Immunohistochemical detection of E7 human papillomavirus protein in pre-malignant and malignant lesions of the uterine cervix

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Abstract. Human papillomavirus (HPV) E7 protein expression is caused by HPV viral DNA integration into human cellular DNA, and is a prerequisite for the development and progression of cervical cancer. The present study aimed to evaluate the role of E7 protein as a biomarker for identification of transformed cervical epithelial cells during the early stages of cervical cancer. Specific monoclonal antibodies to the E7 protein of high-risk HPVs were generated and characterized for applications in immunocytochemistry and immunohistochemistry using cervical epithelial cells or biopsy tissue slides. The specificity and feasibility for detecting precancerous cells in cervical exfoliated epithelial cells was demonstrated. In addition, antibody staining of cervix biopsies indicated the pathological grades of cervical cancer and precancerous lesions. The results of the present study demonstrated the potential benefit of using E7 protein as a novel and specific clinical diagnostic marker to distinguish transient HPV infections from malignant and pre-malignant lesions.

Introduction

Cervical cancer is the second most common malignant tumor in women worldwide, and a leading cause of cancer deaths of females in developing countries (1). Human papillomavirus (HPV) infection is the etiologic agent for virtually all cases of cervical squamous cell carcinoma (SCC) and a large portion of endocervical adenocarcinoma. Among >200 human papillomavirus phenotypes, 14 high-risk HPV (hrHPV) phenotypes have been reported to be closely associated with the initiation and progression of cervical cancer (2). Two genotypes of hrHPV, including HPV 16 and 18, are responsible for ~75% of all cases of cervical cancer (2). Continuous production of the E7 protein from oncogenic genotypes of HPV is required for progression of malignancy (2). Thus, sensitive and specific detection of E7 HPV protein expression in the clinical samples of exfoliated epithelial cells or biopsies from cervix may provide a clinical benefit for early detection of precancerous conditions.

The HPV genome consists of six early open reading frames (E1, E2, E4, E5, E6, and E7), two late open reading frames (L1 and L2). Among six proteins encoded by early open reading frames, E6 and E7 are critical for the development of cervical cancer by regulating cervical epithelial cell immortalization (3). Transient viral infection usually resolves spontaneously within 6 to 12 months without increasing the risk of cervical cancer (4). However, in certain cases, viral DNA can integrate into the host genome to cause persistent HPV infection, resulting in an abnormal accumulation of HPV E6 and E7 proteins within host cells (5). Overexpression of E7 protein can competitively bind to the tumor suppressor protein, retinoblastoma, to cause the disassociation of E2F proteins, leading to the abnormal proliferation of cervical cells (6,7) and indispensably contributing to the development of cervical intraepithelial neoplasia (CIN), adenocarcinoma *in situ* and invasive cervical carcinoma (4,8).

Viral genotyping analysis to identify the oncogenic HPV has not produced good predictive values for the development of intraepithelial CIN lesions (8). It has been reported that 12-14% of low-grade squamous intraepithelial lesions (LSIL) progress to high-grade squamous intraepithelial lesions (HSIL) (9); without treatment, a few of those lesions progress to invasive cancer (10). Further investigation is required to identify other parameters with clinically meaningful predictive values.

Protein biomarkers involved in the development of cervical cancer, including SCC antigen, serum fragments of cytokeratin, carcinoma embryonic antigen, soluble CD44 and matrix metalloproteinases, have been considered as potential diagnostic markers for cervical cancer screening (11). However, these potential protein biomarkers are encoded by the human genome; therefore, diagnostic use is inhibited by the endogenous expression of these biomarkers within noncancerous cervical cells. The oncoprotein E7 encoded by the hrHPV genome with specific expression in HPV-transformed human cervical cells may serve as an ideal biomarker for the early detection of cervical cancer (12).

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In the present study, investigation of the E7 oncoprotein marker via immunostaining of exfoliated epithelial cells and formalin-fixed paraffin-embedded biopsies for the identification of pre-malignant and malignant lesions in squamous cervical carcinoma was performed.

Materials and methods

Cervical cancer cell lines. CaSki cell line (HPV16 positive; CRL-1550™), HeLa cell line (HPV18 positive; CCL-2™) and C-33A cell line (HPV negative; HTB-31™) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). CaSki cells were cultured in RPMI-1640 medium (SH30027; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/m penicillin and 100 g/ml streptomycin. HeLa and C-33A cells were cultured in Minimum Essential Medium (SH30024; Hyclone; GE Healthcare Life Sciences) containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 g/ml streptomycin. For immunocytochemical (ICC) staining, cells ($3x10^4$/well) were transferred to a 24-well plate and were cultured to reach approximately 60-70% confluency. Then cells were rinsed twice with 10 mM PBS (pH 7.4) and fixed in 4% paraformaldehyde for 15 min at room temperature. For other assays, adherent cells were harvested following digestion by trypsin (25200; Gibco; Thermo Fisher Scientific, Inc.). Following two washes with PBS, the harvested cells were fixed in 4% paraformaldehyde for 10 min at room temperature for liquid based cytology (LBC).

