Detection of human papillomavirus oncoprotein E7 in liquid-based cytology

Maria Lidqvist,1,2 Olle Nilsson,1 Jan Holmgren,2 Sebastian Hölters,3 Eva Röijer,1 Matthias Dürr3 and Christian Førmer1

Correspondence
Maria Lidqvist
maria.lidqvist@fdab.com

1Fujirebio Diagnostics AB, Elof Lindéls gata 13, SE-414 55 Gothenburg, Sweden
2Institute of Biomedicine, Department of Microbiology and Immunology, Gothenburg University, SE-405 30 Gothenburg, Sweden
3Department of Gynaecology and Obstetrics, Jena University Hospital, Friedrich-Schiller-University, Jena, 07743 Jena, Germany

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The selection and characterization of a set of mouse mAbs against high-risk human papillomavirus (HPV) E7 oncoprotein and the development of protocols for immunocytochemistry (ICC) are described here. A large number of antibodies raised towards HPV16 and 18 E7 were tested for high-risk specificity by ELISA using a panel of HPV E7 proteins. Antibodies detecting low-risk E7 were discarded, resulting in 38 high-risk HPV E7-specific antibodies. The corresponding epitopes were mapped using overlapping HPV E7 fragments displayed on phage particles. Functionality in ICC against formalin-fixed cervical cancer cell lines was demonstrated for ten mAbs; their high-risk specificity was confirmed by Western blot analysis and ICC on transiently transformed cells expressing high- or low-risk HPV E7. These mAbs were specific for one or several of the high-risk strains HPV16, 18, 31, 35 and 45. Specific E7 staining of liquid-based cytology (LBC) samples was demonstrated for seven mAbs and optimized protocols were established. The E716-41 and E718-79 mAbs demonstrated particularly strong and specific staining of cells stored in LBC fluid for at least 6 months. It is proposed that the high-risk HPV E7 staining protocols established in this study may have the potential to be included in a complementary test for the detection and identification of malignantly transformed cells, in for example atypical squamous cells of undetermined significance samples.

INTRODUCTION

Cervical cancer is the second most common cancer in the world among women, with half a million new cases and 270 000 deaths from the disease every year (WHO/ICO Information Centre on HPV and Cervical Cancer, 2007). The causative agent for cervical cancer is high-risk types of human papillomavirus (HPV); viral DNA can be found in nearly all cervical cancer tumours (Walboomers et al., 1999). To date, 120 HPV types have been identified (Bernard et al., 2010), and among these, about 40 infect the genital tract. Twelve genital HPV types are considered high-risk, known to cause cervical cancer (Bouvard et al., 2009). Geographical variations have been found, but HPV16 and 18 are the two most common types in all regions, being present in about 70% of all cases, followed by HPV45, 33, 31, 52, 58, 35, 39, 51, 59 and 56 in the worldwide distribution (de Sanjose et al., 2010).

Infection with high-risk HPV is a prerequisite for cervical cancer, but for transformation to occur the infection must become persistent (often for several years) (zur Hausen, 1996). A majority of all women are infected with genital HPV during their life time (Koutsy, 1997) and the overall prevalence of genital HPV infection is as high as 10.4% worldwide (de Sanjose et al., 2007). Most infections are cleared by the immune system, often within 1–2 years, and even infections that persist and progress to cervical neoplasia regress spontaneously (Schiffman & Castle, 2003). However, approximately 20% of low-grade lesions will progress to high-grade lesions, and if left untreated, 30% of those will progress to more severe neoplasia (Doorbar, 2006).

