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RESEARCH ARTICLE

Population-Based Evaluation of a Novel E6/E7 Immunocytochemistry Assay for Cervical Cancer Screening and Triage of HPV-Positive Women

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ABSTRACT

The E6 and E7 oncoproteins are significant contributors to HPV-induced cervical cancer and promising candidates for screening methods. However, clinical evidence for detecting E6/E7 by immunocytochemistry (ICC) remains limited. This study evaluated the diagnostic accuracy of a novel E6/E7 immunocytochemistry assay for primary cervical cancer screening and triage of women with high-risk human papillomavirus (hrHPV). In this cross-sectional diagnostic study conducted in 2023, 3,108 women aged 21–81 from Moyu County, Xinjiang, China, underwent HPV testing, cytology, and E6/E7 ICC. Women with abnormal results were referred for colposcopy and biopsy as indicated, and histologically confirmed CIN2+ served as the gold standard. Among 3,049 participants with complete primary screening results, E6/E7 ICC positivity was 7.4% ($n = 226$), intermediate between SureX hrHPV positivity (8.5%, $n = 258$) and cytology ASC-US+ (6.1%, $n = 185$). E6/E7 positivity was strongly associated with HPV16/18 infection (OR = 20.2, 95%CI:13.1–31.0), high-grade cytology (OR = 73.4, 95%CI:20.5–262.8), and increasing histology-confirmed CIN grades (OR: 14.2 ~ 149.0). A total of 3,039 women had evaluable screening outcomes within 12 months, and 36 cases of CIN2+ were identified. The E6/E7 ICC alone detected 86.1% (31/36) of CIN2+ lesions with 93.6% specificity, yielding a 7.4% referral rate. A combined strategy—referring all HPV16/18 positive women to colposcopy and triaging other hrHPV positive women using E6/E7 ICC—detected 88.9% (32/36) of CIN2+ cases with 96.2% specificity and a 4.8% colposcopy referral rate. This first population-based, cross-sectional evaluation provides preliminary evidence that the E6/E7 ICC, particularly in combination with HPV16/18 genotyping, may be an effective strategy for cervical cancer screening and triage. Prospective studies are essential to confirm its long-term predictive value before clinical implementation.

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1 | Introduction

Cervical cancer remains a significant global health burden, with an estimated 604,127 new cases and 314,831 deaths in 2020, corresponding to an age-standardized incidence of 13.3 cases per 100,000 women-years and a mortality rate of 7.2 deaths per 100,000 women-years [1]. While widespread implementation of human papillomavirus (HPV) DNA testing has significantly improved screening sensitivity and provided longer reassurance after a negative test compared to cytology-based methods [2], critical challenges persist in optimizing triage strategies for HPV-positive women—a population representing 10%–20% of screened individuals in routine practice [3].

The high sensitivity of HPV DNA testing comes at the cost of specificity, as most infections (90%) are transient and clear spontaneously within 1 ~ 2 years [4]. This necessitates effective triage methods to identify the minority of individuals who progress to cervical intraepithelial neoplasia grade 2 or worse (CIN2 +). Current clinical triage strategies, including reflex cytology or partial genotyping (HPV16/18), demonstrate suboptimal sensitivity (~ 71.5% for cytology and ~62.7% for HPV16/18) and positive predictive values (~31.8% for cytology and 44.5% for HPV16/18) in recent published syntheses of evidence by WHO [5]. Consequently, 60%–70% of HPV-positive women undergo unnecessary colposcopies, leading to psychological distress, overtreatment, and increased healthcare costs.

The molecular pathogenesis of HPV-driven cervical carcinogenesis hinges on sustained expression of E6 and E7 oncoproteins. These proteins drive malignant transformation through p53 degradation (E6) and inactivation of the Rb pathway (E7), inducing genomic instability and immortalization of epithelial cells [6]. Crucially, the integration of HPV DNA into the host genome during progression to high-grade lesions results in constitutive E6/E7 overexpression, making these proteins ideal biomarkers for identifying true precancers [7].

A recent systematic review summarized the accuracy of E6/E7 oncoprotein tests to detect underlying cervical precancer and cancer [8]. Sensitivity and specificity estimates ranged from 54.2% to 69.5% and from 82.8% to 99.1%, respectively, across the population irrespective of HPV status. This systematic review furtherly highlighted the high specificity of the E6/E7 oncoprotein test, making it a promising triage tool for HPV positive women. However, the lack of large-scale and longitudinal studies leaves a research gap for future investigation.

In this study, we aimed to evaluate the screening accuracy of a novel immune-cytochemistry (ICC) based E6/E7 oncoprotein test in a population-based screening cohort, with rigorous comparison to established screening and triage methods.

2 | Materials and Methods

2.1 | Study Design and Population

This cross-sectional diagnostic study was conducted in 2023 within a general population of 3,108 women aged 25–81 years recruited from Moyu County, Xinjiang, China. The primary aim was to evaluate the performance of a novel HPV E6/E7 oncoprotein immunocytochemistry assay (E6/E7 ICC) for primary cervical cancer screening and the triage accuracy of HPV-positive women. Inclusion criteria include non-pregnant women who have ever been sexually active, aged over 25 years, with an intact cervix and no history of cervical cancer or hysterectomy, while those women who were pregnant, or had prior treatment for cervical precancer, were excluded.

2.2 | Study Procedures

After informed consent, participants completed a standardized questionnaire to capture demographic characteristics (age, education), sexual/reproductive history, and HPV vaccination status. Then, professional gynecologists collected cervical cells first using a liquid-based cytology (LBC) brush (PreservCyt, Hologic, USA) and, secondly, using Attobio E6/E7 cytology preservation solution (Atto-LBC), and stored them at 4°C for subsequent testing.