Preparation of E7 recombinant proteins. DNA fragments encoding E7 proteins in hrHPV16, 18, 31, 33, 35, 39, 45, 52, 58 and 59 strains, and low-risk HPV (lrHPV) 6 and 11 strains were cloned and incorporated into pET-21a(+) (Novagen; Merck KGaA, Darmstadt, Germany) or pGEX-4T-1 (GE Healthcare Life Sciences) plasmids, and then were expressed in *Escherichia coli* (*E. coli*) BL21/DE3 (American Type Culture Collection, Manassas, VA, USA) to purify His-tagged or glutathione S-transferase-tagged fusion protein. Protein purification was performed as described previously (13,14). The aforementioned proteins tagged with green fluorescent protein were prepared by transient expression in 293 mammalian cell line (ATCC; CRL-3216). Coding sequences of E7 proteins were optimized from original HPV genomic sequences. The accession numbers in Genbank were as follows: AHK23257.1 (HPV16), AGM34461.1 (HPV18), AGM34454.1 (HPV31), AGM34459.1 (HPV33), ACV53985.1 (HPV35), AGU90520.1 (HPV39), AGM34464.1 (HPV45), AET07150.1 (HPV52), AFO63477.1 (HPV58) and ACL12335.1 (HPV59).

Preparation of E7 monoclonal antibodies (mAbs). The present study was approved by the ethics committee of The First Affiliated Hospital of Soochow University (Soochow, China). mAbs against hrHPV E7 proteins were generated by immunizing mice or rabbits with purified HPV16 or 18 recombinant proteins, followed by multistage procedures of established technologies for screening antibody clones from hybridoma (15) and from phage displayed antibody libraries (16-18). Individual clones of mAbs were analyzed by various assays including, ELISA with *E. coli*-derived recombinant hrHPVs and lrHPVs E7 proteins, western blot analysis using *E. coli* or mammalian-expressed E7 proteins or endogenous E7 proteins from cervical cancer cell lines, ICC using cervical cancer cell lines, and immunohistochemistry (IHC) using human cancer tissue sections. Immunoreactivity of antibodies generated from HPV16 E7 or HPV18 E7 immunized mice or rabbit were tested against E7 proteins from other HPV strains by ELISA using the aforementioned recombinant proteins. Subsequently, mAb clones with desirable properties from either murine or rabbit origins were selected for molecular cloning of immunoglobulin G (IgG) genes. Cloning of IgG genes was accomplished by isolating the coding sequence of immunoglobulin heavy chain and light chain from hybridoma cells, or scFv sequences in the M13 phage, as described previously (19). The intact IgG molecules for mAb from murine (clone no. E7MuB6 and E7MuH1) and from rabbit (clone no. E7Rb04 and E7Rb19) were produced by large-scale 293 cell transfection using Lipofectamine® LTX reagent (Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. Secreted IgG protein in culture medium was subjected to affinity purification by chromatography using a Protein-A Sepherose 4B column (cat. no. 101041; Thermo Fisher Scientific, Inc.). Protein concentration of purified IgG was determined by measuring the absorbance at 280 nm and the purity was analyzed via SDS-PAGE. Immunological activities of antibodies were analyzed by ELISA, and ICC and IHC staining.

The cyclin dependent kinase inhibitor 2A ($p16^{INK4A}$; clone no. E6H4; cat. no. 705-4713) antibody was purchased from Ventana Medical Systems, Inc. (Oro Valley, AZ, USA) and secondary antibody reagent EnVision+ Peroxidase system (cat. no. K5007) was from Dako (Agilent Technologies, Inc., Santa Clara, CA, USA).

Specimens. The use of human tissue in the present study was approved by the Institutional Review Board at the First Affiliated Hospital of Soochow University (Soochow, China). Tissue slides (three slides from each sample) used for the evaluation of the IHC staining were prepared using formalin-fixed and paraffin-embedded (FFPE) tissue blocks. Specimens with cervical LSIL (45 samples), HSIL (64 samples) and invasive SCC (7 samples) were selected from the tissue archives in the Pathology Department at the First Affiliated Hospital of Soochow University. The age of the patients ranged from 28-66 years with an mean age of 41.8 years. All specimens were cut into 4-5 mm sections. Three slides from each tissue sample were selected for hematoxylin and eosin staining, E7 antibody immunostaining and $p16^{INK4A}$ antibody immunostaining.

Exfoliated cervical epithelial cells were obtained from 13 patients with HSIL at the Cancer Hospital at the Chinese Academy of Medical Sciences (Beijing, China). Samples from 10 healthy individuals were obtained from Peking University Hospital (Beijing, China). Cells were preserved in an LBC fixative solution and used within 6 months post-fixation. Those specimens were previously stained in Pap-stain and graded according to the 2001 Bethesda System (20) by the pathologists at Peking University First Hospital and Cancer Hospital Chinese Academy of Medical Sciences (Beijing, China). Slides were treated with a graded series of ethanol

(100, 70, 50% and water; 1 min each), followed by staining with hematoxylin solution, Harris modified (Sigma-Aldrich; Merck KGaA) at room temperature for 5 min. Slides were washed with water for 10 sec and incubated with 0.5% hydrochloric acid for 8 sec, followed by a further wash with tap water for 5 min. Subsequently, slides were treated with a graded series of ethanol (50, 70, 80 and 96%; 30 sec each), followed by EA-50 staining at room temperature for 2.5 min and washing with 95% ethanol for 1 min and 100% ethanol for 1 min. Finally, slides were washed twice with xylene for 2 min each and mounted with VectaMount™ Permanent Mounting Medium (Vector Laboratories, Burlingame, CA, USA). The study protocol was proved by Institutional Review Board at the Peking University First Hospital and Cancer Hospital Chinese Academy of Medical Sciences.