The two HPV oncogenes, E6 and E7, play a crucial role in the transformation process and are expressed early in cervical carcinogenesis. The genes are expressed at increasing levels during cancer development, often due to the integration of viral DNA into the host genome and loss of expression control, and are then abundantly expressed throughout the epithelia in high-grade cervical dysplasia and cervical cancer (Doorbar, 2006; von Knebel Doeberitz, 2002). This makes the E7 protein a suitable marker for high-grade lesions and cervical cancer.
In countries with effective cytology screening programmes, the incidence and mortality rates of cervical cancer have been reduced dramatically (zur Hausen, 2002). Most population-based cervical cancer screening programmes use the Pap smear test, by which the morphology of cells from the cervix is examined. The Pap test is partly subjective and relatively insensitive, and the test has to be repeated frequently to be effective (Kitchener et al., 2006). In some programmes, the Pap smear test has been replaced with liquid-based cytology (LBC), which allows automated testing and provides residual cellular material for analysis of HPV infection (Arbyn et al., 2008). The cytology can be complemented by an HPV DNA test to detect the presence of high-risk viruses. Although HPV DNA tests have greater sensitivity than the Pap smear test, they cannot distinguish a transient infection from a persistent one (Castle, 2008). Therefore, sensitive and specific tests are needed to identify malignant progression without detecting transient infections. Tests for the detection of HPV oncoprotein mRNA and secondary biomarkers (e.g. K1, p67 and p16) are available, but yet mostly used for triage (Schiffman et al., 2011).

This paper describes the establishment and characterization of mouse mAbs detecting oncogenic HPV E7. The mAbs were carefully characterized and protocols for their use in immunocytochemistry (ICC) were established. We propose that these protocols may be useful to resolve inconclusive cytology or HPV testing, since detection of the E7 oncoprotein with the mAbs indicates that the oncogene is expressed and that the stained cells have been malignantly transformed. However, additional clinical studies are required to exploit the clinical utility of the mAbs described in this paper.

RESULTS

Cloning and expression of recombinant E7 protein

A panel of high- and low-risk HPV E7 proteins were produced and used for the establishment and characterization of the HPV E7 mAbs. When analysed by SDS-PAGE, all preparations were judged as at least 95% pure based on band intensities, with only trace amounts of other proteins. In Western blot with anti-GST polyclonal antibody (pAb), all GST E7 preparations gave a distinct band of the expected size (approx. 38 kDa).

Selection of high-risk HPV E7-specific mAbs with functionality in ICC

In total, 60 hybridomas secreting HPV E7 antibodies were selected in the primary screening process: 25 from mice immunized with HPV16 E7 and 35 from immunizations with HPV18 E7. The selection process used to identify high-risk HPV E7-specific mAbs suitable for use in ICC was then done in a step-wise manner. High-risk HPV E7-specific antibodies were identified by ELISA against a panel of E7 proteins and the epitopes were mapped using overlapping fragments of HPV16 and 18 E7 displayed on phage. Finally, the functionality of the antibodies in ICC was evaluated. This resulted in 10 high-risk HPV E7-specific antibodies with functionality in ICC. The specificity of the mAbs in ELISA is presented in Fig. 1 and the epitope mapping in Fig. 2.

The majority of the HPV16 E7 hybridomas (21/25) were specific to high-risk HPV E7 when tested by ELISA with high- and low-risk HPV E7 in solution. In addition to detecting HPV16 E7, several antibodies (12/21) detected E7
from other high-risk HPV strains, as expected from the amino acid sequence similarity of the high-risk strains. The majority of the high-risk HPV E7-specific antibodies (17/21), as well as the four low-risk antibodies, detected epitopes within the N-terminal region of HPV16 E7 (aa 1–12). Four high-risk HPV E7-specific hybridomas recognized epitopes within aa 37–53 region. Three of these, E716-1, E716-2 and E716-9, were raised through immunization with aa 33–60 of HPV16 E7 expressed on phage particles and, thereby, directed to this part of the antigen. The specificity of these three antibodies has been studied in detail (Lidqvist et al., 2008).

