

## Expression of the high-risk human papillomavirus type 18 and 45 E7 oncoproteins in cervical carcinoma biopsies

Marc Fiedler,<sup>1,2†</sup> Sigrun Ressler,<sup>1,2†</sup> Beatriz Campo-Fernández,<sup>1,2</sup> Andreas Laich,<sup>1,3</sup> Lars Jansen,<sup>4</sup> Andreas Widschwendter,<sup>5</sup> Hans-Peter Viertler,<sup>2</sup> Nicole Bacher,<sup>1,2</sup> Dieter Morandell,<sup>1,2</sup> Elisabeth Müller-Holzner,<sup>5</sup> Matthias Dürst,<sup>4</sup> Pidder Jansen-Dürr<sup>2</sup> and Werner Zwerschke<sup>1,2</sup>

### Correspondence

Werner Zwerschke  
werner.zwerschke@oeaw.ac.at

<sup>1</sup>Tyrolean Cancer Research Institute, Innrain 66, A-6020 Innsbruck, Austria

<sup>2</sup>Institute for Biomedical Ageing Research of the Austrian Academy of Sciences, Rennweg 10, A-6020 Innsbruck, Austria

<sup>3</sup>Amynon BioTech, Innrain 66, A-6020 Innsbruck, Austria

<sup>4</sup>Division Gynaekologische Molekularbiologie, Abteilung Frauenheilkunde, Frauenklinik der Friedrich-Schiller-Universität Jena, Germany

<sup>5</sup>Departments of Obstetrics and Gynaecology, University of Innsbruck, Innsbruck, Austria

E7 proteins are major oncoproteins of high-risk human papillomaviruses (HPVs), which play a key role in cervical carcinogenesis. These proteins have been shown to immortalize primary human cells. Due to the absence of antibodies with suitable sensitivity and specificity, little is known about expression of the E7 oncoproteins in naturally infected tissues. Recently, high-level expression of the E7 protein of HPV-16, the most prevalent oncogenic HPV type, was demonstrated in cervical carcinomas by immunohistochemistry; however, approximately 15 additional high-risk HPV types are known to be associated with cervical carcinoma. It is unknown whether the E7 oncoproteins of HPV-18 and -45, the second and third most prevalent HPV types, are expressed in cervical cancers. Using antibodies against HPV-18 and -45 E7 proteins, it is shown here for the first time that the HPV-18 and -45 E7 proteins can be detected in cervical carcinoma biopsies. Together with anti-HPV-16 E7 antibodies, this could create the possibility of detecting E7 oncoproteins in approximately 80 % of all cervical cancers.

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## INTRODUCTION

Cervical cancer is considered the second most common gynaecological cancer worldwide (Pisani *et al.*, 2002). Epidemiological studies have shown that human papillomaviruses (HPVs) are the main aetiological factors for cervical cancer (reviewed by zur Hausen, 2000). HPVs are small DNA viruses that infect basal proliferating epithelial cells of either the skin or mucosa, and more than 100 different HPV genotypes have been described to date. On the basis of epidemiological and biochemical data, the mucosal genotypes are subdivided into two groups: HPVs of the high-risk group, associated with squamous intraepithelial lesions with a high potential for progression to invasive squamous cell carcinoma, and papillomaviruses of the low-risk group, which cause benign hyperplasias (reviewed by Lowy & Howley, 2001). PCR-based studies have shown that more than 99 % of invasive cervical cancers contain high-risk

HPV DNA (Walboomers *et al.*, 1999), HPV-16 being the most prevalent type with a worldwide incidence of 50–60 %, followed by HPV-18 (approx. 15 %) and HPV-45 (approx. 9 %) (reviewed by Muñoz, 2000; Bosch *et al.*, 2002).

The factors that determine viral persistence and tumorigenic progression are not completely defined. It was found that the high-risk viral DNA frequently integrates into the host genome in cervical cancer cell lines (Schwarz *et al.*, 1985) and HPV-related cervical carcinomas (Luft *et al.*, 2001). These events are frequently correlated with dysregulated expression of the early viral genes E6 and E7 (Jeon *et al.*, 1995), which exhibit the major oncogenic activity of the HPV DNA (Crook *et al.*, 1989). E7, in cooperation with E6, can efficiently immortalize human primary keratinocytes, the natural host cell of HPV, as shown for the oncoproteins of HPV-16 (Münger *et al.*, 1989) and HPV-18 (Barbosa & Schlegel, 1989; Hudson *et al.*, 1990). Given the central role of the HPV-16 E7 protein in cell immortalization and

†These authors contributed equally to this work.