Western blotting. Western blot analysis of endogenous E7 proteins was performed by immunoprecipitation of cell lysates. Protein was extracted from CaSki, HeLa and C-33A cervical cancer cell lines using radioimmunoprecipitation lysis and extraction buffer (Thermo Fisher Scientific, Inc.). Immunoprecipitation was performed as described previously (21). Protein concentration was determined with the bicinchoninic acid method. Protein (20 μg/lane) was subjected to 10% SDS-PAGE, transmembrane and immunoblotting with anti-E7 antibodies. Membranes were blocked at room temperature for 10 min with TBS-Tween (TBST; 50 mM Tris-HCl, 150 mM NaCl, 0.5% Tween 20, pH 7.6) containing 5% non-fat milk. Following washing, membranes were incubated with the aforementioned primary monoclonal E7 antibodies (1:1,000) overnight at 4˚C. Following washing, membranes were incubated with the horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody (1:1,000; cat. no. A2554, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or HRP-labeled mouse anti-rabbit secondary antibody (1:1,000; cat. no. A1949, Sigma-Aldrich; Merck KGaA) at room temperature for 2 h. Finally, signals were detected by adding HRP substrate solution containing 3,3'-diaminobenzidine chromogen (cat. no. ZLT-9033; OriGene Technologies, Inc., Beijing, China).

ICC. For ICC analysis, the prepared Pap-smear slides and LBC (ThinPrep®; Hologic China, Inc., Beijing, China) slides prepared according to the manufacturer's protocols were immediately fixed in 99% ethanol at room temperature for 1 h, and were air-dried at room temperature overnight. Prior to antibody staining, slides were rehydrated in 50% ethanol for 10 min and distilled water for ≥30 sec. Antigen retrieval was performed by heat treatment in epitope retrieval buffer (Tris-EDTA, pH 9.0) at 95-99˚C for 10 min and then slides in epitope retrieval buffer were maintained at room temperature for 20 min until the temperature decreased below 50˚C. Cells were fixed in 4% paraformaldehyde at room temperature for 2 h. and permeabilized in 0.3% Triton X-100 for 15 min at room temperature, followed by washing twice with TBST. ICC staining was then conducted. Briefly, slides were rinsed once in TBST and treated with 3% H_2O_2 at room temperature for 10 min to block endogenous peroxidase. Slides were blocked in 10% newborn bovine serum (Thermo Fisher Scientific, Inc.) at room temperature for 1 h, followed by incubation with primary E7 antibody, E7MuB6 or E7MuH1 (1:1,000) at room temperature (20-25˚C) for 4 h. Slides were subsequently treated with secondary antibody reagent EnVision+ Detection Systems Peroxidase/DAB, Rabbit/Mouse (K5007; Dako; Agilent Technologies, Inc.) according to the manufacturer's protocols. Finally, slides were stained with hematoxylin (0.5%) at room temperature for 2 h, and dehydrated in a series of graded ethanol concentrations (75, 85, 95 and 100%; 10 min each) at room temperature. Finally, slides were mounted with coverslips for observation under an optical microscope (Olympus Corporation, Tokyo, Japan). Positive cervical cancer cells revealed staining of the cytoplasm. CaSki was used as the positive control, and C-33A was used as the negative control.

IHC. IHC staining with anti-E7 rabbit mAbs (clone nos. E7R04 and E7R19) or with anti-$p16^{INK4A}$ antibody (clone no. E6H4; cat. no. 705-4713; Ventana Medical Systems, Inc.) were performed on tissue sections. Briefly, slides carrying cervical tissue sections were dewaxed in xylene for 5 min and rehydrated in a descending ethanol gradient at room temperature (95, 85, 50 and 30%; 2 min each). Epitope retrieval was performed by heating the slides at 121˚C for 2 min in citrate buffer (10 mmol/l, pH 6.0). Endogenous peroxidase activity was blocked with treatment with 3% H_2O_2 solution at room temperature for 20 min. Blocking was performed by incubation with TBST containing 10% newborn bovine serum at room temperature for 1 h. Following washing, primary antibodies (1:1,000) were incubated with the slides at 20-25˚C for 1 h, followed by washing with TBST and incubation with HRP-labeled secondary antibody (Dako EnVision+ Peroxidase system) at room temperature for 30 min. DAB chromogen system (Dako; Agilent Technologies, Inc.) was used for color development according to the manufacturer's protocols. Slides were washed with xylene three times, for 3 min each time. Subsequently, slides were treated with a series of graded ethanol (100, 80, 50 and 30%; 2 min each) at room temperature. Then slides were stained with hematoxylin (0.5%) at room temperature for 20 min. Slides were washed with tap water for 5 min and then treated with acid alcohol (1% HCl in 70% EtOH) until the slides turned pink. Following a further wash with tap water for 5 min, slides were treated with ammonia water (1 ml NH_4OH in 1l H_2O) until the sections noticeably darkened. Slides were washed again with tap water for 5 min and stained with Eosin Y (0.5%) at room temperature for 1 min. Following rinsing in tap water for 1 min, slides were dehydrated in a graded ethanol series (50, 80, 90 and 100%; 2 min each) and mounted on coverslips. The HPV-positive cervical cancer tissue was used as a positive control, and sham-treated slide treated with blank blocking buffer instead of primary antibody was used as negative control.

