Predictive value of p16^{INK4a}, Ki-67 and ProExC immuno-qualitative features in LSIL progression into HSIL

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Abstract. The current nested case-control study was conducted to explore the prognostic value of cyclin-dependent kinase inhibitor 2A (p16^{INK4a}), marker of proliferation Ki-67 (Ki-67) and immunohistochemical cocktail containing antibodies directed against topoisomerase IIα (TOP2A) and minichromosome maintenance 2 (MCM2) proteins (ProExC) immuno-qualitative features to predict low-grade squamous intraepithelial lesion (LSIL) progression. A total of 92 LSIL patients were followed-up for 2 years, where those with high-grade squamous intraepithelial lesion (HSIL) or persistent LSIL were designated as the case group and those who spontaneously regressed were designated as the control group. The infection status of human papillomavirus (HPV) was evaluated using flow-through hybridization and gene chip, whilst the expression of p16^{INK4a}, Ki-67 and ProExC were tested in LSIL patient biopsies by immunohistochemistry. All data were collected at the beginning of the follow-up and patient outcomes were diagnosed by histopathological examination. To analyze the risk factors for LSIL progression, sensitivity, specificity, positive-negative predictive value (PPV-NPV), positive-negative likelihood ratio (PLR-NLR), Youden's index (YI) and multinomial logistic regression analysis was performed. The expression rates of p16^{INK4a}, Ki-67, and ProExC were found to be higher in the progression group compared with those in the persistence and regression groups. Only p16^{INK4a} expression significantly associated with high-risk HPV infection. With respect to predicting HSIL, p16^{INK4a} staining was the most sensitive but Ki-67 staining was found to be the most specific. YI was the highest (42.1%) for p16^{INK4a} expression in the present study, followed by ProExC (39.5%) and Ki-67 (28.3%). However, the expression of ProExC was found to be an independent risk factor for LSIL progression into HSIL. In conclusion, whilst immunohistochemical staining for p16^{INK4a}, Ki-67, and ProExC can be used to predict HSIL progression, only ProExC expression can be applied an independent risk factor for LSIL progression.

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Introduction

Cervical cancer is the second most common female malignancy in China (1), where Shanxi Province is a particularly high-risk (HR) area; with a detection rate of 55-100,000 in Jiexiu (2), which is higher compared with the average incidence of 13.4-100,000 in China (3). Therefore, strategies to prevent the occurrence of cervical cancer in China, especially in Shanxi, are urgently required. Cervical cancer generally develops from pre-existing, non-invasive and squamous precursor lesions, referred to as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL), which also includes low-grade (LSIL) and high-grade SIL (HSIL). Since SIL develops gradually, early diagnosis is an important means of preventing cervical cancer. Although 60% of LSIL cases are transient and spontaneously regress within 12-24 months, LSIL do persist in 30% of the cases, where ~10% of LSIL develop into HSIL within 2 years (4). Since only a small percentage of patients with LSIL progress into HSIL, it is therefore preferable to identify patients with LSIL so that resection can be performed at an early stage, where they can be safely followed up using cervix cytology and colposcopy until the lesions regress. Although loop electrosurgical excision (LEEP) procedures can be performed on LSIL patients to avoid LSIL progression, this treatment can be considered excessive in addition to causing an economic burden on the healthcare system (5). Therefore, it is of medical and economical relevance to identify biomarkers that can distinguish patients with LSIL that are at high risk of progressing into HSIL and cervical cancer (6,7).

Among human papillomavirus (HPV)-infected individuals, only 1% will progress to cervical cancer (8-11). Investigations into the relationship between HPV and the host cell cycle have identified a number of biomarkers, including cyclin-dependent kinase inhibitor 2A (p16^{INK4a}), marker of

proliferation Ki-67 (Ki-67) and immunohistochemical cocktail containing antibodies direct against topoisomerase IIα (TOP2A) and minichromosome maintenance 2 (MCM2) proteins (ProExC), which can be measured to improve the detection and grading of HPV-associated SIL, as they were found to be overexpressed in HPV-infected cells (12). p16^{INK4a} is a kinase inhibitor and tumor suppressor that regulates cell cycle progression in the G_1-S phase and inhibits cell proliferation through a reciprocal relationship with another tumor suppressor protein, retinoblastoma protein (pRb) (13). Ki-67 is a nuclear protein expressed only during the active phases of the cell cycle, namely in the late G_1, S, G_2 and M phases, but not during the resting phases (G_0 and early G_1) (14). By contrast, ProExC, a specific marker of S phase-induced abnormalities, is associated with transcriptional dysregulation and abnormalities caused by the HPV E7 oncoprotein through the E2F transcription factor pathway, which serves an important role in the development and progression of cervical cancer (15,16). As ProExC is predominantly localized in the nucleus and has demonstrated high sensitivity and specificity for HSIL diagnosis (17). Furthermore, it is easier to identify compared with p16^{INK4a} (18). It is therefore possible that p16^{INK4a}, Ki-67 and ProExC can be applied as early HSIL and cervical cancer risk indicators in early cervical lesions. However, to the best of our knowledge, there have been limited studies on the predictive value of p16^{INK4a} and Ki-67 for LSIL prognosis with inconsistent results (19-21), whilst the use of ProExC staining as a biomarker for LSIL prognosis has not been previously reported.