2.3 | Laboratory Procedures

2.3.1 | HPV DNA Genotyping

HPV DNA genotyping was performed using the SureX HPV genotyping assay (SureX® HPV 25X Genotyping Kit, Health Gene Technologies, Ningbo, China) to detect 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and 11 other HPV types (6, 11, 26, 42, 43, 44, 53, 73, 81, 82, and 83) using 2 mL residual cervical samples collected in ThinPrep PreservCyt. The details of the test procedure have been described previously [9, 10]. In brief, specific primers targeting the early genes *E6* and *E7* of HPV were designed to perform specific amplification and capillary electrophoresis separation of different HPV genotypes. Based on the varying lengths of the amplified fragments, 25 HPV types can be simultaneously detected and genotyped in a single reaction. In this study, we focused on the 14 hrHPV types and referred them to colposcopy if positive. The SureX HPV test was performed in a central PCR laboratory according to the manufacturer's instructions.

2.3.2 | careHPV

careHPV DNA test (careLYFE, China) is an in vitro nucleic acid hybridization assay targeting 14 hrHPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) in bulk without genotyping. A test result is considered positive if RLU/CO value ≥ 1.0 pg DNA/mL; otherwise, it is classified as hrHPV negative.

2.3.3 | Cytology Testing

Liquid-based cytology (LBC) slides were evaluated by certified cytopathologists who were blinded to other screening results, using the Bethesda System 2014, and categorized into NILM (Negative for intraepithelial lesion or malignancy), ASC-US (Atypical squamous cells of undetermined significance), LSIL (Low-grade squamous intraepithelial lesion), ASC-H (Atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion), HSIL (High-grade squamous intraepithelial lesion), SCC (Squamous cell carcinoma). Any findings categorized as Atypical Squamous Cells of Undetermined Significance or worse (ASC-US+) were considered cytology positive and referred for colposcopy.

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2.3.4 | *E6 and E7 Immunocytochemical Staining (E6-ICC, E7-ICC)*

Clinical samples of exfoliated cervical epithelial cells collected in Atto-LBC preserve solution were analyzed for E6 or E7 oncoprotein by immunostaining using antibody kits as previously reported [11, 12]. Essentially, cytology slides were stained with commercial antibody reagent kits, E6-ICC (a cocktail of rabbit monoclonal antibodies) and E7-ICC (a murine monoclonal antibody) from Attogen Biomedical Incorporated (Suzhou, China). An automated immunohistochemical stainer, AttoStar 4800 A (Attogen, Suzhou, China), was employed to perform the whole procedure of immunostaining, which included antigen retrieval, incubation with primary antibodies and with secondary antibodies-conjugated with horse-reddish peroxidase, and finally with chromogenic dyes of 3,3′-diaminobenzidine (DAB) and hematoxylin. Standard control slides were used to calibrate the instruments and immunoreagents when performing the E6-ICC and E7-ICC test according to the manufacturer's instructions. Finally, immunochemically stained slides were mounted with coverslips, and staining results were observed under an optical microscope with 20x or 40x objective lenses (Olympus Corporation, Tokyo, Japan).

To improve the accuracy and speed of analyses, whole-slide digital images were acquired using an automated slide scanner, AttoScan 400i (Attogen, Suzhou, China). The AttoScan 400i features several AI-driven tools specifically designed for analyzing E6 and E7 ICC-stained samples. The Cell-AI algorithms, which utilize neural networks and deep learning, were trained on over one million E6/E7 ICC cellular images [13] (China Patents No. CN110956605A, April 3, 2020; CN110956605B, July 14, 2023). An example of the Cell-AI-assisted pre-screening of positively stained cells is provided in Supplementary Figure 1. The Cell-QC application was developed for quality control in accordance with The Bethesda System (TBS) standards. It includes a cell-counting module that automatically flags slides with fewer than 5,000 cells by displaying a red warning signal. This feature serves as a practical tool for ensuring the validity of cytology tests (China Patent No. CN 120070298 A, May 30, 2025). An example of the Cell-QC application is shown in Supplementary Figure 2.

2.4 | Analysis of E6 or E7-stained Cells and Interpretation of Results

Immunochemical localizations on stained epithelial cells were characterized by the staining patterns for E6 and E7. E6 positive cells exhibited signal localization in the cytoplasmic, nuclear, or both, as shown in Figure 1A (left). Whereas the E7 staining pattern was mostly localized in the cytoplasm, as shown in Figure 1B (right). Other features of staining were also characterized, including the number of positively stained cells and the types of cells that exhibited staining, i.e., dysplastic, atypical, metaplastic, and endocervical. The immunocytochemical staining of E6 and E7 on representative clinical samples of exfoliated cervical cells was presented in Supplementary Figure 3.

In this study, all tested samples underwent large-scale image analysis, starting with pre-screening using the Cell-AI image analysis tool, as illustrated in Supplementary Figure 1. This process aimed to identify positively stained cells on E6- or E7-ICC-treated slides. Cytological pathologists, who were blinded to HPV and cytology results, established a definitive positive E6 or E7 ICC result. This required at least one epithelial cell with distinct, specific immunocytochemical staining and appropriate cellular localization. The staining had to be clearly distinguishable from background or nonspecific signal, and observed in morphologically intact epithelial cells. To manage inter-observer variability and ensure reliability of results, a standardized adjudication procedure was applied. Specifically, two qualified cytological pathologists independently reviewed all slides, including those flagged positive or preliminarily classified as negative by AI pre-screening. Cases with concordant interpretations were accepted. For discordant cases, a third senior cytopathological expert performed an additional review, and a final consensus diagnosis was reached through discussion. Slides lacking specific E6 or E7 staining and with fewer than 5,000 evaluable cells were classified as invalid and excluded from negative scoring.

2.5 | Colposcopy and Histopathology

Women with any positive result (hrHPV positive, or cytology ≥ ASC-US, or E6/E7 ICC positive) underwent colposcopy.