Interpretation of E7 and $p16^{INK4A}$ immunostaining. Stained slides were analyzed by two pathologists for variable representation of epithelial and stromal components and immunoreactivity. Squamous epithelial cells, with brown-colored cytoplasmic staining for E7 protein, or with nuclear and/or cytoplasmic staining for $p16^{INK4A}$ protein, were identified and graded as follows: 0, no staining; 1, 1-25% staining; 2, 26-50% staining; 3, 51-75% staining; and 4, 76-100% staining. Cases were classified as positive staining (2+ for E7; 3+ for $p16^{INK4A}$)

vs. negative or focal staining. The staining intensity for each marker was not taken into consideration. The examination and scoring of immunohistochemistry slides were confirmed by a panel of more than two certified professionals in pathology.

In situ hybridization (ISH) analysis for HPV E7 mRNA. ISH analysis for HPV E7 mRNA was performed using RNAscope HPV kit (Advanced Cell Diagnostics, Inc., Newark, CA, USA) according to the manufacturer's protocols. Briefly, FFPE tissue sections (4 mm-thick) were heated (65˚C) for 5 min and pretreated with protease K (10 μg/ml) at 37˚C for 20 min prior to hybridization. For each case, three slides from adjacent tissue sections were separately hybridized with probes targeting ubiquitin C (cat. no. 310041; Advanced Cell Diagnostics, Inc.), DapB (cat. no. 310043; Advanced Cell Diagnostics, Inc.) and a cocktail of 18 genotypes of hrHPV (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82; cat. no. 312591). A HRP-based signal amplification system was subsequently used to hybridize the target probes using a peroxidase activity assay kit (Sigma-Aldrich), and signal generation followed by incubation with the chromogenic substrate 3,3'-diaminobenzidine (0.05%) at room temperature for 30 min. Counter staining with hematoxylin was performed using the method described above. A ubiquitin C probe was used as an endogenous control for RNA integrity assessment. The bacterial gene DapB was used as a negative control to assess background staining. A positive stain was defined by a typical pattern of punctate brown colored granules (precipitates) in or around the nucleus, or in the cytoplasm of the cells. For the paired IHC analysis, adjacent tissue sections were processed by staining with the aforementioned anti-E7 antibody cocktail.

Results

E7 mAb recognizes endogenous E7 protein in cervical cancer cells. mAbs with high specificity towards E7 recombinant proteins from the hrHPV strains (HPV16, 18, 31, 33, 35, 39, 45, 52, 58 and 59), and without cross-reactivity to E7 proteins from the lrHPV strains (HPV6 and 11) were selected via ELISA. Positive antibody clones were further characterized by their abilities to bind the endogenous E7 protein in cervical cancer cells. As presented in Fig. 1, antibodies immunoprecipitated the endogenous E7 protein from cell lysates prepared from cervical cancer cell lines CaSki (HPV16 positive) and HeLa (HPV18 positive). The C-33A cell lysate was included in the parallel experiment as a negative control. Protein samples from immunoprecipitation were analyzed by western blotting. A single specific band with expected molecular weight was detected in CaSki cell lysate (Fig. 1A) and in HeLa cell lysate (Fig. 1B), indicating that mAbs may specifically recognize endogenous E7 proteins expressed in cancer cell lines. Two upper bands in each lane with apparent molecular weight of 55 and 28 kDa respectively represented the IgG heavy chain and light chain of mAb used in the immunoprecipitation.

E7 mAb specifically stained cervical cells by ICC. For further confirmation of the specificity of mAbs against E7 proteins expressed in cervical cells, three cervical cancer cell lines C-33A, HeLa and CaSki were cultured and analyzed via ICC (Fig. 2). HeLa cells exhibited high expression of HPV18

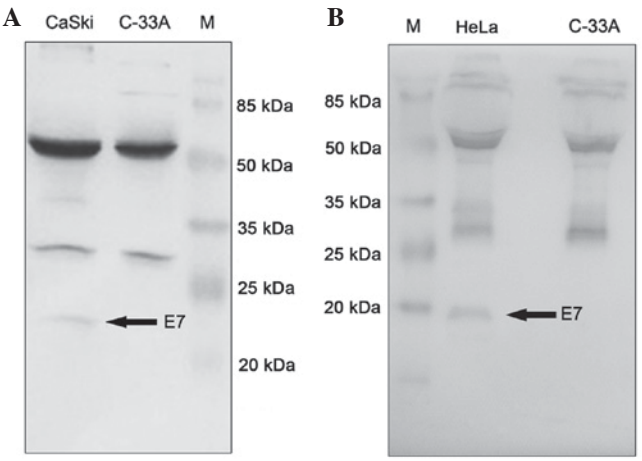

Figure 1. Western blot analyses for the specificity of anti-E7 monoclonal antibodies. E7 proteins in cancer cell lysates were immunoprecipitated and immunoblotted with murine monoclonal antibodies. (A) E7MuB6 protein of HPV16 origin in (A) CaSki and (B) E7MuH1 protein of HPV18 in HeLa were marked with arrows with an apparent molecular weight of 20 kDa. C-33A was the negative control used in parallel experiments. The upper bands in each lane with apparent demonstrated molecular weights of 55 and 28 kDa are the heavy and light chains of the monoclonal antibodies used in the immunoprecipitation, respectively. M, protein molecular weight ladder. HPV, human papillomavirus.