The central aim of the present study was to investigate the expression profiles of p16^{INK4a}, Ki-67, and ProExC in LSIL that progressed into HSIL and those that regressed or exhibited stable LSIL. Additionally, the prognostic value of p16^{INK4a}, Ki-67 and ProExC as potential markers for LSIL progression was evaluated.

Materials and methods

Study design and patient selection. The present study followed a nested case-control design. Patients were recruited in Jiexiu, Shanxi between October and December 2014 and were willing to participate in the screening program. The inclusion criteria were as follows: i) Of Han Chinese ethnicity; ii) married; iii) had resided in Shanxi for ≥1 year; iv) had current or past sexual activity; v) not pregnant; vi) no prior history of cervical cancer or precancerous lesions; vii) no prior history of treatments associated with the cervix including LEEP, conization and adnexectomy; viii) agreed to participate in the present study. A total of 6,257 women aged 19-65 years were included and completed a demographic characteristic-related questionnaire and underwent thin-prep cytologic test (TCT) testing. All participants with abnormal cervical cytology results were referred to colposcopy and histopathological examination. Of the 438 women diagnosed with atypical squamous cells of undetermined significance and above according to the TCT (ASC-US+), 118 women were excluded; with 61 due to refused consent, 53 due to incomplete medical examination and 4 due to inadequate information, including outliers in the questionnaire regarding age and pregnancy history. Of a total of 320 women who received the final diagnosis, 194 were diagnosed with normal cervix, 96 with LSIL, 28 with HSIL and 2 with SCC. Of the 96 women diagnosed with LSIL, 16 progressed to HSIL and 24 exhibited persistent LSIL which were classified as the case group, whilst 52 patients whose LSIL regressed spontaneously were considered the control group. A total of 4 patients were excluded due to insufficient material in the consecutive follow-up (Fig. 1).

All patients satisfying the inclusion criteria, including those diagnosed with HSIL, were followed-up every 6 months for 2 years with colposcopy and histopathological examinations. Any patients diagnosed with HSIL were treated by professional clinicians using LEEP or cervical conization, where endocervical curettage was performed simultaneously. and their follow-up was completed. All subjects provided written informed consent; the present study was approved by the Ethical Committee of Shanxi Medical University (Shanxi, China) and performed in accordance with the Declaration of Helsinki.

Liquid-based cytology and HR-HPV testing. All subjects were requested to provide two cervical tissue specimens, obtained by exfoliation using a brush during gynecological examination. One sample was automatically prepared for TCT using the Cytyc Thinprep® 2000 (Cytyc Corporation). Cytological classifications of disease grade were made by 2 cytopathology physicians under double-blinded conditions using parameters defined by the current 2001 Bethesda System (22). The other sample was processed for HPV genotyping by Hybri-Max™ using an HPV GenoArray Diagnostic kit (Guangdong Hybribio Biotechnology Co., Ltd.). A total of 21 types of HPV, including 15 HR-HPV serotypes and 6 low-risk serotypes can be identified by flow-through hybridization using a SLAN®-96S Real-Time PCR System (cat. no. SN 161403401; Shanghai Hongshi Medical Technology Co., Ltd.) and HHM-2 fast nucleic acid molecule hybridization instrument (cat. no. 20152400604; Guangdong Hybribio Biotechnology Co., Ltd.) (23).

Colposcopy and histopathological examination. Colposcopy was performed by gynecological specialists at the Second Hospital of Shanxi Medical University (Shanxi, China) using the Preventive Oncology International micro-biopsy protocol of directed and random biopsies (24), which results in ≥4 cervical biopsies received from patients with or without endocervical curettage (2). The histology slides were interpreted and the diagnosis was agreed upon by the two pathologists.

Immunohistochemical (IHC) detection of p16^{INK4a}, Ki-67 and ProExC. All specimens were fixed in 10% formalin for 24 h at room temperature, embedded in paraffin, cut into continuous 4-μm sections and subsequently dewaxed. Following alcohol dehydration, the sections were incubated in 3% hydrogen peroxide for 20 min at room temperature to block endogenous peroxidase activity. Antigen retrieval was then performed in 10 mM citrate buffer (Dako; Agilent Technologies, Inc.) for 3 min at 100˚C and PBS washing, with this step was repeated for 3 times. The sections were blocked with 10% normal goat serum (ZSGB-BIO; OriGene Technologies, Inc.) for 10 min at room temperature followed