About half of the antibodies raised against HPV18 E7 (17/35) proved to be high-risk HPV E7-specific in ELISA: most of these (12/17) detected HPV18 E7 and the closely related HPV45 E7, while four were entirely specific for HPV18 E7 and one detected E7 from several high-risk strains. Nine of the high-risk HPV E7 antibodies showed reactivity to aa 1–22 and eight to epitopes within aa 22–53. All low-risk antibodies reacted to the N terminus (aa 1–22) of the HPV18 E7 protein.

Of the 38 hybridomas specific for high-risk HPV in the ELISA with E7 in solution, ten detected E7 in cervical cancer cell lines in ICC and were selected for further evaluation. The hybridomas were cloned by limiting dilution and purified on prosep A. All mAbs were of isotype IgG.

Only 2/17 of the high-risk HPV E7-specific antibodies, recognizing epitopes within aa 1–12 of HPV16 E7, detected HPV16 E7 in ICC, while 4/4 mAbs, recognizing internal epitopes of HPV16 E7, were positive in ICC. Within the high-risk HPV-specific antibodies raised against HPV18 E7, 1/9 detecting epitopes in the N-terminal region, and 3/8 detecting internal epitopes, recognized E7 in ICC. This indicates that the E7 N-terminal is highly immunogenic in mice, but it is not as accessible for ICC analysis.

**Confirmation of high-risk specificity in Western blot and E7-transfected cells**

The results of the further specificity analyses of the ten high-risk HPV E7-specific mAbs suitable for ICC are summarized in Table 1. All mAbs demonstrated specific staining of proteins of the expected molecular mass (approx. 11 kDa for endogenous E7 and 38 kDa for recombinant GST E7 protein) in Western blot analysis, without cross-reactivity to other cellular proteins or GST (Fig. 3). The Western blot results confirmed the results from the ELISA with the rHPV E7 protein for all mAbs but E716-41, which also showed weak cross-reactivity to rHPV35 E7 (Fig. 3a), and E718-79 to HPV45 E7.

The mAbs were further evaluated on formalin-fixed COS cells transiently transfected with an expression vector for E7. In this system, mAbs E716-1, E716-2, E716-9, E716-41 and E716-81 were highly specific for HPV16, staining about 5% of the E7-expressing COS culture. The mAb E716-74 produced background and could not be evaluated in this test system. The mAbs E716-1, E716-2 and E716-9 did not stain HPV31 E7-transfected cells, although they did detect rHPV31 E7 in ELISA and Western blot. The HPV35 E7 was not included in the transient transfection experiments.

The mAbs E718-27, E718-38 and E718-79 were specific for HPV18 E7 expressed by COS cells, with the exception that clone E718-38 showed some cross-reactivity to HPV45 E7, as expected from the ELISA and Western blot results.

**Evaluation of mAbs in ICC**

The ten selected, high-risk HPV E7-specific mAbs were carefully evaluated in ICC on formalin-fixed cells and cells

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**Fig. 2.** Schematic presentation of the overlapping E7 fragments, together covering the whole HPV16 (a) and 18 (b) E7 proteins. The fragments were displayed on phage particles and used for epitope identification. The reactivity of the antibodies to the E7 fragments was determined by ELISA, and the epitopes of the individual mAbs were estimated from the binding pattern. The mAbs E716-1 and E716-2 bind the same fragment as E716-9.
stored in LBC solution. Cervical cancer cell lines CaSki, HeLa and C-33A were used for all experiments.

All ten mAbs functioned well in ICC with formalin-fixed cells. The HPV16 mAbs stained CaSki cells and the HPV18 mAbs stained HeLa cells. In addition, mAbs E716-74 and E718-27 showed some cross-reactivity to HeLa and CaSki, respectively, in contradiction with the ELISA and Western blot results. No staining of the HPV-negative C-33A cells was observed with any of the mAbs. Fifteen minutes of incubation with 0.3 % Triton X-100 prior to mAb incubation was optimal for all mAbs, while heat-induced epitope retrieval (HIER), or no antigen retrieval (AR), resulted in a weaker, or undetectable, signal for all mAbs.

