulation of LINE-1 transcription is the first line of defense against mutagenic somatic retrotransposition. At the organism level, LINE-1 transcription is suppressed by epigenetic mechanisms already in the germline and early embryo due to the action of the piRNA system.[57,58] Heterochromatin established on LINE-1 loci in early developmental stages is then passed on to somatic tissues during individual development by cell divisions to maintain the transcriptionally repressed state of LINE-1 elements. However, sporadically in epithelial cells, leaky transcription of some LINE-1 copies can occur potentially in response to stimuli that may interfere with these epigenetic mechanisms. Presumably, this phenomenon is what we and McKerrow and colleagues have observed in epithelial cells. For this reason, we classified ORF1p immunostaining as negative when only less than 10% weakly reactive epithelial cells were present (see Materials and Methods). Future research is needed to show whether such weak sporadic expression observed in normal epithelium leads to new retrotransposition events, as there are many posttranscriptional and post-translational mechanisms for LINE-1 regulation[31,32] that may yet be able to prevent this. Although, it is clear that the stronger LINE-1 expression observed in tumors leads to retrotransposition events.[29]

The potential utility of ORF1p as a solid tumor biomarker is demonstrated by the fact that its detection for diagnostic purposes is currently even being considered from body fluids. Recently, Taylor et al have demonstrated that ORF1p detection from plasma may serve as a

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multi-cancer biomarker with potential utility for disease detection and monitoring.[59] Another recent study by Sato et al have revealed the detectability of ORF1p in ascites and plasma samples from patients with high-grade serous ovarian cancer, also underscoring the potential of ORF1p as a promising candidate serum biomarker.[38]

While our study provides promising insights into ORF1p as a novel biomarker for CIN diagnosis, it is important to acknowledge certain limitations. The relatively small sample size, especially in specific subgroups, might impact the statistical power and robustness of the findings. In addition, the utilization of Tissue Microarray technology, despite our efforts to ensure core representativeness, introduces a limitation in capturing the full heterogeneity within complex lesions. It is noteworthy that ORF1p staining exhibits a more uniform distribution within cervical lesions compared with the often segmental and patchy patterns seen in p16 staining. We established that 2 mm tissue cores from the selected target lesions were most appropriate for constructing our TMAs, which is in line with previous studies.[60–63] Two-millimeter cores are large enough to minimize problems of representativity but small enough to allow all samples to be treated with identical staining protocol during analysis. While our findings are promising, further large-scale studies are essential to fully validate the diagnostic and prognostic utility of ORF1p across diverse demographics and clinical settings.

Our findings support that the use of ORF1p alongside Ki67 and p16 markers enhances the diagnostic capability and potential reproducibility in distinguishing dysplasia from normal epithelium and differentiation among the CIN grades. ORF1p appears as a valuable candidate biomarker that offers a more reliable diagnosis for improved CIN patient management.

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