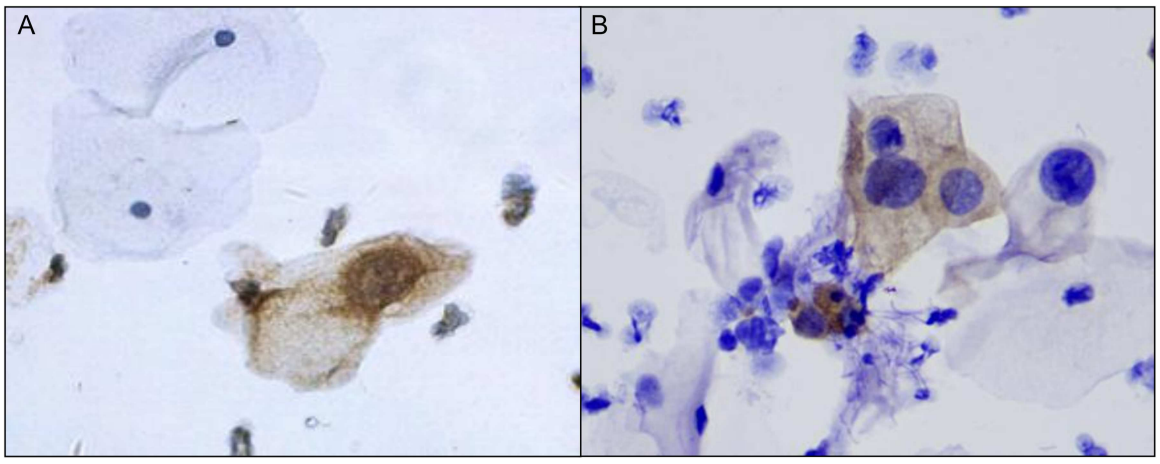


FIGURE 1 | Microscopic photos of immunochemically stained positive cells illustrate E6 or E7 staining pattern and cellular localization. (A) E6 staining shown as brown color in the cytoplasm and nucleus of dysplasia squamous epithelial cells. No brown staining on the normal epithelial cells on the same slide. (B) E7 positive staining is localized in the cytoplasm of dysplasia squamous epithelial cells, whereas no brown staining on the normal epithelial cells.

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Directed biopsies were taken from acetowhite lesions, and were sent to the pathology department of the Affiliated Cancer Hospital of Xinjiang Medical University for diagnosis by the senior pathologists specializing in gynecological tumors. Final histopathological diagnoses were classified as: normal, cervical intraepithelial neoplasia grade 1 (CIN1), CIN2, CIN3, or invasive cancer. Pathologists were blinded to all other screening results.

2.6 | Statistical Analysis

Logistic regression models assessed associations between E6/E7 positivity and different demographic and clinical characteristics. Overall prevalence of hrHPV (14 types) detected by both SureX HPV and careHPV tests was initially reported as a percentage. While evaluating the triage performance of different algorithms, SureX HPV test results were used. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) with 95% confidence intervals (CIs) for hrHPV, cytology (ASC-US+), and E6/E7 ICC in detecting CIN2+ and CIN3+ were calculated against histopathology as the gold standard. Pairwise comparisons of colposcopy referral rates, sensitivity, and specificity between the reference algorithm (hrHPV screening without triage) and each of the other algorithms were conducted using McNemar's test. To control the family-wise error rate across this set of pre-planned comparisons against a common reference, *p*-values were adjusted using the Bonferroni method with $n = 13$. The number of comparisons ($n = 13$) corresponds to the 13 primary hypotheses, each testing a new algorithm against the reference, rather than all possible pairwise combinations. Consequently, statistical significance was evaluated against an adjusted alpha level of $\alpha = 0.05/13$, rather than the conventional $\alpha = 0.05$ (Supplementary Table 1). Furthermore, the relative ratios of colposcopy referral rates, sensitivity, and specificity were computed with 95% CIs to further demonstrate increase or reduction in these indicators. Analyses were conducted using [Stata SE 15.1].

3 | Results

As shown in Figure 2, a total of 3,108 women were recruited; 3,049 had complete primary screening results, and 3,039 had evaluable screening outcomes within 12 months and were included for further analysis. 8.5% ($n = 258$) of women tested positive for hrHPV by SureX HPV, and 6.1% ($n = 185$) of women had cytology ASC-US + , whereas the positivity rates for E6 ICC and E7 ICC were 4.8% ($n = 147$) and 5.1% ($n = 156$), respectively. A total of 514 women with any positive results were referred to colposcopy, and 485 underwent initial colposcopy (attendance rate 94.4%). Women with an initial normal colposcopy or negative/CIN1 histology results, as well as those lost to initial colposcopy, were recalled for 1-year follow-up. Overall, 36 CIN2+ cases were detected during baseline screening and 1-year follow-up.

3.1 | Demographic and Clinical Characteristics of the Study Population

Participants' characteristics are shown in Table 1. The median age of women was 46 years (IQR 40–54). The overall positivity of E6 or E7 ICC (E6/E7) was 7.4% (226/3049) and showed an increasing trend with age ($P_{trend} = 0.004$). Post-menopause women showed a higher risk of being E6/E7 positive than pre-menopause women (OR: 1.7, 95%CI: 1.3–2.3). There is no significant difference in E6/E7 oncoprotein positivity between subgroups by other demographic characteristics. 49.5% of