Seven mAbs stained cells stored in the Thinprep LBC fluid. The results for the individual mAbs are summarized in Table 1. The mAbs E716-41 and E718-79 were superior, showing strong specific staining of CaSki and HeLa, respectively. Different ratios of target cells (CaSki and HeLa) were added to HPV-negative cells (C-33A) to mimic clinical samples with infected cells mixed with normal cells. As little as 1 % of target cells could easily be detected when using a mAb concentration of 1 μg ml⁻¹. The cells were stored for at least 6 months in the LBC fluid prior to mAb staining, without loss of signal or adding background (Fig. 4). Treatment for 15 min with 0.3 % Triton X-100 gave the strongest staining for LBC samples, while HIER, or no AR, reduced the signals. In all ICC experiments, HIER with Tris-EDTA had a negative effect on cell morphology.

**DISCUSSION**

During the past 50 years, cervical cytology screening programmes, sometimes complemented by HPV testing, have reduced the rates of cervical cancer dramatically.

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### Table 1. Summary of specificity of the high-risk specific mAbs

<table>
<thead>
<tr>
<th>mAb</th>
<th>ELISA*</th>
<th>Western blot†</th>
<th>ICC COS cells‡</th>
<th>ICC LBC sample§</th>
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<td>E716-1</td>
<td>16, 31, 35</td>
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</tr>
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<td>18</td>
<td>–</td>
<td>16</td>
</tr>
<tr>
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<td>18</td>
<td>18 (45)</td>
<td>18</td>
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</tr>
</tbody>
</table>

*ELISA with recombinant HPV1, 6, 11, 16, 18, 31, 33, 35, 45 E7.
†Western blot analysis with recombinant HPV1, 6, 11, 16, 18, 31, 33, 35, 45 E7.
‡COS cells expressing HPV16, 18, 31, 33, 45, 52, 58 E7.
§CaSki (HPV16 E7), HeLa (HPV18 E7) and C-33A (HPV negative) cells.

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Fig. 3. The specificity of the mAbs was confirmed with denatured protein by Western blot analysis. Recombinant GST E7 of nine HPV strains and lysates of E7-expressing cervical cancer cell lines were separated on SDS-PAGE and analysed by Western blot using the high-risk HPV E7-specific mAbs. (a) The mAb E716-41, raised against HPV16 E7, strongly recognized HPV16 E7 in cellular extract and recombinant protein preparations, with a weak, but specific cross-reactivity to rHPV35 E7. (b) The mAb E718-68, raised against HPV18 E7, strongly recognized HPV18 E7 in cellular extracts and rHPV18 and 45 E7. M, Marker.
However, cervical cytology screening and treatment are not available in all countries, and cervical cancer remains the second most common cancer in the world among women (WHO/ICO Information Centre on HPV and Cervical Cancer, 2007). Also in countries where screening programmes are in place, cervical cancer still occurs. The screening methods used today generate a large proportion of inconclusive results (Schiffman et al., 2011) and complementary methods to clarify suspicious samples would be of great value. There is also an obvious need for efficient low-cost tests to reach women in developing countries. Since oncogenic HPV is the causative agent of cervical cancer, this is an obvious target for diagnosis. In this study, ten mAbs detecting oncogenic types of HPV E7 proteins were identified and selected for their usefulness to identify E7 in ICC. Protocols providing highly specific staining of E7-expressing cells in an LBC medium were designed.

Fig. 4. Immunostaining of cervical cancer cell lines stored in Thinprep LBC liquid for 6 months, using the optimized staining protocols. The target cells were mixed with HPV DNA-negative cell line C-33A. (a) Staining of HPV16-positive cell line CaSki with mAb E716-41. (b) Staining of HPV18-positive cell line HeLa with mAb E718-79.