virus-associated oncogenesis, the molecular interactions of HPV-16 E7 have been studied intensively. Immortalization by E7 is, at least in part, caused by its ability to interact directly with and functionally inactivate cell-cycle regulators such as pRb (retinoblastoma protein) (Dyson *et al.*, 1989) and several other nuclear and cytoplasmic factors (reviewed by Zwierschke & Jansen-Dürr, 2000; Münger & Howley, 2002). Recently, high-level expression of the HPV-16 E7 oncoprotein in cervical cancers was demonstrated (Fiedler *et al.*, 2004). However, no information is yet available about expression of HPV-18 and -45 E7 oncoproteins in cervical carcinomas. We generated polyclonal antibodies against the HPV-18 and -45 E7 oncoproteins and analysed their expression levels in paraffin sections of cervical cancer biopsies.

## METHODS

### Generation of polyclonal antisera to high-risk HPV proteins.

Purified preparations of E7 proteins were used to produce polyclonal anti-HPV-18 and -45 E7 antibodies in goats (Charles River). For immunization, 1 mg each of the respective E7 protein was mixed with 1·1 ml complete Freund's adjuvant (Sigma). The first, second and third boosts were each done 28 days apart with 1 mg E7 protein mixed with 1·1 ml incomplete Freund's adjuvant. Two weeks after the third boost, the first production bleeding was performed. Blood (300 ml) was taken from the jugular vein and collected into a vessel containing 2 ml 32% sodium acetate solution per 100 ml blood. Blood was processed to citrate plasma and stored at  $-20^{\circ}\text{C}$ . To purify type-specific polyclonal anti-E7 antibodies, E7 proteins from HPV-18 and -45 were coupled separately to NHS-activated Sepharose 4 (NHS-activated Sepharose HiTrap columns; General Electric); the final E7 concentration coupled to the column was adjusted to  $1\text{--}25\text{ mg (ml gel)}^{-1}$ . For antibody purification, citrated plasma was diluted 1:2 in running buffer (PBS containing 200 mM NaCl, 5 mM EDTA, 0·05%  $\text{NaN}_3$ , pH 7·4), adjusted to 0·64% sodium citrate and filtrated through a  $0\text{--}22\text{ }\mu\text{m}$  filter. Upon equilibration with 10 column volumes (CVs) of running buffer, diluted plasma was passed at a flow rate of  $0\text{--}5\text{ ml min}^{-1}$  through the respective affinity column. After loading, the columns were washed with 10 CVs washing buffer (PBS containing 1 M NaCl, 5 mM EDTA, 0·05%  $\text{NaN}_3$ , pH 7·4) and 10 CVs running buffer at a flow rate of  $1\text{ ml min}^{-1}$ . Antibodies were eluted with 3 M potassium thiocyanate, 150 mM KCl, 10 mM potassium phosphate, pH 7·4, at a flow rate of  $0\text{--}2\text{ ml min}^{-1}$ . The fraction size was 1 ml. Antibody-containing fractions were pooled and passed through a HiLoad 16/60 Superdex 75 gel-filtration column (General Electric), thereby performing a buffer exchange into 166 mM potassium phosphate, 83 mM glycine, 0·05%  $\text{NaN}_3$ , pH 7·2. Finally, antibody-containing fractions were pooled, concentrated to  $1\text{ mg ml}^{-1}$  (using concentrators with a cut-off of 10 kDa; Vivascience/Sartorius), aliquotted and lyophilized (Chris Alpha 1-4 freeze dryer; Osterode).

**Western blot analysis.** Cells were lysed in PBS containing 1% Triton X-100 using an ultrasonic cell disruptor (Branson). Lysates were separated by SDS-PAGE (12·5% gel) and transferred to a PVDF membrane (NEN). The membrane was blocked in blocking buffer (PBS containing 0·2% Tween 20 and 5% milk powder) and incubated with the affinity-purified polyclonal goat antibodies against HPV-18 and -45 E7 for 1 h. After washing, a peroxidase-conjugated anti-goat IgG (Promega) was applied. The membrane was washed and the bound antibodies were visualized by using a chemiluminescence Western blotting detection system (NEN).

**Immunofluorescence experiments.** Confocal immunofluorescence microscopy was performed essentially as described previously (Mannhardt *et al.*, 2000; Fiedler *et al.*, 2004).