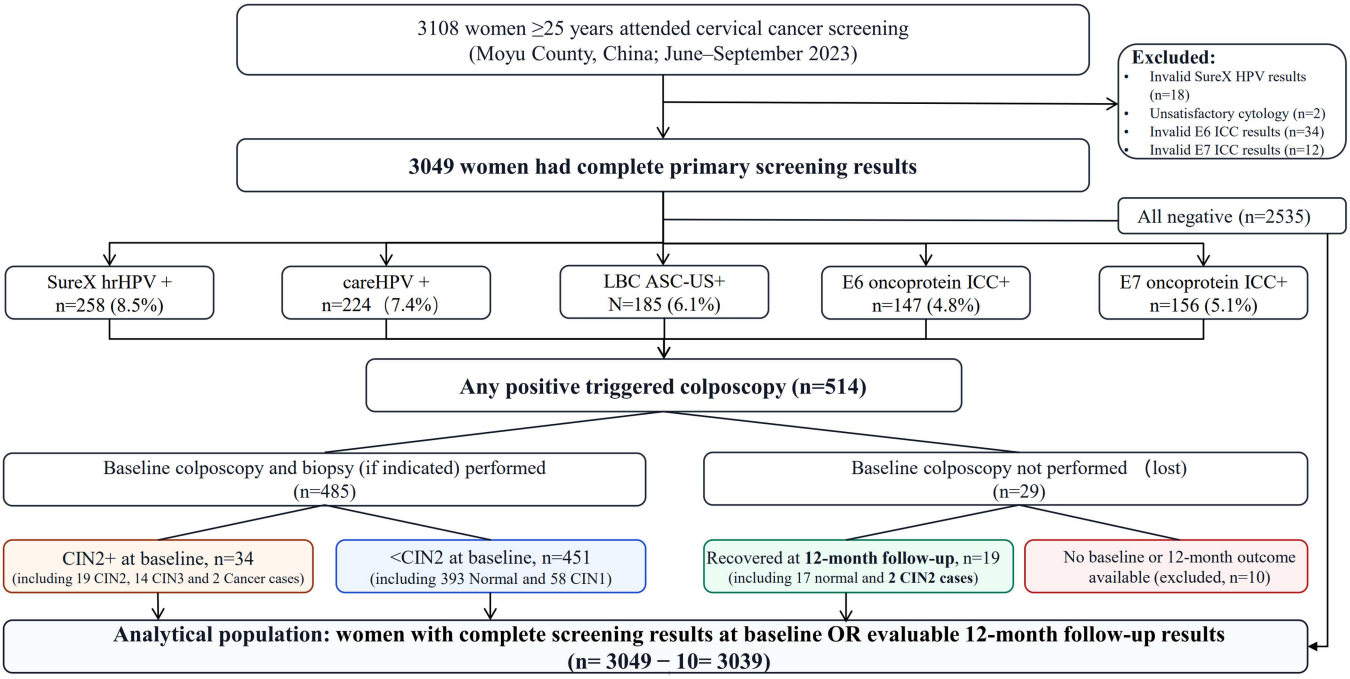


FIGURE 2 | Study population and screening process. *Note:* Two new cases detected during the 12-month follow-up were from the group lost to baseline colposcopy examination. ASC-US+, atypical squamous cells of undetermined significance or worse; CIN, cervical intraepithelial neoplasia; hrHPV, high-risk human papillomavirus; ICC, immunocytochemical staining; LBC, liquid-based cytology.

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TABLE 1 | Characteristics of the study population and prevalence of high-risk HPV infection and E6/E7 oncoprotein ($N = 3049$).

Characteristics	No. of women	Prevalence of high-risk HPV[a], ($n = 258$, %)	E6/E7 ICC positivity ($n = 226$, %)	Odds ratios (ORs) for E6/E7 ICC positivity
Age, y				
Median (IQR)	46 (40–54)	NA	NA	
25–34	280 (9.2)	22 (7.9)	17 (6.1)	1
35–45	1126 (36.9)	90 (8.0)	72 (6.4)	1.1 (0.6–1.8)
46−55	1141 (37.4)	101 (8.9)	82 (7.2)	1.2 (0.7–2.1)
56–65	435 (14.3)	37 (8.5)	49 (11.3)	**2.0 (1.1–3.5)**
> = 66	67 (2.2)	8 (11.9)	6 (9.0)	1.5 (0.6–4.0)
p(trend) value		$p < 0.001$	$p = 0.004$	
Menopause				
Pre-menopause	1878 (61.6)	152 (8.1)	116 (6.2)	1
Peri-menopause	173 (5.7)	12 (6.9)	8 (4.6)	0.7 (0.4–1.5)
Post-menopause	998 (32.7)	94 (9.4)	102 (10.2)	**1.7 (1.3–2.3)**
p value		$p = 0.363$	$p < 0.001$	
Age of sexual debut, y				
< 18	1338 (43.9)	115 (8.6)	101 (7.6)	1
> = 18	1711 (56.1)	143 (8.4)	125 (7.3)	1.0 (0.7–1.3)
p value		$p = 0.815$	$p = 0.799$	
Lifetime sexual partners				
1	2117 (69.4)	163 (7.7)	144 (6.8)	1
> = 2	932 (30.6)	95 (10.2)	82 (8.8)	1.2 (1.0–1.8)
		$p = 0.023$	$p = 0.053$	
Pregnancies, No.				
0	40 (1.1)	6 (15.0)	5 (12.2)	1
1–3	937 (30.7)	89 (9.5)	69 (7.4)	0.6 (0.2–1.5)
4−6	1687 (55.3)	136 (8.1)	125 (7.4)	0.6 (0.2–1.5)
> = 7	385 (12.6)	27 (7.0)	27 (7.0)	0.5 (0.2–1.5)
HPV status		$p = 0.179$	$p = 0.659$	
HPV16/18+	101 (3.3)	/	50 (49.5)	**20.2 (13.1–31.0)**
Other 12-hrHPV+	157 (5.1)	/	48 (30.6)	**9.1 (6.2–13.3)**
hrHPV-	2791 (91.5)	/	128 (4.6)	1
p value	/	/	$p < 0.001$	
Cytology status				
NILM	2863 (93.9)	200 (7.0)	148 (5.2)	1
ASC-US/LSIL	170 (5.6)	67 (39.4)	66 (38.8)	**11.5 (8.1–16.4)**
High grade cytology	15 (0.5)	14 (93.3)	12 (80.0)	**73.4 (20.5–262.8)**
p(trend) value		$p < 0.001$	$p < 0.001$	
Histology status				
Benign	2928 (96.0)	160 (5.5)	158 (5.4)	1
CIN1	58 (1.9)	48 (82.8)	26 (44.8)	**14.2 (8.3–24.5)**
CIN2	19 (0.6)	15 (79.0)	17 (89.5)	**149.0 (34.1−650.6)**
CIN3	14 (0.5)	14 (100)	11 (78.6)	**64.3 (17.8–232.7))**
Cancer	1 (0.03)	1 (100)	1 (100)	/
Invalid	29 (1.0)	20 (69.0)	13 (44.8)	/
p(trend) value		$p < 0.001$	$p < 0.001$	

Note: (a) hrHPV positive by SureX PCR HPV test. Bold values are used to indicate statistical significance ($p < 0.05$).