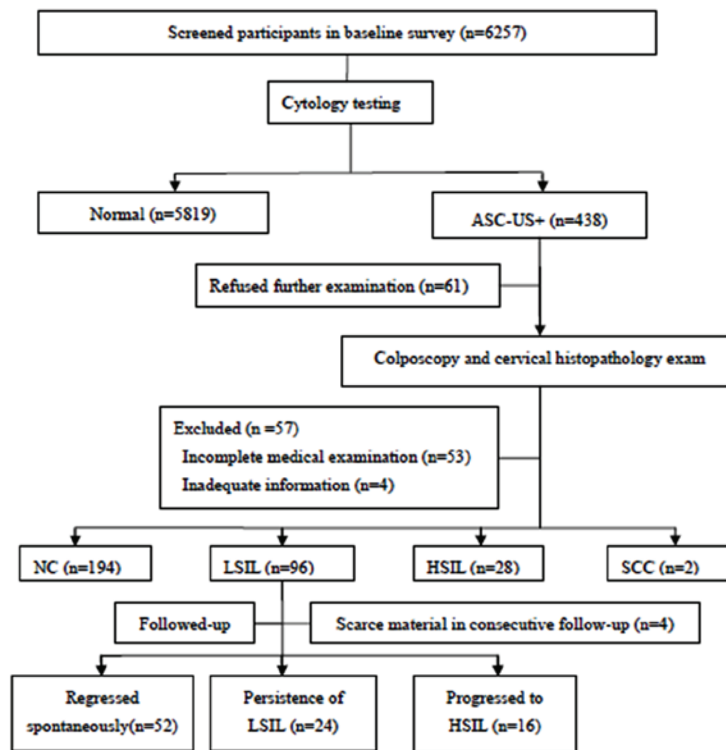

Figure 1. Schematic representation of the study design, from the recruitment of subjects to final analysis. NC, normal cervix; LSIL, low-grade cervical intraepithelial neoplasia; ASC-US, atypical squamous cells of undetermined significance; HSIL, high-grade cervical intraepithelial neoplasia; SCC, squamous cell carcinoma.

by incubation overnight at 4˚C with the following primary antibodies: Monoclonal mouse anti-p16^{INK4a} (1:100; cat. no. TA500036; OriGene Technologies, Inc.), monoclonal mouse anti-Ki-67 (1:100; cat. no. RMA-0542; Fuzhou Maixin Biotech Co., Ltd.) and ProExC, formed by mixing mouse monoclonal anti-MCM2 at (1:100; cat. no. sc-373702) with anti-TOP2A antibody (1:50; cat. no. sc-365916; both Santa Cruz Biotechnology, Inc.). The following day, the sections were treated in accordance with manufacturer's protocol of Histostain-SP kit (cat. no. SP-9002; ZSGB-BIO; OriGene Technologies, Inc.). Briefly, the sections were incubated with biotinylated goat anti-mouse immunoglobulin G secondary antibodies for 15 min at room temperature and horseradish peroxidase-conjugated streptavidin for 15 min at room temperature. The slides were then incubated with 3,3-diaminobenzidine for 1-2 min, washed three times using PBS, and stained with hematoxylin-eosin for 1 min. Following dehydration, the sections became transparent and were covered with neutral balsam (cat. no. G8590; Beijing Solarbio Science & Technology Co., Ltd.). Human prostate carcinoma tissues provided by the Pathology Department of the Second Hospital of Shanxi Medical University (Shanxi, China) were applied as positive controls and PBS solution without primary antibodies was used as negative control (Fig. 2).

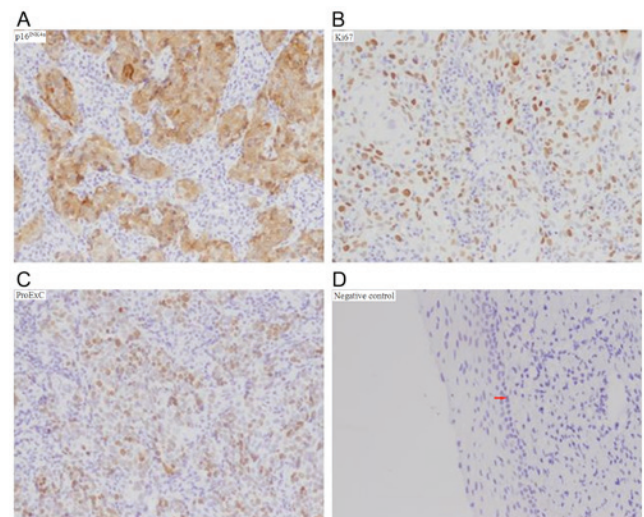

Figure 2. Positive and negative controls. (A) Positive controls for p16^{INK4a}, (B) Ki67, (C) ProExC in prostate cancer tissues. (D) Negative control for p16^{INK4a}, Ki67 and ProExC in cervical cancer tissues. Magnification, x200.