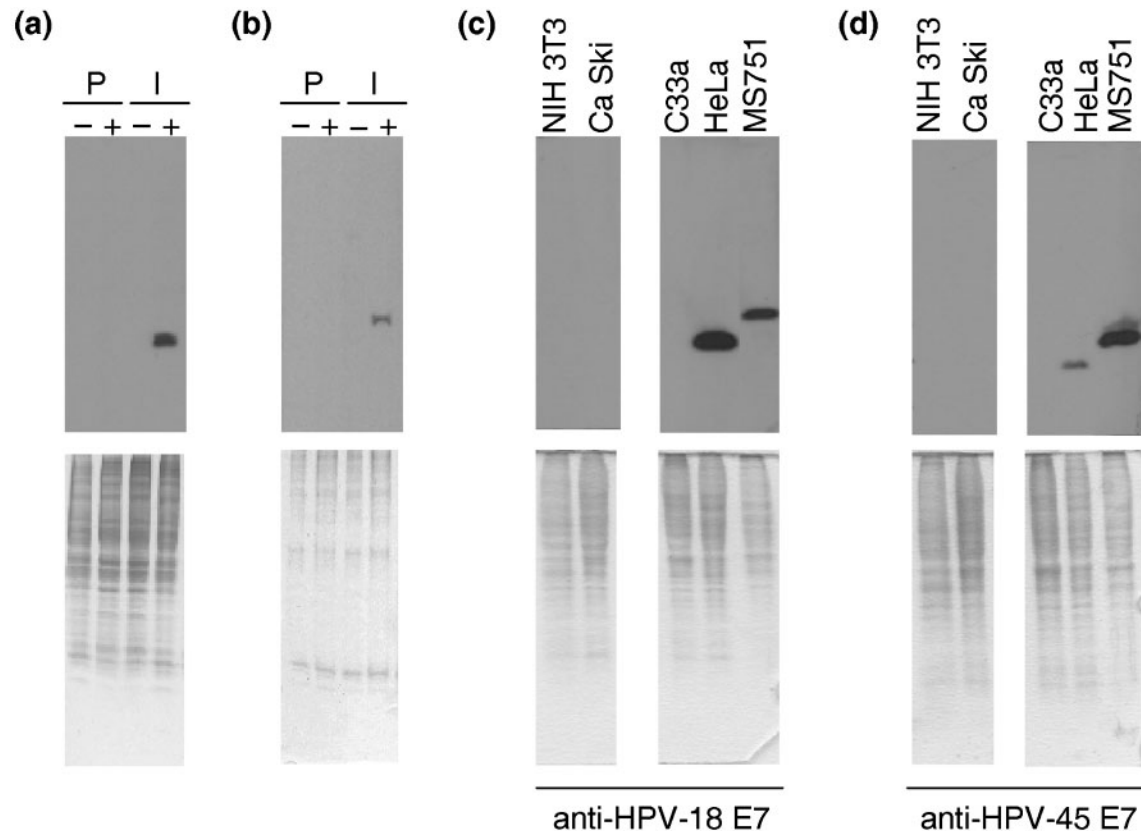
**PCR-based HPV typing.** Formalin-fixed, paraffin-embedded tissues were processed with a QIAamp Tissue kit according to the manufacturer's instructions (Qiagen). Total cellular DNA was used in the GP5<sup>+</sup>/GP6<sup>+</sup> general primer PCR and the amplicons were used for HPV typing using an enzyme immunoassay with different HPV type-specific oligonucleotides (Fiedler *et al.*, 2004).

**Immunohistochemical detection of HPV E7 proteins and p16<sup>INK4a</sup> in cervical biopsies.** Immunohistochemical detection of HPV-18 and -45 E7 proteins and p16<sup>INK4a</sup> in cervical biopsies was performed essentially as described previously (Fiedler *et al.*, 2004). For the detection of p16<sup>INK4a</sup>, we used the anti-p16<sup>INK4a</sup> antibody, clone JC8 (Neomarkers). The goat anti-HPV-18 and -45 E7 antibodies were from Amynon Biotech.