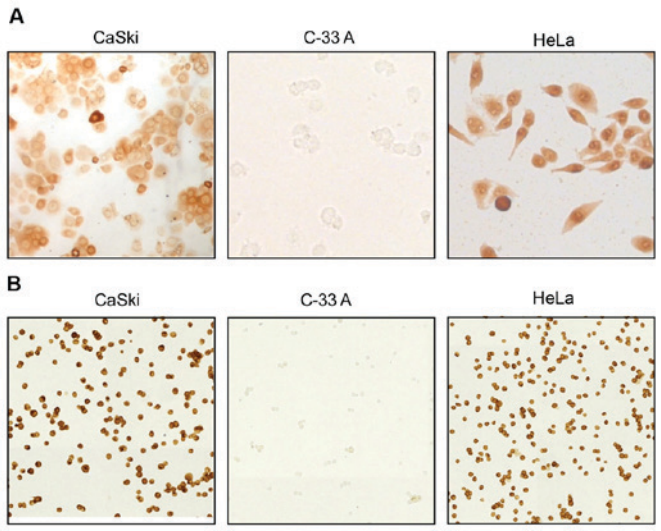

Figure 2. Analyses of the specificity of E7 monoclonal antibodies in cervical cancer cell lines by immunocytochemistry. (A) Intense staining was observed in the cytoplasm of HPV-positive CaSki cells with E7MuB6 antibody in adherent cells (left panel), no staining was observed within the HPV-negative C-33A cells (middle panel); E7MuH1 revealed cytoplasmic and nuclear staining within adherent HeLa cells (right panel). (B) Antibody E7MuH1 stained suspended CaSki cells (left panel), no staining with antibody within the HPV-negative C-33A cells (middle panel), E7MuH1 staining in suspended HeLa cells (right panel). Magnification, x20.

E7 protein, while CaSki cells demonstrated overexpression of HPV16 E7 proteins. C-33A cells were used as a negative control as HPV16 E7 and HPV18 E7 proteins are not expressed within these cells. HeLa cells were exclusively stained by mAb E7MuH1 against HPV18 E7 protein, while HPV16 E7 protein was specifically detected in CaSki cells using mAb E7MuB6 against HPV16 E7 proteins; neither antibody recognized C-33A cells. Collectively, the results of the present study indicated

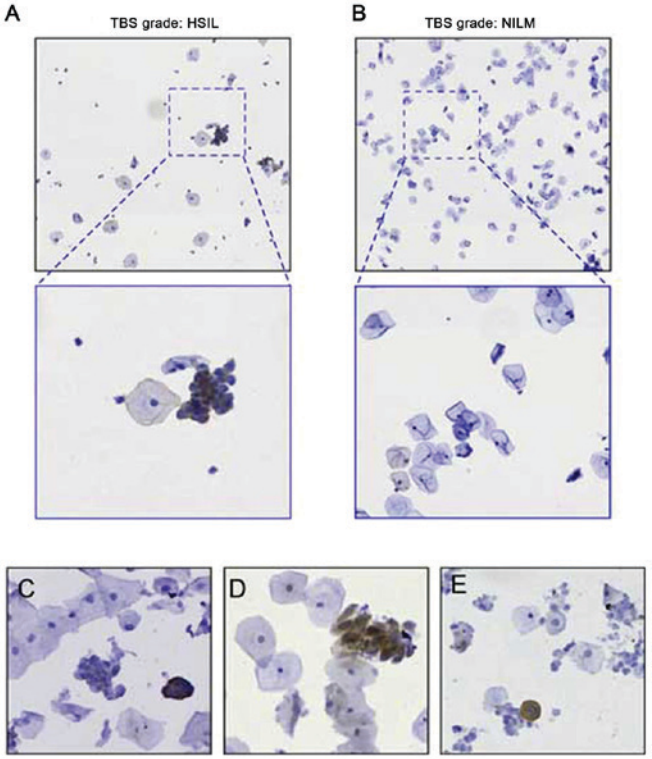

Figure 3. Representative immunocytochemistry staining results of squamous intraepithelial cells using anti-HPV E7 mAbs. Anti-E7 mAb-stained neoplasia cells were observed in (A) HSIL, but not in (B) NILM. Positive staining of E7 in various lesions of dysplastic cells. TBS grade (C) HSIL, (D) low-grade squamous intraepithelial lesion and (E) atypical squamous cells of undetermined significance. mAb, monoclonal antibody; TBS, 2001 Bethesda System; HSIL, high-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion and malignancy.