Light microscopic evaluation of p16^{INK4a}, Ki-67 and ProExC staining. Nuclear or nuclear plus cytoplasmic staining was considered to be positive for p16^{INK4a} expression, whilst cytoplasmic staining alone was recorded as negative. Staining for Ki-67 and ProExC was exclusively nuclear. For p16^{INK4a}, a lack of staining, staining of isolated cells or small cell clusters, and a focal staining pattern were considered negative; whereas continuous, diffuse cellular staining in the basal and parabasal cell layers was considered positive (Fig. 3) (25). For Ki-67, <50% staining or staining only in the lower half of the epithelium was interpreted as negative; >50% staining or staining in more than half of the epithelium was considered positive (Fig. 4) (26). For ProExC, minor adjustments were made to the protocols previously reported by Shi *et al* (27) and

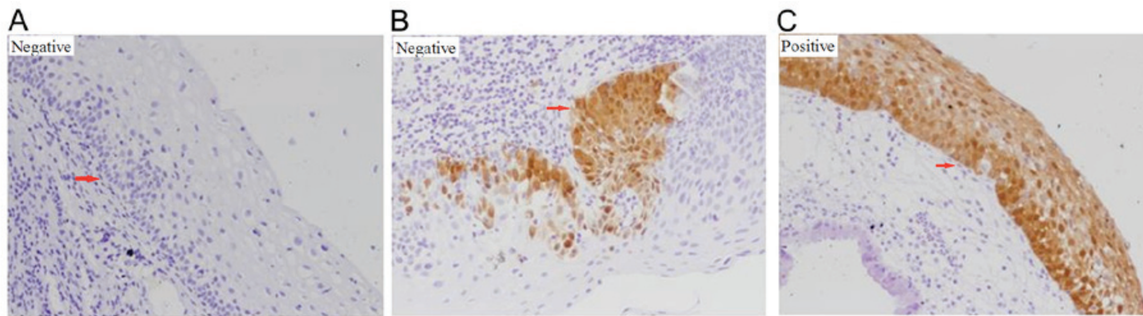

Figure 3. Expression of p16INK4a in low-grade cervical intraepithelial neoplasia tissues. (A) Representative image of a specimen exhibiting negative staining. (B) Representative image of a specimen exhibiting focal staining patterns, which were regarded as negative. (C) Representative image of a specimen exhibiting continuous, diffuse staining, which was regarded as positive. Magnification, x200. p16^{INK4a}, cyclin-dependent kinase inhibitor 2A.

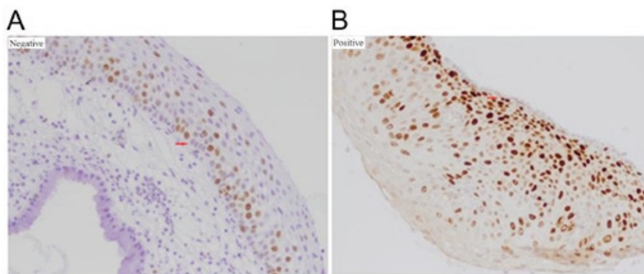

Figure 4. Expression of Ki67 in low-grade cervical intraepithelial neoplasia tissues. (A) Representative image of a specimen exhibiting staining representing <50%, interpreted as negative. (B) Representative image of a specimen exhibiting staining representing >50%, considered as positive. Magnification, x200. Ki-67, marker of proliferation Ki-67.

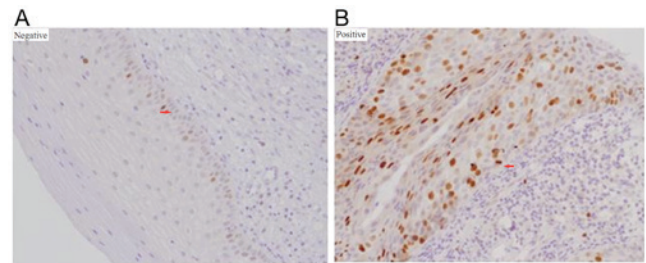

Figure 5. Expression of ProExC in in low-grade cervical intraepithelial neoplasia tissues. (A) Cell staining occupying <33% of squamous epithelium was regarded as negative. (B) Cell staining occupying more thana third of squamous epithelium was regarded as positive. Magnification, x200. ProExC, DNA topoisomerase IIα and minichromosome maintenance complex component 2 antibody cocktail.

Pinto *et al* (28), where staining was assessed in accordance with the distribution of positive cells in the vertical plane of the squamous epithelium. No positive cells or positive cells occupying <33% of the squamous epithelium was interpreted as negative, whilst positive cells occupying >33% of the squamous epithelium was interpreted as positive (Fig. 5). One tissue section was analyzed per patient. Blinded analysis of all sections was conducted by two pathologists using light microscopy (BX46; Olympus Corporation) according to the protocol described previously (29).