## RESULTS AND DISCUSSION

### Characterization of anti-HPV-18 E7 and -45 E7 antibodies

To generate polyclonal antisera against the HPV-18 and -45 E7 oncoproteins, we immunized goats with highly purified HPV-18 and -45 E7 proteins, respectively. The antisera were affinity-purified and the appearance of E7 immunoreactivity was analysed by Western blotting, using extracts from human osteosarcoma (U-2 OS) cells transiently transfected with cytomegalovirus promoter-driven expression vectors for each of the E7 proteins. As shown in Fig. 1, the antisera derived against HPV-18 E7 (Fig. 1a) and HPV-45 E7 (Fig. 1b) specifically detected E7 in the lysates of the transiently transfected cells as a single band and gave no signal with mock-transfected cell extracts. Use of pre-immune serum did not yield any signal. To establish the detection of clinically relevant amounts of the HPV-18 E7 protein, we analysed extracts of the HPV-18 DNA-positive cervical carcinoma-derived cell line HeLa (Schwarz *et al.*, 1985) (Fig. 1c). The anti-HPV-18 E7 antibodies recognized a single band in HeLa cell extracts (Fig. 1c). No signal was recognized in extracts of C33a cells (Fig. 1c), a cervical carcinoma cell line that is devoid of any HPV DNA, and in extracts of the unrelated mouse NIH 3T3 cells (Fig. 1c), underlining the specificity of the anti-HPV-18 E7 antibodies. To establish the detection of clinically relevant amounts of the HPV-45 E7 protein, we analysed extracts of the HPV-45 DNA-positive cervical carcinoma-derived cell line MS751 (Geisbill *et al.*, 1997) (Fig. 1d). The antibodies generated against HPV-45 E7 specifically detected a single band in MS751 cells and did not cross-react with C33a proteins (Fig. 1d) or NIH 3T3 proteins (Fig. 1d). As HPV-18 E7 and HPV-45 E7 belong to the same subfamily of E7 proteins, showing high amino acid sequence similarity (Ullman *et al.*, 1996), we wondered whether the anti-HPV-18 E7 antibodies would recognize HPV-45 E7 protein and vice versa in Western blot experiments. The anti-HPV-18 E7 antibodies strongly recognized HPV-45 E7 as a single band in lysates of the HPV-45-positive MS751 cervical carcinoma cells (Fig. 1c); however, these antibodies detected no band in



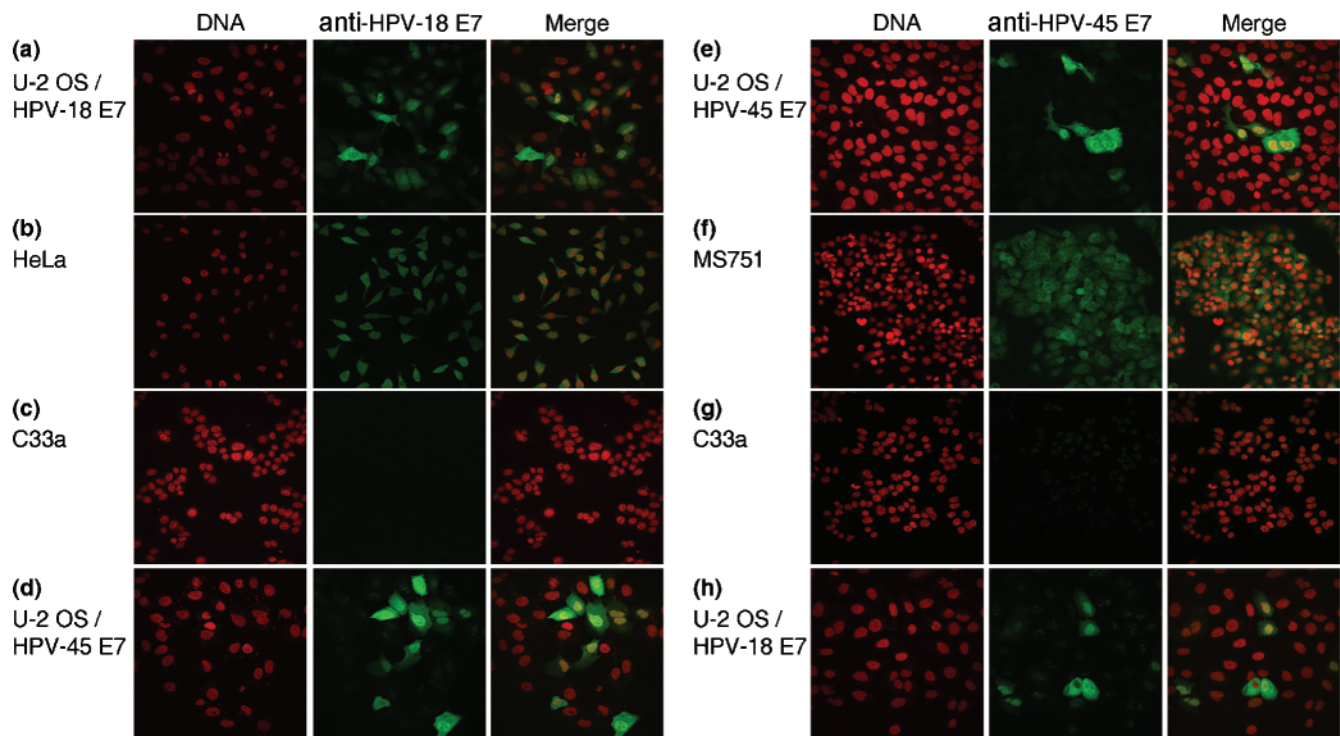
**Fig. 1.** Characterization of affinity-purified anti-HPV-18 and anti-HPV-45 E7 antibodies. (a) U-2 OS cells were either mock-transfected (–) or transiently transfected with an expression vector for HPV-18 E7 (+). Cellular extracts were separated by SDS-PAGE and analysed by Western blotting, using goat anti-HPV-18 E7 antibodies (I) or pre-immune serum (P) as indicated. (b) U-2 OS cells were either mock-transfected (–) or transiently transfected with an expression vector for HPV-45 E7 (+). Cellular extracts were separated by SDS-PAGE and analysed by Western blotting using goat anti-HPV-45 E7 antibodies (I) or pre-immune serum (P) as indicated. (c) HPV-18 E7-expressing HeLa cells, HPV-45 E7-expressing MS751 cells, HPV-16 E7-expressing Ca Ski cells and additional negative-control cells (NIH 3T3 and C33a) were lysed, separated by SDS-PAGE and analysed by Western blotting using anti-HPV-18 E7 antibodies. (d) HPV-45 E7-expressing MS751 cells, HPV-18 E7-expressing HeLa cells and negative-control cells [NIH 3T3, Ca Ski (HPV-16-positive) and C33a] were lysed, separated by SDS-PAGE and analysed by Western blotting using anti-HPV-45 E7 antibodies.