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HPV16/18 positive women were tested positive by E6/E7 ICC, significantly higher than the other 12hrHPV positive women (*vs.* 30.6%, $p < 0.001$) and the hrHPV negative women (*vs.* 4.6%, $p < 0.001$), with ORs at 20.2 (95%CI: 13.1–31.0) and 9.1 (95%CI: 6.2–13.3). Women with high-grade cytology had a significantly increased risk of E6/E7 ICC positivity (OR: 73.4, 95% CI: 20.5–262.8).

3.2 | Positivity of E6 and E7 Oncoproteins by Cytology and Histology Results

Figure 3 separately shows the E6 ICC and E7 ICC by cytology and histology lesions. E6/E7 ICC positivity increased with cytology severity ($P_{trend} < 0.001$), reaching 88.9% among HSIL cytology women (Figure 3). Similarly, E6/E7 ICC positivity steadily increased from 5.4% among normal and 44.8% among CIN1 lesions to 81.0% and 80.0% among CIN2 and CIN3+ lesions by histology ($P_{trend} < 0.001$).

3.3 | Type-Specific E6/E7 Oncoprotein Expression

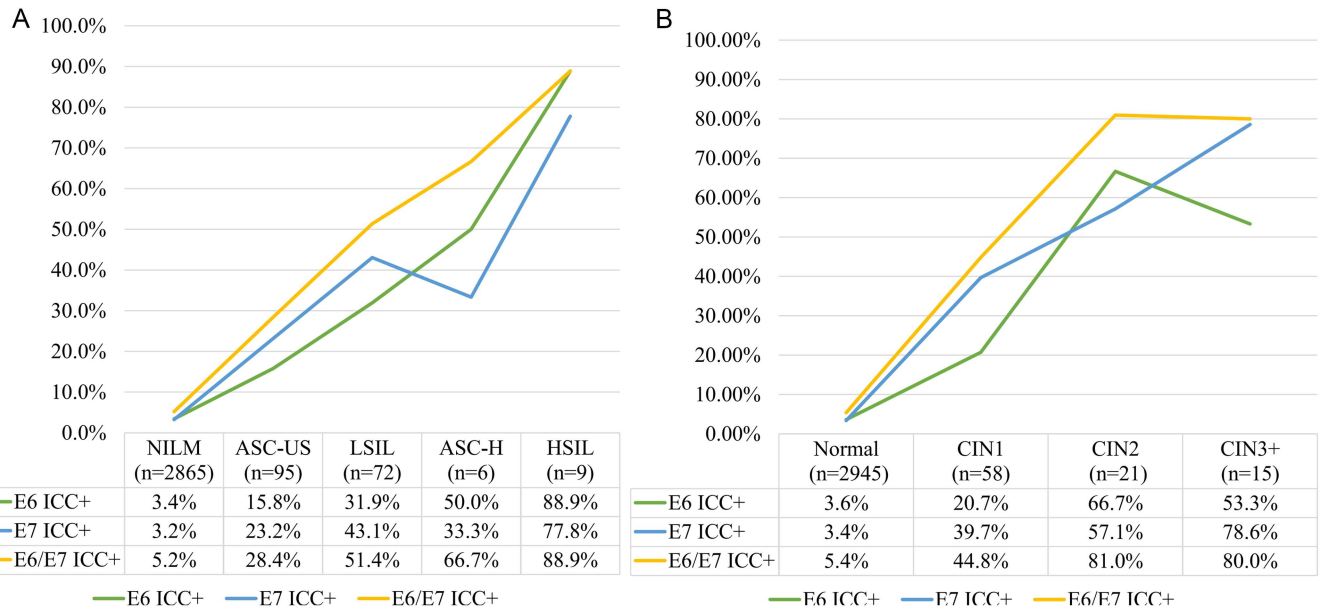
As Figure 4 presented, the most prevalent HPV type in our study was HPV 16 ($n = 99$), of which 49.0% ($n = 48$) were E6/E7 ICC positive. Although HPV18 ($n = 5$) and HPV33 ($n = 4$) were less prevalent, the E6/E7 ICC positivity were the highest (4/5 for HPV18, 2/4 for HPV33) among these types. 50% (10/20) of HPV51 and HPV58 infected cases were E6/E7 ICC positive.

3.4 | Diagnostic Performance of E6/E7 Oncoprotein Among the Screened Population

As shown in Table 2, in primary screening methods, E6 ICC and E7 ICC showed the highest specificity over 95% with the lowest colposcopy referral rates (4.8 ~ 5.1%), but the sensitivity was suboptimal (66.7% for both). While E6/E7 ICC screening yielded a colposcopy referral rate of 7.4% ($n = 224$), detecting 86.1% (31/36) of CIN2+ lesions with a specificity of 93.6% (2810/3003), resulting in a PPV of 13.8% and a NPV of 99.8%. Supplementary Table 1 showed the comparison between different algorithms, where the differences between E6/E7 ICC and hrHPV primary screening did not show statistical significance. While E6 ICC and E7 ICC showed significantly lower colposcopy rates, with significantly higher specificity.

Among "screen-triage" algorithms, hrHPV primary screening with E6/E7 ICC triage demonstrated improved efficiency, achieving an even lower colposcopy referral rate of 3.2% ($n = 96$) with a significantly higher specificity of 97.7% (2934/3003) compared to hrHPV screening without triage ($p < 0.001$ for both), but the sensitivity was only at 75.0% (27/36). This enhanced specificity translated into substantially higher PPV (28.1%) and NPV (99.7%). When using the combined algorithms, in which HPV16/18 women were referred directly to colposcopy and the other 12-hrHPV positive women were triaged with E6/E7 ICC, the highest sensitivity was observed, similar to hrHPV screening alone, detecting 88.9% (32/36) of CIN2+ cases. But this algorithm exhibited a significantly lower referral rate of 4.8% ($n = 145$) and an increased specificity of 96.2% (2890/3003) compared to hrHPV screening ($p < 0.001$), with a PPV of 22.1% and an NPV of 99.9%.