Since the HPV E7 mAbs were intended for diagnostic use, the high-risk specificity had to be carefully investigated. Antibodies raised against E7 often detect several strains, due to the sequence homology of the E7 proteins from different HPV strains. Almost half of the antibodies (22 of 60) raised in the study cross-reacted with low-risk HPV1, 6 and 11 E7 and were discarded. All these antibodies recognized epitopes within the N terminus of the protein, a region with high sequence similarity between high- and low-risk strains.

In a phylogenetic tree, based on the E7 amino acid sequence, two populations of high-risk HPV strains with high sequence similarity appear (Fig. 5): the HPV16 species including HPV31 and 35, and the HPV18 species including HPV45, in agreement with used classifications (Bernard et al., 2010; Schiffman et al., 2011). The high-risk HPV E7 mAbs established in this study were raised against HPV16 or

Fig. 5. Phylogenetic tree inferred from the E7 amino acid sequence of 12 high-risk HPV types, three low-risk types and the probably carcinogenic HPV68. The phylogenetic analysis is based on the multiple alignments of the E7 amino acid sequences in CLUSTAL W. Evolutionary analyses were conducted in MEGA5. The eight most common high-risk strains, found in almost 90% of all cervical cancer cases worldwide, are in bold letters. All established mAbs only react with closely related HPV strains.
18 E7, but most of them also detect one or two of the most closely related high-risk strains, though with lower affinity (Table 1, Fig. 1). Specificity to other strains is not likely, based on sequence analysis of the binding regions for each mAb.

The purpose of this study was to identify antibodies suitable for ICC and the possible influence by different fixatives had to be considered in the evaluation. Formalin is a cross-linking fixative, known to alter the three-dimensional structure of proteins by forming methylene bridges between certain amino acids, thereby modifying many epitopes. Antigen retrieval has the capability to reverse some of the cross-linking, though the knowledge about the underlying mechanisms of this process is limited. HIER with citrate pH 6 is the most widely used AR method and was evaluated without success in this study. The alcohol in the LBC solutions denatures many proteins as compared with the cross-linking of proteins by formalin. As a consequence, different AR methods are optimal for different fixatives, and they have to be evaluated for each antibody–antigen pair and fixative (van der Loos, 2007). In our hands, pre-treatment with Triton X-100 resulted in the most intense staining, in agreement with earlier results (Jeon et al., 2007).

It was especially encouraging that seven mAbs stained E7 in LBC samples, since LBC is gradually replacing the conventional Pap smear test. The LBC techniques provide more uniform slides and leave residual cell material for further tests. The E716-41 and E718-79 mAbs demonstrated particularly strong and specific staining of target cells, even when the cells had been kept for 6 months in LBC preservative fluid. The mAbs could easily identify single HPV E7-positive cells from HPV-negative cells, which is a probable situation in a clinical sample, containing only a few infected cervical cells and many uninfected cells (Fig. 4).

There is a need for convenient tests for early detection of cervical cancer in order to introduce screening in the developing countries, as well as to be able to distinguish malignantly transformed cells from transient infections. The straightforward and quick staining protocols established in this study have the potential to become a part of such a test. The antibodies in this study were raised against HPV16 and 18 E7, and detected HPV16 and 18 E7 with high sensitivity and HPV31, 35 and 45 E7 with lower sensitivity in ELISA and Western blot analysis. To improve the clinical utility for triage of atypical squamous cells of undetermined significance (ASCUS) cytology results, antibodies with high affinity to other high-risk strains would need to be included. So far, the antibodies have been evaluated on cervical cancer cell lines. The antibodies have high sensitivity and specificity in these systems, indicating that they may have diagnostic potential for the identification of E7 expression in HPV16 and 18 DNA-positive samples and, combined with antibodies with other specificities, to resolve ASCUS cytology samples of unknown HPV genotype. In order to evaluate the clinical utility of the established reagents, studies on HPV-genotyped cervical LBC samples and biopsies are under way.

METHODS

**Cell lines.** Cervical cancer-derived cell lines CaSki (expressing HPV16 E7), HeLa (expressing HPV18 E7) and C-33A (Negative for HPV) were grown in Dulbecco’s modified Eagle medium (DMEM; Sigma-Aldrich), 5 % FBS (HyClone) and 1 % DMEM supplement (Gibco). All cell lines were obtained from ATCC.