lysates of the HPV-16 E7 protein-expressing cervical carcinoma cell line Ca Ski (Fig. 1c), suggesting that the anti-HPV-18 E7 antibodies cross-react specifically with HPV-45 E7. The anti-HPV-45 E7 antibodies, which strongly recognize HPV-45 E7 (Fig. 1d), showed only weak cross-reactivity with the HPV-18 E7 protein in HeLa cell lysates (Fig. 1d) and did not recognize HPV-16 E7 in Ca Ski cells (Fig. 1d), suggesting that these antibodies are highly specific for HPV-45 E7. Taken together, these results demonstrate that the anti-E7 antibodies generated here are of sufficient quality to detect HPV-18 E7 and -45 E7 proteins specifically in Western blots.

#### Detection of HPV-18 and -45 E7 proteins in cervical cancer cell lines

To establish a reliable *in situ* detection procedure for HPV-18 and -45 E7 proteins, we first studied the subcellular distribution of these E7 proteins in cell lines by indirect

immunofluorescence. Human osteosarcoma (U-2 OS) cells were transiently transfected with an expression vector for HPV-18 E7. The transfected cells were fixed with 4% paraformaldehyde (PFA)/0.1% Triton X-100 and co-stained with the DNA stain TO-PRO-3 and affinity-purified anti-HPV-18 E7 antibodies (Fig. 2a). Staining was visualized as either green (E7) or red (DNA) fluorescence, using a confocal laser-scanning microscope. Under these conditions, we were able to establish specific immunofluorescence detection of the HPV-18 E7 protein. In most of the transiently transfected cells, the anti-HPV-18 E7 antibodies predominantly stained structures in the nucleus and, to a minor extent, in the cytoplasm. In contrast, no signal was obtained in mock-transfected cells and pre-immune serum did not recognize the E7 antigen (data not shown). In the HPV-18-positive cervical carcinoma cell line HeLa, the anti-HPV-18 E7 antibodies stained the nucleus and cytoplasm (Fig. 2b), as shown previously for HPV-16 E7 (Smotkin & Wettstein,



**Fig. 2.** Detection of HPV-18 and -45 E7 proteins in human cells. Cells were stained with TO-PRO-3 (left panels) and anti-HPV-18 (middle panels, a–d) or anti-HPV-45 (middle panels, e–h) E7 antibodies and images were merged (right panels). (a) U-2 OS cells transiently transfected with an expression vector for HPV-18 E7; (b) HeLa cells; (c) C33a cells; (d) U-2 OS cells transiently transfected with an expression vector for HPV-45 E7; (e) U-2 OS cells transiently transfected with an expression vector for HPV-45 E7; (f) MS751 cells; (g) C33a cells; (h) U-2 OS cells transiently transfected with an expression vector for HPV-18 E7. All cells were fixed with 4% PFA/0.1% Triton X-100, processed for indirect immunofluorescence microscopy and viewed by using a confocal laser-scanning system.