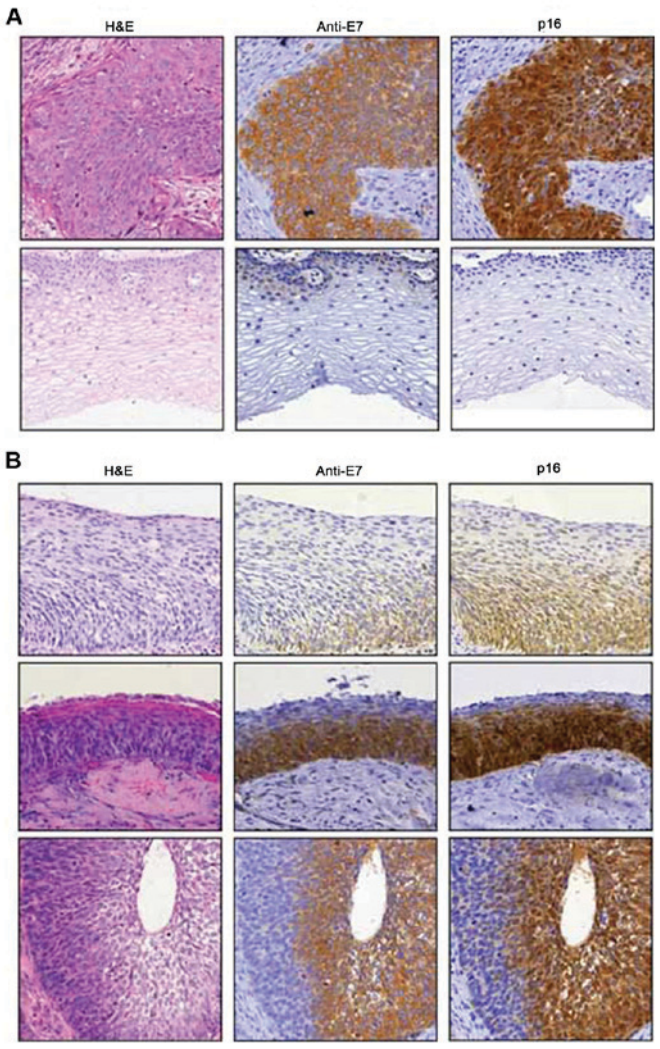

Figure 4. Representative results of immunohistochemistry staining with E7 mAbs and $p16^{INK4A}$ antibody. (A) Cervical carcinoma lesions (top panels) and normal cervical tissues (bottom panels) were stained with H&E, E7 mAbs and $p16^{INK4A}$ antibody (left to right). (B) Representative CIN1 (top panels), CIN2 (middle panels) and CIN3 (bottom panels) lesions were stained with H&E, E7 mAbs and $p16^{INK4A}$ antibody. Magnification, x20. mAbs, monoclonal antibodies; CIN, cervical intraepithelial neoplasia; H&E, hematoxylin and eosin; $p16^{INK4A}$, cyclin dependent kinase inhibitor 2A.

that the mAbs generated have high specificity for E7 proteins expressed in cervical cancer cells.

Application of mAb in ICC staining of exfoliated cervical epithelial cells. LBC or thin-layer cytology has been widely applied in clinical testing for the examination of exfoliated cervical epithelial cells. To investigate the compatibility of E7 mAb for immunostaining on LBC slides, fixed cervical epithelial cells were treated with mAb E7MuB6. Antibody activity and specificity were demonstrated using positive samples of HSIL and positive HPV16 genotyping results. Normal cervical cell slides negative for intraepithelial lesion and malignancy (NILM) were used as negative controls. As presented in Fig. 3, the slides treated with murine mAb revealed dark brown colored cells, indicating E7 positive expression within the transformed cells, consistent with their HSIL cytological morphology features (Fig. 3A). None of cells on the slides NILM were stained by the E7 mAb (Fig. 3B). In addition, LBC slides with varying lesion grades [HSIL (Fig. 3C), LSIL (Fig. 3D), and atypical squamous cells of undetermined significance (ASC-US; Fig. 3E)] could be identified via a direct evaluation based on the expression of the oncoprotein E7. This demonstrated the feasibility of using a specific biomarker for the interpretation of the cytopathological results. As presented in Fig. 3E, a case classed as ASC-US case by the conventional cytology test was identified as positive for precancerous cells based on the identification of E7 oncoprotein expression.

IHC reactivity with E7 protein and comparison with surrogate $p16^{INK4A}$ marker. Pathology tests are considered to be the most important test for the clinical diagnosis of cervical cancer or precancerous lesions. To evaluate the utility of E7 antibodies for histopathological examination of cervical cancer, the IHC staining capacity of the rabbit monoclonal anti-E7 cocktail was investigated and compared with the anti-E7 immunostaining patterns of the established surrogate protein marker $p16^{INK4A}$. As presented in Fig. 4A, rabbit monoclonal anti-E7 mAbs exhibited intense granular staining in cytoplasm of human cervical carcinoma lesions (top), while no brown staining in normal cervical tissues was observed (bottom). In addition, E7 mAbs demonstrated a similar staining pattern to that of anti-$p16^{INK4A}$ antibody in different premalignant and malignant lesions (Fig. 4A and B).

It has been speculated that the tumorigenic functions of E7 protein may be associated with its expression levels during the development and progression of cancer. To further investigate whether the E7 antibodies used in the present study may reveal

Table I. E7 and p16^{INK4A} immunostaining in tissue slides via immunohistochemistry.