Statistical analysis. All statistical analyses were performed using the SPSS software package (version 22.0; IBM Corp.). The data are expressed as the mean ± standard deviation or percentages. One-way ANOVA and Pearson's Chi-squared test were applied to compare continuous and categorical factors, respectively. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), negative likelihood ratio (NLR) and Youden's index (YI) were calculated based on p16^{INK4a}, Ki-67, and ProExC staining using the formulae of Galen and Gambino (30): Sensitivity = true positive-(true positive + false negative); specificity = true negative-(false positive + true negative); PPV = true positive-(true positive + false positive); NPV = true negative-(false negative + true negative); PLR = sensitivity-(1-specificity); NLR = (1-sensitivity)-specificity; and YI = sensitivity + specificity-1. Multinomial logistic regression analysis was used to analyze the association between the potential predictor variables and LSIL prognosis. Data from the LSIL regression group served as the reference category. The odds ratios (OR) with 95% confidence intervals (CI) were calculated based on Wald Chi-squared statistics. All statistical tests were two-tailed and P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. A total of 92 cases of LSIL were found; of which 16 patients progressed to HSIL, 24 patients were diagnosed with persistent LSIL and 52 patients exhibited LSIL that regressed spontaneously. Table I displays the characteristics of the subjects in the progression, persistence and regression groups. The median ages in the progression, persistence, and regression groups were 42.8 (29-55 years), 45.9 (26-60 years) and 44.9 years (24-60 years), respectively. No significant differences were observed in age, age at menarche, age at marriage, age at first intercourse, age at first pregnancy, age at delivery, TCT, HR-HPV infection, spouse smoking history, gravidity, parity, menstrual regularity, condom use, intrauterine device use, vaginitis history, pelvic inflammation disease history, chronic disease history, or gynecological operation history among the regression, persistence and progression groups. However, there were significant differences in menopause status, where significantly fewer post-menopausal subjects were found in the progression group compared with those in the persistence and regression groups.

Table I. Characteristics among the study population (n=92).

Characteristic	Progression (n=16)	Persistence (n=24)	Regression (n=52)	P-value[a]
Age (years)	42.8±7.3	45.9±9.2	44.9±9.4	0.188
Age at menarche (years)	14.6±1.5	14.9±2.2	14.5±2.1	0.794
Age at marriage (years)	23.3±2.7	23.3±4.4	23.1±2.2	0.963
Age at first intercourse (years)	23.0±2.7	22.8±4.3	22.9±2.4	0.984
Age of first pregnancy (years)	23.9±2.6	23.0±6.3	23.7±2.5	0.726
Age at delivery (years)	25.2±3.0	23.5±6.5	24.3±2.5	0.445
TCT[b] (1-2-3-4-5) %	0-25.0-62.5-12.5-0	4.2-33.3-37.5-20.8-4.2	4.0-16.0-68.0-10.0-2.0	0.461
HR-HPV infection (% yes)	81.3	62.5	48.1	0.054
Spouse smoking history (% yes)	87.5	75	59.6	0.08
Gravidity (%, <2 times)	37.5	45.8	46.2	0.822
Parity (%, <3 times)	75	79.2	88.5	0.348
Menstrual regularity (% yes)	68.8	87.5	90.4	0.091
Menopausal status (% post)	6.3	41.7	34.6	0.047
Condoms use ever (% yes)	6.3	8.3	11.5	0.793
IUD use ever (% yes)	50	62.5	42.3	0.261
Vaginitis history (% yes)	43.8	29.2	36.5	0.911
Pelvic inflammatory disease history (% yes)	12.5	0	23.1	0.113
Chronic disease history (% yes)	12.5	16.7	15.4	0.936
Gynecological operation history (% yes)	25	8.3	11.5	0.274

[a]P-values were based on ANOVA for continuous variables and Chi-squared for categorical variables. [b]Including five types: 1=normal, 2=inflammation, 3=ASC-US, 4=LSIL and 5=HSIL. LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; IUD, Intrauterine device; TCT, thin-prep cytologic test; ASC-US, atypical squamous cells-undetermined significance.

p16^{INK4a}, Ki-67 and ProExC staining and analysis of association with HR-HPV infection. According to IHC, positive p16^{INK4a} was defined as observation of nuclear or nuclear plus cytoplasmic staining, whereas positive Ki-67 and ProExC expression were defined as exclusively nuclear staining. The p16^{INK4a} expression rates in the progression, persistence, and regression groups were found to be 75.0, 41.7 and 28.8%, respectively. The Ki-67 expression rates in the progression, persistence, and regression groups were 37.5, 12.5 and 7.7%, respectively. The rates of positive ProExC staining in the progression, persistence, and regression groups were 75.0, 41.7 and 32.7%, respectively (Table II). The differences in p16^{INK4a}, Ki-67 and ProExC staining between the 3 groups were statistically significant (χ^2=10.87, 9.03, 8.98; P<0.05). Specifically, the rates of p16^{INK4a}, Ki-67, and ProExC staining were higher in the progression group compared with those in the persistence and regression groups (P<0.05), but the differences between the persistence and regression groups were not statistically significant (P>0.05; Table II).