1987; Fiedler *et al.*, 2004). No staining was observed with the anti-HPV-18 E7 antibodies in the HPV-negative cervical carcinoma cell line C33a (Fig. 2c), underlining the specificity of the anti-HPV-18 E7 antibodies. To test for antibody cross-reactivity, U-2 OS cells were transiently transfected with an expression vector for HPV-45 E7, which is structurally related to the HPV-18 E7 protein. Co-staining with TO-PRO-3 and affinity-purified anti-HPV-18 E7 antibodies showed that the anti-HPV-18 E7 antibodies cross-reacted with the native HPV-45 E7 protein in cells fixed with the mild fixative PFA (Fig. 2d).

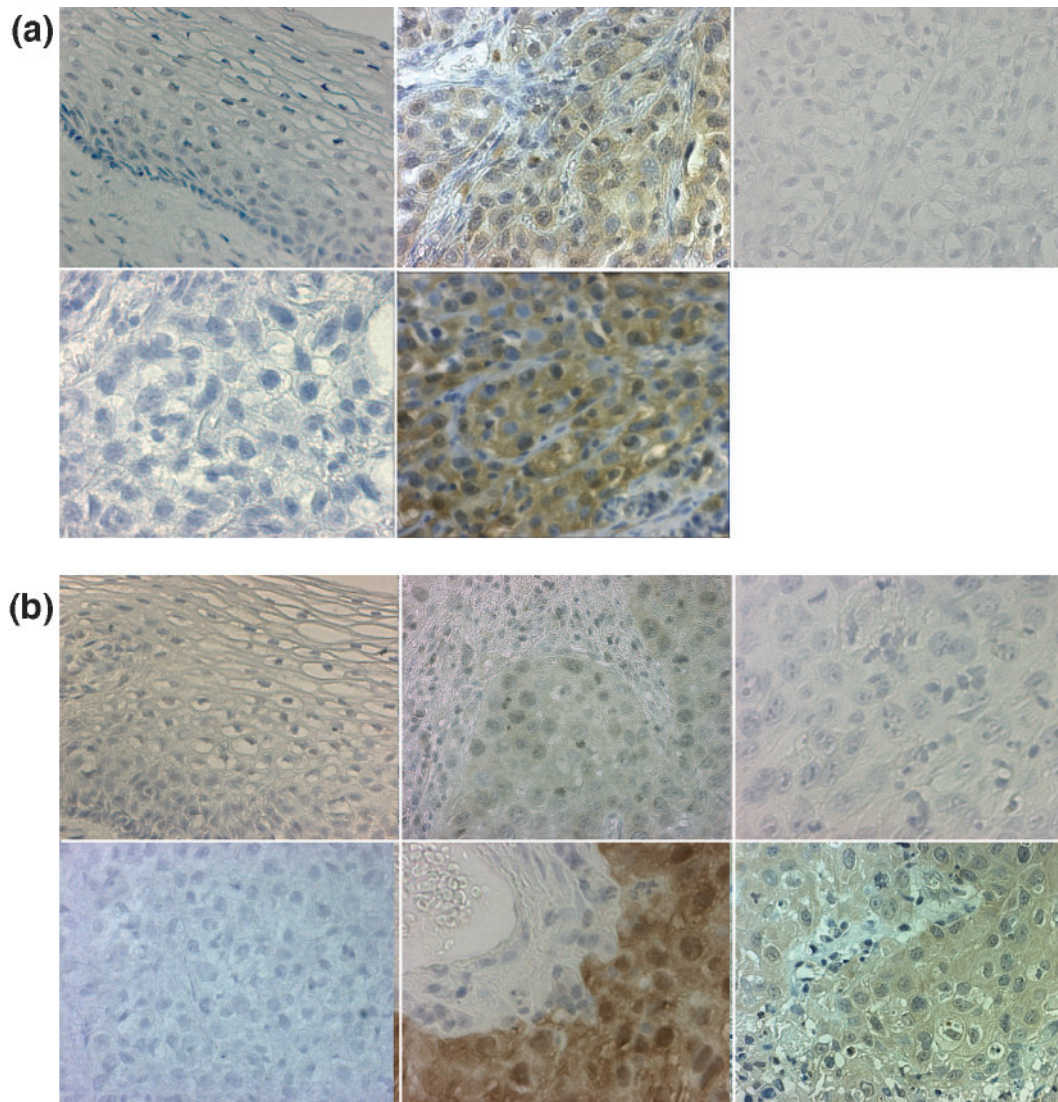
U-2 OS cells transiently expressing HPV-45 E7 were co-stained with TO-PRO-3 and affinity-purified anti-HPV-45 E7 antibodies (Fig. 2e), showing predominantly stained structures in the nucleus and, to a minor extent, in the cytoplasm. Similar to HPV-16 E7 (Fiedler *et al.*, 2004) and HPV-18 E7 (see above), the HPV-45 E7 protein was detected in the nucleus and cytoplasm in the HPV-45-positive cervical carcinoma cell line MS751 (Fig. 2f). Virtually no staining was observed with the anti-HPV-45 E7 antibodies in the HPV-negative cervical carcinoma cell line C33a (Fig. 2g), underlining the specificity of the anti-HPV-45 E7 antibodies. Finally, HPV-45 E7 antibody recognition of