	LSIL		HSIL		SCC	
Protein expression	Cases	%	Cases	%	Cases	%
E7						
Negative	31	68.9	6	9.4	0	0
Positive	14	31.1	58	90.6	7	100
Total	45		64		7	
p16^{INK4A}						
Negative	35	77.8	4	6.3	0	0
Positive	10	22.2	60	93.7	7	100
Total	45		64		7	

p16^{INK4A}, cyclin dependent kinase inhibitor 2A; HSIL, high grade squamous intraepithelial lesions; LSIL, low grade squamous intraepithelial lesions; SCC, squamous cell carcinoma.

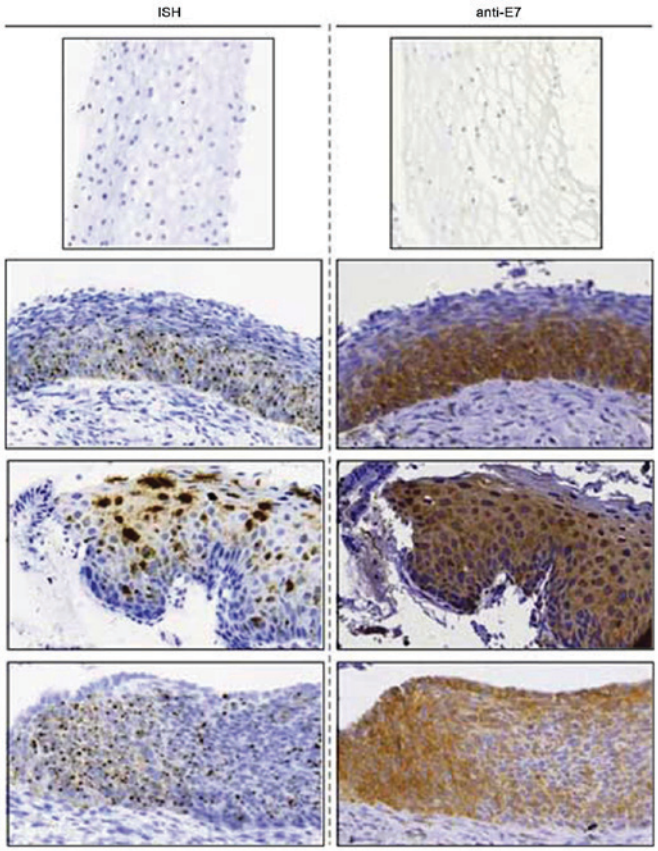

Figure 5. Representative results of ISH and IHC staining of cervical carcinoma lesion. Adjacent sections of high-grade squamous intraepithelial lesion tissue were analyzed via ISH with E7 mRNA probes (left panels) or IHC with E7 monoclonal antibodies cocktail (right panels). Top panel demonstrated the negative controls with normal cervix tissue in both assays. Magnification, x20. IHC, immunohistochemistry; ISH, *in situ* hybridization.

the expression levels of HPV E7 proteins in cervical lesions of increasing pathological grades, tissue sections from cervical intraepithelial neoplasia (CIN)1, CIN2 and CIN3 samples were subjected to IHC staining using the novel rabbit anti-E7 mAbs (Fig. 4B). The staining results indicated an increased amount of E7 positive cells on the slides as the pathological stage was increased. This observation of E7 staining is consistent with the increased severity of pathological stages, from CIN1 to CIN2, and then to CIN3 lesions. Comparison of the E7 and p16^{INK4A} antibody staining results in normal tissue (negative) and cervical carcinoma (positive) demonstrated similar staining patterns as presented in Fig. 4A. A notable difference is that the p16^{INK4A} antibody stained numerous foci in morphologically normal cervical cells, whereas the E7 antibody demonstrated an overall similar staining pattern, but with a distinct specificity to the cervical cancer lesions (Fig. 4B).

To assess the robustness of the E7 immunostaining in a larger number of pathology specimens, an antibody reagent cocktail of anti-E7 mAbs was optimized and used to test tissue slides from 116 cases with pathology reports in a double-blind experiment. For comparison, p16^{INK4A} immunostaining was performed in parallel experiments. The results were analyzed against the gold standard of pathology. As presented in Table I, the staining of E7 among the three pathological groups, LSIL, HSIL and SCC exhibited a trend of increasing E7-positive rates: 31.1, 90.6 and 100%, respectively. The p16^{INK4A} group (Table I) revealed similar results to those of the anti-E7 test group. Highly similar results were reported between the two protein biomarkers.

ISH analysis of HPV E7 mRNA. In the present study, antibody-binding specificity to E7 antigen was investigated via IHC staining. In addition, HPV E7 mRNA expression levels were investigated via ISH using adjacent sections from the same tissue blocks used for E7 antibody staining by IHC. As presented in Fig. 5, E7 mAbs and E7 mRNA probes revealed similar localization of positively stained loci or regions on the slides, indicating the accurate recognition of the antibodies to the endogenous E7 antigen in the immunohistochemistry assay. Morphologically aberrant cells exhibited the typical punctate dots following the application of the E7 mRNA probe via ISH; staining intensity also appeared to be increased with the increasing degree of cancer. In this set of experiments, the mRNA and protein staining patterns appeared to be co-localized in the same areas in the lesions. Antibody

staining indicated that the E7 mRNA expression levels were associated with the intensity and location of E7 protein expression. The findings of the present study further confirmed the specific immunoreaction of E7 antibodies and the endogenous tumor antigen E7 within cervical lesion tissue.