**Cloning and expression of recombinant E7 protein.** cDNA was prepared from CaSki and HeLa cells, and the ORFs of HPV16 and 18 E7 were amplified in PCRs with E7-specific primers, including BamHI/EcoRI sites. The entire reading frames of HPV1, 6, 11, 31, 33, 35 and 45 E7 flanked by BamHI/EcoRI sites were synthesized by GenScript. All E7 ORFs were cloned into expression vector pGEX-6P-3 (GE Healthcare) using the BamHI/EcoRI cloning sites in fusion with the glutathione S- transferred (GST) ORF. Sequence verified clones were used for the expression of recombinant full-length HPV E7 (rHPV E7) protein and for construction of phage clones. For details see Lidqvist et al. (2008).

The GST E7 fusion proteins were purified on glutathione sepharose (GE Healthcare). Prior to immunization, the GST tag was removed with PreScission protease (GE Healthcare) according to the manufacturer’s instructions. The concentration of all protein preparations were determined from the absorbance at 280 nm, using theoretical absorbance factors calculated from the amino acid sequence (ExPASy ProtParam tool). The purity of the samples was studied by analysing 0.75 μg protein in SDS-PAGE (NuPAGE). The identity of the GST-tagged proteins was confirmed by Western blot analysis using a rabbit anti-GST pAb (Z-5; Santa Cruz Biotechnology) as described below.

**Selection of high-risk HPV E7-specific mAbs with functionality in ICC**

**Establishment of HPV16 and 18 E7 antibodies.** Twelve-week-old female BALB/c mice (B&K Universal) were immunized with 25 μg rHPV16 or rHPV18 E7 oncoprotein in 100 μl saline solution and 100 μl Ribi Adjuvant (Corixa) by intraperitoneal injection followed by additional injections of 10 μg antigen together with adjuvant, every 3–4 weeks for 2–5 months. Three mAbs (E716-1, E716-2 and E716-9) were raised through immunization with HPV16 E7 aa 33–60 peptide displayed on phage particles as previously described (Lidqvist et al., 2008).

Serum antibody tities against rHPV E7 were continuously measured by ELISA. Serial dilutions of pre-immune and immune sera in Blocker casein in PBS (Pierce) were added to Reacti-Bind glutathione-coated wells (Pierce), coated with recombinant GST-tagged HPV16 or 18 E7. After 1 h incubation, wells were washed six times in 5 mM Tris/HCl pH 7.8, 150 mM NaCl, 0.005 % Tween 20 and anti-E7 antibodies were traced by the HRP-conjugated rabbit anti-mouse (Dako) for another hour. After an additional washing step, HRP-substrate Enhanced K-Blue (Neogen) was added for the detection of binding and plates were analysed by measuring absorbance at 620 nm (Versa Molecular Corporation). All animal experiments were performed in accordance with the Animal Welfare Ordinance and the Animal Welfare Act of Sweden and were approved by an animal ethics committee (approval no. 310-2005 and 445-2008).

B-lymphocytes from mice with high anti-E7 serum antibody titres were fused with mouse myeloma cell line P3X63Ag8.653 (ATCC; CRL-1580) essentially as described previously (de St Groth & Scheidegger, 1980). Hybridomas producing antibodies with reactivity to rHPV16 or 18 E7 proteins were selected by ELISA. The medium from individual hybridomas was incubated overnight in microtitre wells coated with goat anti-mouse IgG (Jackson Immunoresearch Laboratories). After washing, the wells were incubated with GST HPV16 E7 or GST HPV18
E7 protein for 2 h. An unrelated GST fusion protein was used as a negative control to exclude antibodies with reactivity to GST or the GST linker. Antigen binding was detected by incubations with, first, rabbit anti-GST (Z-5; Santa Cruz Biotechnology) for 1 h and, finally, with HRP-conjugated swine anti-rabbit IgG (Dako) for 1 h. Following each incubation, the plates were washed three times in 5 mM Tris/HCl pH 7.8, 150 mM NaCl, 0.005 % Tween 20. Reactivity was measured at 450 nm after visualization with O-phenylenediamine substrate (Sigma-Aldrich). In the screening process, signals of at least five times the absorbance of the negative control were considered as positive. Antibody isotypes were determined by ELISA using isotype-specific antibodies (Zymed Laboratories).