To better demonstrate the utility of E6/E7 ICC among hrHPV positive women, the triage performances of different algorithms were evaluated and restricted to the hrHPV positive population and presented in Supplementary Table 2. E6/E7 ICC detected 84.4% (27/32) of CIN2+ with a colposcopy referral rate of 38.6% ($n = 96$) with a specificity of 68.2% (148/217), a PPV of 28.1%, and an NPV of 96.7%. Among two-step triage strategies, HPV16/18 with reflex E6/E7 ICC achieved maximal sensitivity (100%, 32/32) and NPV (100%), but required the highest referral rate (58.2%, $n = 145$) and exhibited the lowest specificity (47.9%, 104/217) and PPV (22.1%).



	NILM (n=2865)	ASC-US (n=95)	LSIL (n=72)	ASC-H (n=6)	HSIL (n=9)
E6 ICC+	3.4%	15.8%	31.9%	50.0%	88.9%
E7 ICC+	3.2%	23.2%	43.1%	33.3%	77.8%
E6/E7 ICC+	5.2%	28.4%	51.4%	66.7%	88.9%

	Normal (n=2945)	CIN1 (n=58)	CIN2 (n=21)	CIN3+ (n=15)
E6 ICC+	3.6%	20.7%	66.7%	53.3%
E7 ICC+	3.4%	39.7%	57.1%	78.6%
E6/E7 ICC+	5.4%	44.8%	81.0%	80.0%

FIGURE 3 | Positivity of E6 ICC and E7 ICC by cytology and histology status. (A) Positivity of E6 ICC and E7 ICC by cytology grades. (B) Positivity of E6 ICC and E7 ICC by histology grades. ASC-US+, atypical squamous cells of undetermined significance or worse; CIN, cervical intraepithelial neoplasia; ICC, immunocytochemical staining; LBC, liquid-based cytology.

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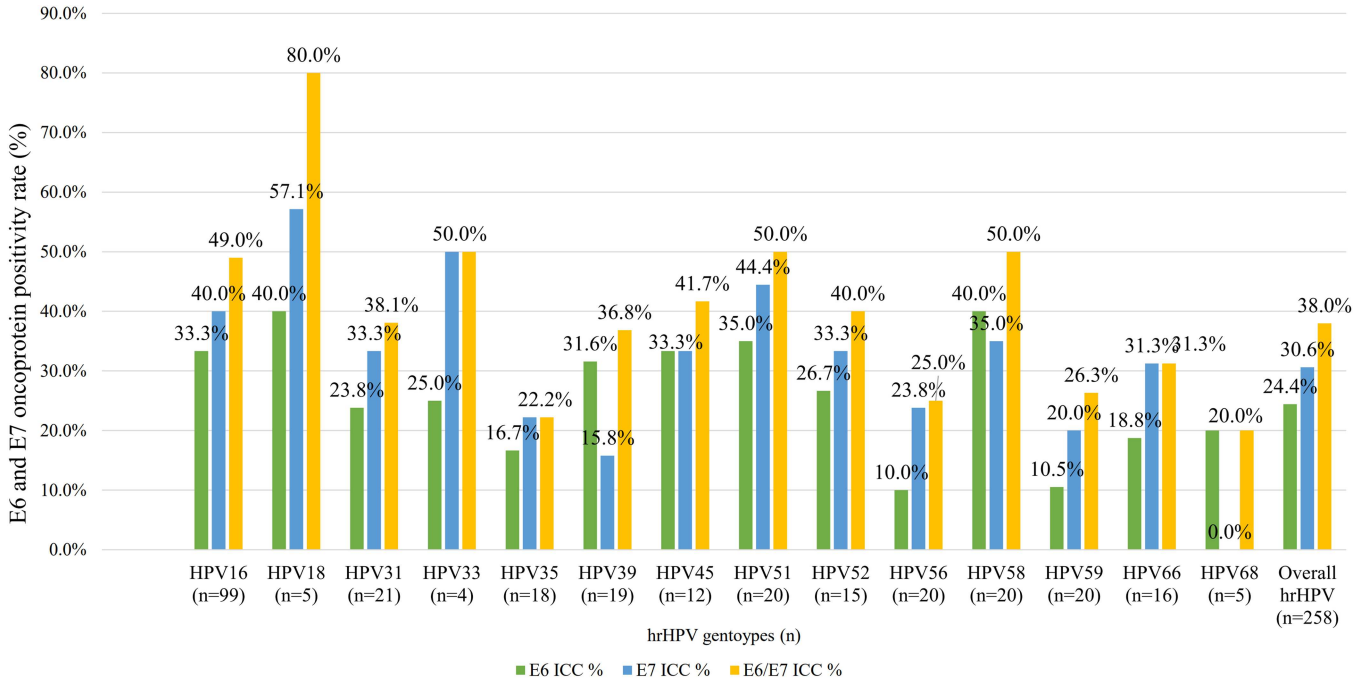


FIGURE 4 | Type-specific E6 and E7 oncoprotein positivity.

4 | Discussion

Despite extensive research on triage strategies for human papillomavirus (HPV)-positive women, the optimal approach remains to be defined. The emerging biomarkers and the advancements in detection methodologies for established markers ensure that HPV triage continues to be a critical focus in cervical cancer prevention. In this study, we evaluated a novel immunocytochemistry assay specifically targeting the E6 and E7 oncoproteins, pivotal drivers of HPV-mediated carcinogenesis. This represents the first assessment of this E6/E7 ICC technology within a population-based cervical screening setting, evaluating its performance both as a primary screening tool and, crucially, for the triage of hrHPV-positive women. Notably, in this cross-sectional assessment, the E6/E7 ICC assay demonstrated an efficient balance of moderate sensitivity (86.1%) and high specificity (93.6%) with a low immediate colposcopy referral rate (7.4%) in the general screening population. Furthermore, the combined approach (HPV16/18 with reflex E6/E7 ICC for other 12-hrHPV positive women) achieved similar sensitivity (88.9%) with only half of the colposcopy referral rates (relative ratio: 0.58, 95%CI: 0.52–0.64) and slightly increased specificity (relative ratio:1.04, 95%CI: 1.03–1.05) compared to hrHPV screening alone. These cross-sectional results provide initial evidence supporting the potential utility of direct E6/E7 oncoprotein detection by immunocytochemistry for the triage of HPV-positive women; however, longitudinal data are required to further confirm its predictive value and clinical applicability.