Discussion

Cervical cancer is a leading cause of cancer-associated mortality in females worldwide and the second most common cancer in females in developing countries (1). According to the data released by the National Central Cancer Registry of China, the incidence of cervical cancer in China was 15/100,000 in 2015, which is still listed as one of the major malignant tumors threatening female health in China (22). Fortunately, cervical cancer is preventable and curable at the early stages due to the relatively slow progression of cervical carcinoma (1). The morbidity and mortality of cervical cancer has been remarkably reduced by the early screening tests, including Pap-smear and liquid-based cytology (9). Low sensitivity and false positive results from the abnormal morphology of ASC-US are major limitations of cytology tests used in primary screening. According a meta-analysis of Pap-smear tests and LBC, the sensitivity and accuracy of cervical cytology exhibited wide variation in different regions and institutions (23). In addition, a study of a large population revealed that 30-60% of patients with invasive cervical cancer were reported to be normal in Pap-smear testing 3-5 years prior to diagnosis, indicating that the cervical cytology test has a poor negative predictive value (24). By contrast, the development of molecular HPV testing has largely improved the detection sensitivity, however, it is limited by its low overall specificity and low positive predictive value (10,25). A desirable improvement in the early screening is to identify the real-time phenotype of precancerous cervical lesions, and to avoid over diagnosis and/or treatment of patients with transient HPV infection only, or ASC-US and a low-grade LSIL cervical lesion.

Biomarkers can potentially improve the diagnostic interpretation of cytology tests for early stage cervical cancer (11). Despite the benefit of decreasing inter-observer variations for the diagnosis of LSILs, the clinical significance of $p16^{INK4A}$ is limited by its intrinsic properties, including the inconsistent expression in tumors, lack of tumor specificity and the lack of uniformity in scoring systems.

E7 is a pivotal oncoprotein, with expression required to maintain the transformed phenotype in hrHPV-induced human cancers. The conformational, structural and biochemical behavior of E7 protein has been extensively characterized (18). Several conformational E7 species have been identified, including a nuclear dimer and high molecular weight spherical oligomers in the cytoplasm of transformed cells (26). It has been hypothesized that the predominant species of E7 oligomers may exist in transformed cells, and contain epitopes that are not exposed in the dimer. The complexity of conformational changes in E7 protein made the 'rational design' and construction of an E7 antibody extremely difficult.

Considering its highly specific expression in transformed cervical cells, HPV E7 protein has been well reported as an appropriate biomarker candidate for the detection of cervical cancer (12). A number of studies reported the detection of E7 proteins using either polyclonal antibodies or mAbs (12,26-32). Lidqvist *et al* (18) described the production of mouse mAbs to HPV E7 protein and use for testing LBC slides. However, data from a clinical evaluation was not reported. Faoro *et al* (26) reported a new E7 antibody with limited antibody specificity data and indiscriminative immunoreactivity in LSIL and HSIL. Collectively, available reports have demonstrated a lack of thorough characterization of antibody specificity, or the evaluation of E7 protein as a biomarker for diagnostic testing.

The present study reported an empirical approach to develop several unique mAbs that recognize E7 proteins from HPV16, HPV18 as well as structurally-associated E7 proteins from other strains of hrHPVs. This may increase the range of diagnostic application for the analysis of clinical specimens. mAb were constructed using protein antigens of E7 from hrHPV16 and 18 and subsequently used to immunize mice and rabbits. mAb clones were screened and selected based on binding specificity to E7 proteins from numerous hrHPV strains, including HPV16 and 18, and structurally-associated hrHPV31, 33, 35, 39, 45, 52, 58, and 59. Antibody candidate clones demonstrating cross-reactivity with low risk strain HPV E7 proteins (HPV6 and 11) were excluded from subsequent analysis in the present study. Using a variety of characterization assays, it was confirmed that the selected mAbs specifically bind to the endogenous hrHPV E7 proteins in cervical cancer cells (CaSki and HeLa). Finally, the applications of these anti-E7 mAbs for the specific recognition of E7-expressing cervical cells in clinical specimens were demonstrated, via LBC slides and FFPE tissue slides by ICC or IHC, respectively. Of particular interest is investigation of the use of anti-E7 staining in ASC-US slides, which may contribute to the interpretation of cytology-based early screening tests. In addition, IHC analysis using the E7 antibody cocktail reagent produce similar results to $p16^{INK4A}$ immunostaining, indicating the feasibility of using E7 as a specific biomarker for identification of premalignant cervical epithelial lesions.

In summary, the HPV E7 protein encoded by the viral genome, but only expressed in transformed human cells, may be considered as an ideal biomarker for the early detection of precancerous lesions. The detection of E7 protein expression may effectively distinguish malignant transformations from transient HPV infections. This E7 protein identification technique may be used in either ICC or IHC analyses of cervical samples, and provide more reliable results for improved efficiency in cervical cancer screening and early diagnosis.

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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

LS and XC designed the experiments. LS, FH and CS performed the experiments. YH and YL analyzed the data. All authors read the manuscript.

Ethics approval and consent to participate

The use of human tissue in the present study was approved by the Institutional Review Board at the First Affiliated Hospital of Soochow University, prior to data collection. Written informed consent was obtained from all participants involved in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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