HPV-18 E7 protein expressed in transiently transfected U-2 OS cells was tested in immunofluorescence experiments. The affinity-purified anti-HPV-45 E7 antibodies clearly recognized the HPV-18 E7 protein (Fig. 2h), suggesting that the anti-HPV-45 E7 antibodies cross-react with the structurally related HPV-18 E7 protein under these conditions.

### High-level expression of HPV-18 and -45 E7 oncoprotein in cervical cancers

To determine whether HPV-18 E7 protein can be detected in cervical carcinoma biopsies, we performed immunohistochemistry experiments with the affinity-purified polyclonal anti-HPV-18 E7 antibodies on paraffin sections of normal cervical tissues and invasive cervical cancers (Fig. 3a). The tumour material was characterized as HPV-18 DNA-positive by PCR analysis essentially as described by Fiedler *et al.* (2004). The anti-HPV-18 E7 antibodies stained almost all cells in the HPV-18 DNA-positive tumour islets, but did not stain cells in adjacent connective tissue (Fig. 3a, upper middle panel). Staining by the anti-HPV-18 E7 antibodies could be blocked by pre-incubation of the antibodies with purified HPV-18 E7 antigen (Fig. 3a, upper right panel). The anti-HPV-18 E7 antibodies did not stain





**Fig. 3.** Immunoperoxidase staining of paraffin sections of cervical biopsies with affinity-purified antibodies against HPV-18 and -45 E7. (a) Detection of HPV-18 E7 oncoprotein in HPV-18 DNA-positive cervical cancers. No staining was observed in normal cervical squamous epithelium with the anti-HPV-18 E7 antibodies (upper left panel). In HPV-18 DNA-positive cervical carcinomas, epithelial tumour cells were stained positive by the anti-HPV-18 E7 antibodies (upper middle panel). Staining by the anti-HPV-18 E7 antibodies was competed out by pre-incubation of the antibodies with purified HPV-18 E7 antigen ( $2.5 \mu\text{g } \mu\text{l}^{-1}$ ) (upper right panel). No staining of HPV-18 DNA-positive cervical carcinomas was obtained by adding goat pre-immune serum (lower left panel). Positive staining of a serial section of an HPV-18 DNA-positive cervical carcinoma was detected by using anti-p16<sup>INK4a</sup> antibodies (lower middle panel). (b) Detection of HPV-45 E7 oncoprotein in HPV-45 DNA-positive cervical cancers. No staining was observed in normal cervical squamous epithelium with the anti-HPV-45 E7 antibodies (upper left panel). In HPV-45 DNA-positive cervical carcinomas, epithelial tumour cells were stained positive by the anti-HPV-45 E7 antibodies (upper middle panel). Staining by the anti-HPV-45 E7 antibodies could be competed out by pre-incubation of the antibodies with purified HPV-45 E7 antigen ( $2.5 \mu\text{g } \mu\text{l}^{-1}$ ) (upper right panel). No staining of HPV-45 DNA-positive cervical carcinomas was obtained by adding goat pre-immune serum (lower left panel). Positive staining of a serial section of an HPV-45 DNA-positive cervical carcinoma was detected by using anti-p16<sup>INK4a</sup> antibodies (lower middle panel). In HPV-45 DNA-positive cervical carcinomas, epithelial tumour cells were stained positive by the anti-HPV-18 E7 antibodies (lower right panel).