Despite the well-established, critical role of HPV E6 and E7 oncoproteins in cervical carcinogenesis, providing a strong theoretical foundation for their use as ideal biomarkers, their translation into widespread screening practice over the past decades has faced significant hurdles [14]. Early efforts focused on technologies like the immunochromatographic rapid test—such as Onco E6^{TM} (Arbor Vita Corporation, US), which targets the E6 oncoprotein of HPV16, 18 (with or without HPV45) [15]. Initial large-scale studies reported the suboptimal sensitivities of E6 oncoprotein (42.8% for CIN2+ and 54.2% for CIN3 +) but a superior specificity around 95.0% among hrHPV positive women [16], and a 3-year prospective study demonstrated the elevated expression of HPV16/18 oncoprotein was associated with persistence of HPV infection (OR, 54.64; 95% CI, 7.19–415.09) [17], subsequent 10-year cohort data demonstrated promising long-term risk prediction capabilities where 10-year cumulative incidence rate (53.0%) of CIN3+ was reported among baseline E6 oncoprotein positive women [18]. However, the suboptimal sensitivity, particularly for detecting prevalent CIN2 + /CIN3+ cases, limited its clinical adoption. Subsequent iterations aimed to improve sensitivity by expanding the spectrum of detectable types from HPV16/18/45 to 8 most prevalent high-risk HPV types (HPV16/18/31/33/35/45/52/58), showing enhanced sensitivity (67.7%) with a minimal loss in specificity (89.5%) [19], but a broader validation and robust prospective evidence confirming the clinical utility and cost-effectiveness of these evolved assays within routine screening programs remain limited. Of note, the ESTAMPA study, an ongoing, large-scale, multicenter cross-sectional study ($n = 42{,}502$) [20], evaluated the triage performance of the 8-HPV-type oncoprotein test as triage of HPV-positive women ($n = 3{,}074$) [21]. The feasibility of the test has been well described in the published paper, while the accuracy of the oncoprotein test needs to be addressed in the following paper, which might provide important evidence for its utility in real-world settings.

Our study evaluates a fundamentally distinct technological approach based on a novel immunocytochemistry assay designed for the direct morphological visualization of E6/E7 oncoprotein expression within cervical cells. This method inherently offers better objectivity and reproducibility than subjective cytological interpretation. Indeed, our results demonstrate that E6/E7 ICC significantly outperformed liquid-based cytology, achieving higher sensitivity (86.1% *vs.* 61.1% in

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TABLE 2 | Screening performance of different algorithms among total population (0–12 M, $N = 3039$, CIN2 + = 36).

	Screening algorithms	TestPos (*N*)	colposcopy referral rates (%)	Sensitivity ($N = 36$)	Specificity ($N = 3003$)	PPV	NPV
1	hrHPV (SureX HPV)	249	8.2	88.9 (32)	92.8 (2786)	12.9	99.9
				(73.9–96.9)	(91.8–93.7)	(9.0–17.7)	(99.6–100)
	hrHPV (careHPV)	217	7.1	80.6 (29)	93.7 (2815)	13.4	99.8
				(64.0–91.8)	(92.8–94.6)	(9.1–18.6)	(99.5–99.9)
2	LBC ASC-US+	183	6.0	61.1 (22)	94.6(2842)	12.0	99.5
				(44.9–75.2)	(93.8–95.4)	(8.1–17.5)	(99.2–99.7)
3	E6 alone	146	4.8	66.7 (24)	95.9 (2881)	16.4	99.6
				(49.0–81.4)	(95.2–96.6)	(10.8–23.5)	(99.3–99.8)
4	E7 alone	154	5.1	66.7 (24)	95.7 (2873)	15.6	99.6
				(49.0–81.4)	(94.9–96.4)	(10.2–22.3)	(99.3–99.8)
5	E6/E7	224	7.4	86.1 (31)	93.6 (2810)	13.8	99.8
				(70.5–95.3)	(92.6–94.4)	(9.6–19.1)	(99.8–99.9)
6	hrHPV, HPV16/18 triage	99	3.3	80.60 (29)	97.70 (2933)	29.30	99.80
				(64.0–91.8)	(97.1–98.2)	(20.6–39.3)	(99.5–99.9)
7	hrHPV, LBC ASC-US+ triage	81	2.7	58.3 (21)	98.0 (2943)	25.9	99.5
				(42.2–72.9)	(97.4–98.4)	(17.6–99.7)	(99.2–99.7)
8	hrHPV, E6 triage	62	2.0	55.6 (20)	98.6 (2961)	32.3	99.5
				(38.1–72.1)	(98.1–99.0)	(20.9–45.3)	(99.1–99.7)
9	hrHPV, E7 triage	77	2.5	58.3 (21)	98.1 (2947)	27.3	99.5
				(40.8–74.5)	(97.6–98.6)	(17.7–38.6)	(99.2–99.7)
10	hrHPV, E6/E7 triage	96	3.2	75.0 (27)	97.7 (2934)	28.1	99.7
				(57.8–87.9)	(97.1–98.2)	(19.4–38.2)	(99.4–99.9)
11	HPV16/18, other 12hrHPV with LBC ASC-US triage	129	4.2	83.3 (30)	96.7 (2904)	23.3	99.8
				(68.1–92.1)	(96.0–97.3)	(16.8–31.3)	(99.6–99.9)
12	HPV16/18, other 12hrHPV with E6 triage	128	4.2	86.1 (31)	96.8 (2906)	24.2	99.8
				(70.5–95.3)	(96.1–97.4)	(17.1–32.6)	(99.6–99.9)
13	HPV16/18, other 12hrHPV with E7 triage	135	4.4	88.9 (32)	96.6 (2900)	23.7	99.9
				(73.9–96.9)	(95.9–97.2)	(16.8–31.8)	(99.6–100)
14	HPV16/18, other 12hrHPV with E6/E7 triage	145	4.8	88.9 (32)	96.2 (2890)	22.1	99.9
				(73.9–96.9)	(95.5–96.9)	(15.6–29.7)	(99.6–100)