To analyze the association between HR-HPV infection and p16^{INK4a}, Ki-67 and ProExC staining, the 92 subjects were divided into 2 groups in accordance with their HR-HPV status. The rate of positive p16^{INK4a} expression in the HR-HPV-positive group was significantly higher compared with that in the HR-HPV-negative group. Specifically, p16^{INK4a} expression was found to significantly positively associated with HR-HPV infection (P=0.001; Table III). However, no significant associations were found between Ki-67 or ProExC expression status and HR-HPV infection (P>0.05; Table III). Of the four LSIL progression cases with negative ProExC immunostaining, three (3-4, 75%) were tested negative for HR-HPV. By contrast, the majority of (10-12, 83%) the LSIL progression cases demonstrating positive ProExC immunostaining were also tested HR-HPV positive (data not shown).

Statistical analysis of the utility of IHC for p16^{INK4a}, Ki-67, and ProExC for the prediction of HSIL progression. Patients with progression into HSIL were classified into the progression group, whereas those with LSIL regression or persistence were assigned into the non-progression group. The rates of positive p16^{INK4a}, Ki-67, and ProExC expression in the progression groups were found to be 75.0, 37.5 and 75.0%, respectively. The rates of positive expression of p16^{INK4a}, Ki-67, and ProExC in the non-progression groups (persistence and regression groups) were found to be 32.9 (25-76), 9.2 (7-76) and 35.5% (27-76), respectively (Table II).

Table IV shows the sensitivity, specificity, PPV, NPV, PLR, NLR, and YI for p16^{INK4a}, Ki-67, and ProExC staining for the prediction of progression to HSIL. The sensitivity and NPV were calculated to be the highest for p16^{INK4a} (75.0 and 92.7% respectively), whereas the specificity, PPV, PLR and NLR were the highest for Ki-67 (90.8%, 46.2%, 4.08 and 0.69, respectively). YI, which is considered a more comprehensive measure of sensitivity and specificity (31), was found to be the highest for p16^{INK4a} (42.1%), followed by ProExC (39.5%) and Ki-67 (28.3%).

Risk factors affecting LSIL progression. HR-HPV infection, and staining for p16^{INK4a}, Ki-67 and ProExC were examined

Table II. Comparison of p16INK4a, Ki67, ProExC expression in the progression, persistence and regression groups.

A, p16INK4a

Expression status	Progression (n=16)	Persistence (n=24)	Regression (n=52)	χ^2	P-value
Negative	4 (25.0)	14 (58.3%)	37 (71.2%)		0.004
Positive	12 (75.0%)	10 (41.7%)	15 (28.8%)	10.87	

B, Ki-67

Expression status	Progression (n=16)	Persistence (n=24)	Regression (n=52)	χ^2	P-value
Negative	10 (62.5%)	21 (87.5%)	48 (92.3%)		0.011
Positive	6 (37.5%)	3 (12.5%)	4 (7.7%)	9.03	

C, ProExC

Expression status	Progression (n=16)	Persistence (n=24)	Regression (n=52)	χ^2	P-value
Negative	4 (25.0%)	14 (58.3%)	35 (67.3%)		0.011
Positive	12 (75.0%)	10 (41.7%)	17 (32.7%)	8.98	

p16INK4a, cyclin-dependent kinase inhibitor 2A; Ki-67, marker of proliferation Ki-67; ProExC, DNA topoisomerase IIα and minichromosome maintenance complex component 2 antibody cocktail.

Table III. Association analysis between p16INK4a, Ki67 and ProExC expression with HR-HPV infection.

A, p16INK4a

	HR-HPV infection			
Expression status	Negative (%)	Positive (%)	χ^2	P-value
Negative	31 (79.5)	24 (45.3)	10.933	0.001
Positive	8 (20.5)	29 (54.7)		

B, Ki-67

	HR-HPV infection			
Expression status	Negative (%)	Positive (%)	χ^2	P-value
Negative	34 (87.2)	45 (84.9)	0.096	0.757
Positive	5 (12.8)	8 (15.1)		

C, ProExC

	HR-HPV infection			
Expression status	Negative (%)	Positive (%)	χ^2	P-value
Negative	23 (59.0)	30 (56.6)	0.052	0.82
Positive	16 (41.0)	23 (43.4)		

HR-HPV, high-risk human papilloma virus; p16INK4a, cyclin-dependent kinase inhibitor 2A; Ki-67, marker of proliferation Ki-67; ProExC, DNA topoisomerase IIα and minichromosome maintenance complex component 2 antibody cocktail.

Table IV. Values of p16^{INK4a}, Ki67 and ProExC positivity in LSIL specimens to predict HSIL.