Selection of high-risk HPV E7-specific antibodies by ELISA. The E7 antibodies were further analysed by ELISA, as described above, for their reactivity towards HPV E7 proteins from a panel of high-risk (16, 18, 31, 33, 35 and 45) and low-risk (1, 6 and 11) strains. The 706-C5 and 711-13 mAbs (Hytest) were used as positive controls for HPV6, 11, 16 and 31. Signals of at least twice the signal of the negative controls with no antigen were considered as positive. Antibodies with reactivity to E7 from any of the low-risk strains were rejected.

Epitope mapping with fragment phage display. Fragments of HPV16 and 18 E7 were amplified from the constructed pGEX-6P-3 HPV E7 plasmid clones, using the primers described in Supplementary Table S1 (available in JGV Online). The fragments were cloned into one of three vectors: phage display vector c88-4 kindly provided by Professor G. P. Smith (University of Missouri, Columbia, USA), vector M13KE (New England Biolabs) or, together with a recombinant phage gene pVIII, into the arabinose-regulated vector pBAD/Myc-HisA (Invitrogen). Phage particles were amplified according to standard techniques and prepared with double polyethylene glycol precipitation (Lidqvist et al., 2008). Phage particle concentration was calculated from the absorbance at 269 nm (Bonncastle et al., 2001).

Maxisorp wells (Nunc A/S) coated with goat anti-mouse IgG were incubated overnight with antibody-containing growth medium from individual hybridomas, followed by incubation with the phage clones displaying overlapping fragments of the E7 protein (Fig. 2). Bound phage particles were detected with a rabbit anti-M13 antibody (raised against M13 phage particles at Fujirebio Diagnostics AB) and HRP-conjugated swine anti-rabbit IgG (Dako). All incubations were followed by three washes in 5 mM Tris/HCl pH 7.8, 150 mM NaCl, 0.005 % Tween 20. Phage binding was visualized with HRP substrate Enhanced K-Blue. A signal of twice the absorbance of the negative control was considered as positive.

Selection for functionality in ICC. All high-risk HPV E7-specific antibodies were tested for functionality in ICC, to identify antibodies with reactivity to formalin-fixed E7 protein in for example cell smears. Newly harvested CaSki, HeLa and C-33A cells were mounted on polysine slides (Menzel GmbH & Co KG). The cells were fixed for 10 min in 10 % neutral buffered formalin (NBF) and washed for 5 min in TBS 0.05 % Tween 20. To block endogenous peroxidase activity, the slides were incubated in 1 % hydrogen peroxide for 5 min. The slides were incubated with hybridoma growth media for 1 h. Antibody binding was visualized by the REAL EnVision Detection system and diaminobenzidine (Dako). Cells were counterstained with Mayer’s haematoxylin (Dako) to facilitate identification of cell morphology. All incubations were done at room temperature.

Promising antibodies were cloned by limiting dilution. The mAbs were produced by in vitro cultivation in DMEM, 2.5 % FBS, 1 % DMEM supplement in roller bottles for 2 weeks. The antibodies were purified using protein A (Montagé Prosep-A media plug; Millipore) and eluted at pH 4.

Western blot analysis. The reactivity of the selected mAbs with denatured E7 protein was confirmed by Western blot studies using cell lysates of cervical cancer cell lines CaSki (expressing HPV16 E7), HeLa (expressing HPV18 E7) and C-33A (HPV negative) and rHPV E7 protein from HPV1, 6, 11, 16, 18, 31, 33, 35 and 45, prepared as described above.