Note: Of the 3,108 recruited women, 3,049 had complete primary screening results, and after accounting for 29 women lost to baseline colposcopy but 19 of them being successfully evaluated within 12 months, the final analytic cohort was 3,039 women.

primary screening and 75.0% *vs.* 58.3% in the "screen-triage" algorithm) with minimal loss in specificity (93.6% *vs.* 94.6% and 97.7% *vs.*98.0%). The concept of using ICC for objective biomarker detection in cervical cytology is well established, exemplified by the validated dual-stain cytology for p16/Ki-67, which demonstrates robust triage performance in many studies [22–24]. As mentioned, our study introduces an ICC assay targeting a different biological dimension: the direct detection of E6/E7 viral oncoproteins. This represents a theoretical distinction from p16/Ki-67, which detects downstream cellular markers of HPV oncogene activity. While detecting the proximal causative agents could hypothetically influence test characteristics, this remains speculative without direct comparative evidence. A direct head-to-head comparison between E6/E7 ICC and p16/Ki-67 was not performed in the current cohort. Given that p16/Ki-67 consistently shows high sensitivity (approximately 70%–90%) and good specificity (approximately 60%–80%) [25–28], and provides better long-term risk stratification than cytology over 5 years among hrHPV-positive women [29], future research directly comparing the performance, cost-effectiveness, and clinical utility of these two advanced ICC-based approaches within the same population is warranted to define their respective roles in optimized HPV-based cervical cancer screening algorithms.

Data on type-specific E6/E7 expression of the oncoproteins across HPV types remain limited. The ongoing ESTAMPA study, currently the largest investigation, reported preliminary E6/E7 positivity rates of 11% for HPV16, 10% for HPV18, 29% for HPV31, 17% for HPV33, and 12% for HPV52 using a qualitative, lateral-flow immunoassay that detects oncoproteins in a cell lysate [21]. Notably, our findings using the E6/E7 ICC assay

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revealed substantially higher positivity rates: 49.0% for HPV16, 38.1% for HPV31, 50.0% for HPV51 and HPV58, 40% for HPV52. These marked discrepancies, particularly the elevated rates of HPV16, HPV31, HPV51, and HPV58 in our study, warrant careful consideration. Potential explanations include differences in study populations, the distinct detection principles (morphological ICC visualization *vs*. the biochemical method used in ESTAMPA), and variations in the spectrum of HPV types targeted by each assay. Furthermore, specific technical factors—such as antibodies' epitope specificity, staining intensity thresholds for positivity, and the enhanced analytical sensitivity potentially conferred by AI-assisted image analysis in our ICC method—could substantially influence reported positivity rates and contribute to the observed discrepancies. These factors highlight that the two assays measure 'E6/E7 positivity' through distinct technological lenses, making direct numerical comparisons less informative. Given the scarcity of comparable data, generalizability and underlying reasons for these differences require cautious interpretation. These findings also highlight significant gaps in our understanding of type-specific E6/E7 expression patterns and underscore the need for further research to validate them, elucidate the contributing factors, and optimize E6/E7-based detection strategies.

Several limitations of this study warrant consideration. Firstly, the cross-sectional design provides robust estimates of test performance but does not assess longitudinal outcomes, such as the long-term risk of CIN2/3 or cancer development in test-negative women or the predictive value for future progression. Secondly, while the population-based screening cohort ($N = 3{,}039$) provides valuable real-world context, the absolute number of CIN2+ endpoints ($n = 36$), particularly within the hrHPV-positive subset (CIN2+ $n = 32$), limits the precision of some estimates, especially for high-sensitivity algorithms. Thirdly, the evaluation was conducted within a single screening round (within 12 months) in a specific population; further validation across diverse populations and healthcare settings is needed to confirm generalizability.

In conclusion, this first population-based, cross-sectional evaluation provides initial evidence for the potential of a novel E6/E7 oncoprotein-targeted immunocytochemistry assay in cervical cancer screening and triage. Our findings suggest that, while sensitivity was limited when used as a stand-alone screening or triage test in cervical cancer screening, a strategy combining HPV16/18 genotyping with E6/E7 ICC could achieve a favorable balance of sensitivity and specificity with a reduced immediate colposcopy referral rate. The cross-sectional design precludes assessment of long-term predictive value; therefore, longitudinal studies are essential to confirm these findings and evaluate the strategy's true clinical utility before any implementation can be considered.

Author Contributions

Remila Rezhake and Guzhalinuer Abulizi contributed to the conceptualization and design of the study. Guzhanuer Abuduxikuer, Tangnuer Abulimiti, Remila Rezhake, and Kadeliya Muhetaer contributed to data curation, formal analysis, and Interpretation. Tangnuer Abulimiti, Guligeina Abudurexiti, Kadeliya Muhetaer, Qian Zhuo, Jing Yang, Gulixian Tuerxun, and Wenyun Li contributed to project administration and supervision. Guzhanuer Abuduxikuer, Tangnuer Abulimiti, Remila Rezhake, and Guzhalinuer Abulizi contributed to writing the original draft of the manuscript. Guzhalinuer Abulizi and Jing Yang contributed to reviewing and editing. Remila Rezhake, Guzhalinuer Abulizi, and Tangnuer Abulimiti guaranteed the acquisition of funding. All authors approved the manuscript. Remila Rezhake, Guzhanuer Abuduxikuer, and Guzhalinuer Abulizi verified the data underlying this study. All authors were responsible for the decision to submit the paper for publication.

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Ethics Statement

The study protocol was approved by the Ethics Committee of The Affiliated Tumor Hospital of Xinjiang Medical University (Approval number: K-2021064). All recruited women provided written informed consent to participate in this study.

Conflicts of Interest

R.R. received free reagents from AttoBio from China for independent research. The funders had no role in the study design, data analysis, data interpretation, or report writing. The other authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Anonymized data will be shared with a signed data access agreement with publication on reasonable request (remila@xjmu. edu. cn and gzlnr@qq. com).

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Supporting Information

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