Variable	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR	NLR	YI (%)
p16^{INK4a} positivity	75.00	67.10	32.40	92.70	2.28	0.38	42.10
Ki-67 positivity	37.50	90.80	46.20	87.30	4.08	0.69	28.30
ProExC positivity	75.00	64.50	30.80	92.40	2.11	0.39	39.50

PPV, positive predictive value; NPV, negative predctive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; YI, Youden's index; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; p16^{INK4a}, cyclin-dependent kinase inhibitor 2A; Ki-67, marker of proliferation Ki-67; ProExC, DNA topoisomerase IIα and minichromosome maintenance complex component 2 antibody cocktail.

by multivariate logistic regression analysis (significance level, α=0.05; permitted error =0.10) to obtain a statistically significant equation. Menopausal status, which was found to be a significant variable in the present study (Table I), was also included as one of the items in this analysis. The logistic regression models revealed ProExC expression to be an independent risk factor for LSIL progression after 2 years. The risk of progression for LSIL patients with positive ProExC staining was found to be 6.11-fold higher compared with that of patients negative for ProExC staining (95% CI, 1.438-25.997; Table V).

Discussion

In the present study, IHC was performed to detect and measure the expression of p16^{INK4a}, Ki-67, and ProExC in cervical specimens. IHC is a conventional method in clinical diagnosis where there is no strict requirement for the size of tissues.

The present nested case-control study demonstrated that although IHC staining for p16^{INK4a}, Ki-67 and ProExC can be applied to predict HSIL progression, only ProExC staining was an independent risk factor for LSIL progression after 2 years. To the best of our knowledge, the present study was the first to analyze the predictive value of ProExC staining compared with p16^{INK4a} and Ki-67 for the progression of LSIL among Han Chinese women.

The p16^{INK4a} tumor suppressor protein inhibits the cyclin-dependent kinases that regulate progression through the cell cycle by phosphorylating the retinoblastoma protein. In most cervical malignancies, the functional inactivation of pRb by HPV E7 results in the overexpression of p16^{INK4a} (32). Previous studies have demonstrated that p16^{INK4a} can be used as a prognostic marker for disease progression and infection by HR-HPV (33,34). Consistent with this notion, it was found in the present study that p16^{INK4a} expression was associated with HR-HPV infection. The value of p16^{INK4a} in CIN grading has been previously reported. In 2012, the American Society for Colposcopy and Cervical Pathology and the Lower Anogenital Squamous Terminology project recognized the value of p16^{INK4a} by proposing a new 2-stage classification system; specifically for LSIL to include CIN1 and p16^{INK4a}-negative CIN2, and for HSIL to include p16^{INK4a}-positive CIN2 and CIN3 (35). However, studies on the value of p16^{INK4a} in LSIL progression are limited, with inconsistent findings. In the past decade, the majority of studies suggested that patients with p16^{INK4a}-positive LSIL were at higher risks of developing HSIL (36-38). Liao *et al* (19) conducted a prospective population-based study to evaluate if the overexpression of p16^{INK4a} in LSIL biopsies can accurately predict HSIL progression and found that p16^{INK4a} expression in LSIL on initial diagnosis was associated with an increased risk of HSIL in 2 years (OR=1.43; 95% CI, 0.52-3.91). However, Sagasta *et al* (20) showed that HSIL/CIN2-3 exhibited higher positive rates for p16^{INK4a} staining compared with persistent or regressing LSIL/CIN1 lesions (71 vs. 44%), but found that p16^{INK4a} immunostaining was not associated with risk of HSIL [hazard ratio 1.6 (95% CI, 0.9-2.6); P=0.095]. Sagasta *et al* (20) subsequently concluded that p16^{INK4a} overexpression in biopsies from women with LSIL was a poor predictor in LSIL progression, with little or no value as a marker in clinical practice. Results from the present study were consistent with the latter study. The p16^{INK4a} expression rate in the progression group was higher compared with the persistence and regression groups, and the YI for p16^{INK4a} was the highest of the markers tested, suggesting that p16^{INK4a} was the most accurate marker for predicting HSIL progression. However, p16^{INK4a} expression was not an independent risk factor for LSIL progression after 2 years, suggesting that p16^{INK4a} can be used to increase the accuracy of the HSIL diagnosis but not in predicting LSIL progression.

Ki-67 is a DNA-binding enzyme which is utilized widely to measure tumor cell proliferation and evaluate the degree of tumor malignancy and prognosis. A number of studies have previously reported that Ki-67 expression is positively associated with the grade of cervical lesions (39-41). In 2015, the Bethesda guidelines recommended p16^{INK4a}/Ki-67 double staining as part of the cytological diagnostic procedure (42). A dual p16/Ki-67 immunocytochemistry assay is now available for use as an adjunct test for cervical cancer screening (43). In a previous study, the p16^{INK4a}/Ki-67 dual staining strategy was tested in a large, prospective clinical trial involving 27,456 women; where p16^{INK4a}/Ki-67 dual staining cytology testing was found to increase the sensitivity of HSIL diagnosis whilst maintaining high specificity (44). In another study, Kanthiya *et al* (45) found that Ki-67 expression was demonstrated in 75.4% of CIN2-3, 22.6% of CIN1 and 11.3% clinical specimens with non-dysplasia. These results suggest that Ki-67 overexpression can also be used as a marker for the tendency for progression in early cervical lesions. However, only one study, which was conducted by Kruse *et al* (46), included samples from patients with CIN1 (n=25) and CIN2 (n=65) and investigated Ki-67 IHC in CIN progression. They found that the prognostic value of the Ki-67 progression-risk model exceeded the value of the histopathological CIN grade for the prediction of progression to a higher CIN grade. Although it was found that the Ki-67 expression rate was higher in the

Table V. Multivariate analysis of potential risk factors for LSIL progression or persistence in patients with different cervical intraepithelial neoplasia prognosis.