HPV DNA-negative normal squamous epithelium (Fig. 3a, upper left panel). No staining of cervical carcinoma sections was observed when pre-immune serum was used (Fig. 3a,

lower left panel) or by the secondary anti-goat IgG alone (data not shown). It has been shown that the cyclin-dependent kinase inhibitor p16<sup>INK4a</sup> is upregulated in

HPV-positive cervical carcinomas (Sano *et al.*, 1998; Klaes *et al.*, 2001). For this reason, an adjacent section of the HPV-18-positive cervical cancer was stained with anti-p16<sup>INK4a</sup> antibodies (Fig. 3a, lower middle panel), which revealed the same tumour cell pattern as the anti-HPV-18 E7 antibodies, indicating that both antibodies can detect HPV-18-positive invasive cervical cancer cells. We analysed histological sections of HPV-18 DNA-positive cervical carcinomas derived from six different patients, showing that HPV-18 E7 is readily detectable in HPV-18 DNA-positive cervical cancers. In all samples, the tumour areas were stained clearly positive by the anti-HPV-18 E7 antibodies. The use of anti-HPV-45 E7 antibodies did not yield a signal in HPV-18 E7-positive cervical cancer biopsies (data not shown). This suggested that the anti-HPV-45 E7 antibodies did not cross-react with the HPV-18 E7 protein in paraffin sections of the HPV-18 DNA-positive cervical cancers tested in this study.

To analyse whether the HPV-45 E7 protein is detectable in HPV-45-positive cervical carcinomas, we performed immunohistochemistry experiments with affinity-purified polyclonal anti-HPV-45 E7 antibodies on paraffin sections of HPV-45 DNA-positive invasive cervical cancers (Fig. 3b). The anti-HPV-45 E7 antibodies stained almost all cells in the HPV-45 DNA-positive tumour islets, but not in adjacent connective tissue (Fig. 3b, upper middle panel). Staining by the anti-HPV-45 E7 antibodies could be blocked by pre-incubation of the antibodies with purified HPV-45 E7 antigen (Fig. 3b, upper right panel). The anti-HPV-45 E7 antibodies did not stain normal squamous epithelium (Fig. 3b, upper left panel) and no staining of cervical carcinoma sections was observed when pre-immune serum (Fig. 3b, lower left panel) or secondary anti-goat IgG alone (data not shown) was used. Staining of an adjacent section of the HPV-45-positive cervical cancer with anti-p16<sup>INK4a</sup> antibodies (Fig. 3b, lower middle panel), resulted in a staining pattern similar to that obtained with the anti-HPV-45 E7 antibodies, suggesting that both the anti-HPV-45 E7 and the anti-p16<sup>INK4a</sup> antibodies can stain HPV-45-positive tumour cells in invasive cervical carcinoma sections. Finally, we investigated whether the anti-HPV-18 E7 antibodies cross-react with the HPV-45 E7 protein in cervical cancer biopsies. As shown in Fig. 3(b) (lower right panel), the anti-HPV-18 E7 antibodies stained HPV-45 DNA-positive cervical carcinomas strongly and specifically, indicating that the anti-HPV-18 E7 antibodies cross-react with the HPV-45 E7 protein in cervical cancer sections. We analysed paraffin sections derived from five different HPV-45 DNA-positive cervical carcinoma patients and, in all specimens, the tumour areas were stained positive by the anti-HPV-45 E7 antibodies, indicating that the HPV-45 E7 protein is detectable in HPV-45 DNA-positive cervical cancers. In summary, the findings presented in this study indicate that both the HPV-18 and -45 E7 oncoproteins are clearly expressed in natural cervical cancer biopsies.

There is ample evidence that expression of the E7 onco-gene is necessary for induction and maintenance of the

transformed phenotype (reviewed by zur Hausen, 2000); however, the role of the oncoprotein in cervical carcinogenesis has remained elusive, due to the lack of antibodies allowing its detection in clinical samples (reviewed by von Knebel Doeberitz, 2002). Recently, we showed that the HPV-16 E7 oncoprotein is expressed at a high level in cervical cancer biopsies by using rabbit anti-HPV-16 E7 antibodies (Fiedler *et al.*, 2004); however, the expression levels of the HPV-18 and -45 E7 oncoproteins in cervical carcinomas were unknown. In this study, we have generated polyclonal goat anti-HPV-18 and -45 E7 antibodies. Using affinity-purified batches of these antibodies, we have demonstrated, for the first time, that the HPV-18 and -45 E7 oncoproteins are expressed in HPV-18- and -45-positive cervical cancer biopsies. The E7 proteins were detected in both the nucleus and the cytoplasm of the tumour cells, underlining the fact that the E7 oncoproteins act in both cellular compartments (reviewed by Zwerschke & Jansen-Dürr, 2000). Together with the study by Fiedler *et al.* (2004), these findings suggest that the high-risk E7 oncoproteins of HPV-16, -18 and -45 are expressed continuously in cervical carcinomas. These three HPV types are aetiologically linked to approximately 80 % of all cervical cancers worldwide (reviewed by Muñoz, 2000). Thus, the antibodies described here may have diagnostic potential as tumour markers for cervical cancer.

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