Cells were lysed by freeze–thawing in PBS containing 1 % Triton X-100, 1 mM DTT, 0.2 mM PMSF, 1 mM NaF and complete EDTA-free protease inhibitor cocktail (Roche Diagnostics). Cell debris was removed by centrifugation and the total protein concentration of the supernatants was determined by the Bio-Rad protein assay kit 1 (Bio-Rad).

Cell lysates (containing 50 µg cellular protein per lane) and recombinant GST E7 protein (1 ng GST E7 per lane) were separated on NuPAGE Bistris and transferred to PVDF membranes (Bio-Rad) according to the manufacturer’s instruction. Total cell lysate of C-33A and Escherichia coli-expressing GST was included as control for cross-reactivity to cellular proteins and GST. After blocking the membranes overnight in 5 % non-fat dry milk, the E7-antibodies, also pre-incubated in 5 % non-fat dry milk, were added to the membrane at a concentration of 1–2 µg ml⁻¹ and incubated for 1 h. After three 15 min washes with TBS 0.2 % Tween 20, the secondary antibody, HRP-conjugated rabbit anti-mouse immunoglobulin (Dako), was incubated with the membranes for 1 h. After another wash, antibody binding was visualized using chemiluminescent detection (ECL+; GE Healthcare) according to the manufacturer’s instruction. Control experiments with mouse anti-HPV16 E7 (TVG7104; Santa Cruz Biotechnology) and goat anti-HPV18 E7 (N-19; Santa Cruz Biotechnology) was included to control for E7 expression in the cervical cancer cell line lysates and rabbit anti-GST (Z-5; Santa Cruz Biotechnology) to ensure that recombinant GST E7 protein was present in all wells (data not shown). We used HRP-conjugated donkey anti-goat (Promega) and HRP-conjugated swine anti-rabbit (Dako) as secondary antibodies to the pAbs.

ICC on COS transfectants. The specificity of the antibodies was also evaluated by staining transiently transfected COS-7 cells. HPV16, 18 and 45 E7 ORFs were cloned into expression plasmid pCR3.1 (Invitrogen) and HPV31, 33, 52 and 58 E7 into pBK-CMV (Stratagene). Plasmid integrity was confirmed by sequence analyses. The COS-7 cells (ATCC: CRL-1351) were plated on 10 cm dishes and transfected by calcium phosphate co-precipitation with 20 µg E7-expressing plasmid mixed with 2 µg pBK–EGFP. The latter plasmid served to monitor transfection efficiency that was usually in the range of 10–20 %. Two days post-transfection, the cells were trypsinized, neutralized in cell culture medium, centrifuged and resuspended in PBS. Cytospins were then prepared by centrifuging 10⁵ cells onto Superfrost Plus microscope glass slides (Thermo Scientific). The slides were either immediately frozen at −80 °C or fixed in 10 % NBF for 20 min at room temperature followed by a washing step for 5 min in TBS containing 0.05 % Tween 20. The slides were then processed as described above.

Evaluation of mAbs in ICC. The high-risk HPV E7-specific mAbs were carefully evaluated in ICC against cervical cancer cell lines CaSki, HeLa and C-33A. The cells were either formalin-fixed on the slide or stored in Thinprep LBC solution (Cytyc) prior to mounting on slides. The protocol for each mAb was optimized regarding antibody concentration, antibody incubation times and AR. Three different protocols for permeabilization and AR were evaluated: 15 min treatment with 0.3 % Triton X-100 in TBS at room temperature, 10 min boiling in microwave oven in 10 mM citrate pH 6.0, and 10 min boiling in 10 mM Tris, 1 mM EDTA, pH 9.0. Endogenous peroxidases were blocked with 1 % hydrogen peroxide in TBS 0.05 % Tween 20 for 5 min.
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