A, LSIL persistence

						95% CI	
Variable	β	SE	Wald	P-value	OR	Lower	Upper
HR-HPV infection	0.607	0.549	1.223	0.269	1.834	0.626	5.376
menopause status	0.7	0.569	1.517	0.218	2.015	0.661	6.141
$p16^{INK4a}$ staining	0.407	0.584	0.485	0.486	1.502	0.478	4.717
Ki67 staining	0.57	0.867	0.432	0.511	1.767	0.323	9.664
ProExC staining	0.579	0.547	1.121	0.29	1.785	0.611	5.217

B, LSIL progression

						95% CI	
Variable	β	SE	Wald	P-value	OR	Lower	Upper
HR-HPV infection	1.502	0.844	3.167	0.075	4.489	0.859	23.465
menopause status	-0.835	1.159	0.519	0.471	0.434	0.045	4.208
$p16^{INK4a}$ staining	1.038	0.771	1.812	0.178	2.823	0.623	12.791
Ki67 staining	1.526	0.91	2.808	0.094	4.598	0.772	27.386
ProExC staining	1.811	0.738	6.011	0.014	6.114	1.438	25.997

LSIL regression was used as the reference group. OR, odds ratio; CI, confidence interval; Wald, Wald Chi-squared statistic. LSIL, low-grade squamous intraepithelial lesions; $p16^{INK4a}$, cyclin-dependent kinase inhibitor 2A; Ki-67, marker of proliferation Ki-67; ProExC, DNA topoisomerase IIα and minichromosome maintenance complex component 2 antibody cocktail; HR-HPV, high-risk human papillomavirus; SE, standard error.

progression group compared with that in the persistence and regression groups in the present study, Ki-67 expression was not an independent risk factor for LSIL progression after 2 years. Therefore, further studies are crucial to confirm the value of Ki-67 IHC for the prediction of LSIL progression.

ProExC consists of antibodies specific for MCM2 and TOP2A, both of which are overexpressed during cervical dysplasia and neoplasia (14). MCM2 is a member of the DNA licensing factor family and a marker of cell proliferation, whereas TOP2A is an enzyme that unwinds and decatenates DNA in preparation for DNA replication, transcription, chromosome segregation, and cell cycle progression (47,48). Walts and Bose (12) evaluated the efficacy of $p16^{INK4a}$, Ki-67, and ProExC immunostaining, alone and in combination, for the diagnosis of CIN, and provided guidance for instances with discordant staining patterns. ProExC expression was found in 26.0% CIN2-3 and in 6.0% CIN1 cases. Although these findings confirmed that $p16^{INK4a}$, Ki-67, and ProExC are specific and sensitive markers that can be used for the diagnosis of CIN, no prospective studies had previously investigated the predictive value of ProExC for LSIL progression. The present study found that the ProExC staining rate in the progression group was higher compared with those in the persistence and regression groups and that the YI for ProExC staining was higher compared with that for Ki-67, indicating that ProExC IHC was more suitable for the predicting progression to HSIL. The overexpression of MCM2 provides the link between oncogenic HPV infection and the molecular event of cervical dysplasia (49), which is consistent with data from the present study. Of the four LSIL progression cases with negative ProExC immunostaining, three (3-4, 75%) were negative for HR-HPV. By contrast, the majority of (10-12, 83%) the LSIL progression cases demonstrating positive ProExC immunostaining were also tested HR-HPV positive. Additionally, only positive ProExC staining was found to be an independent risk factor for LSIL progression in 2 years, suggesting that ProExC-positive LSIL pose a higher risk of developing into HSIL.

To conclude, to the best of our knowledge, this is the first study to report that compared with $p16^{INK4a}$ and Ki-67, only ProExC staining was an independent risk factor for LSIL progression over 2 years. This study provides a new insight into identifying LSIL patients at a higher risk of malignant progression, potentially facilitating more cost-effective and efficient interventions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LD, LS, WZ and JW conceived and designed the present study. XL, WG and ZQ collected the data and performed the experiments. LD and WZ performed the data analysis and interpretation. LD and LS wrote the manuscript. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Shanxi Medical University Science Research Ethics Committee (Shanxi, China), and written informed consent was obtained from all patients prior to publication.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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