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Subcellular localization and quantitation of the human papillomavirus type 16 E6 oncoprotein through immunocytochemistry detection

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ABSTRACT

Human papillomavirus (HPV) type 16 E6 is a viral oncoprotein essential for host cell transformation. Due to its role in HPV-induced cancers of the genital, head and neck epithelia, reliable protein-level determination of E6 expression would be an invaluable diagnostic tool. Immunocytochemical detection and subcellular localization of HPV16 E6 has been demonstrated with varying success and a comprehensive review of techniques is lacking. To address these issues, we used established monoclonal antibodies and optimized a standard immunocytochemical method for E6 protein detection inside the HPV16 positive cell lines, SiHa and CaSki. E6 oncoprotein was detected primarily in the nucleus. We also refined quantitative analysis with a software to objectively differentiate between HPV16 positive and negative cells. Our analysis was also able to differentiate expression differences between SiHa and CaSki on par with RT-qPCR . Thus, we provide a long-needed, robust protocol for antibody-mediated detection of the HPV16 E6 oncoprotein inside cultured cells.

Introduction

For over two decades, the 18 kDa human papillomavirus (HPV) type 16 E6 protein has been implicated in phenotype transformation of cervical carcinoma-derived cell lines. Unlike other HPV16 proteins, which are differentially expressed throughout the viral life cycle and in the transformed phenotype of the host, E6 is expressed in both pre-malignant and malignant tissues. These criteria make E6 an ideal biomarker with diagnostic potential (Androphy et al., 1987).

Most useful diagnostic tests require the development of antibodies to a key antigen. In the mid-80s, polyclonal HPV18 (second in prevalence to HPV16) E6 antibodies were generated and used for immunoblotting of HeLa cells (Matlashewski et al., 1986), providing the proof-of-concept necessary for the development of polyclonal antibodies for HPV16 E6. Such antibodies raised in rabbits against a bacterial HPV16 E6 fusion protein were used for E6 immunoprecipitation (Androphy et al., 1987). In refining the process, the hunt for monoclonal antibodies (mAb) against E6 began. Interestingly, one of the first HPV18 E6 monoclonal antibodies, mAb C1P5, cross-reacted with HPV16 E6 in an immunoblot of SiHa cells (which contain three HPV16 copies per cell) (Yee et al., 1985; Baker et al., 1987; Banks et al., 1987). The epitope recognized by mAb C1P5 represents a region of immunological conservation between HPV16 and HPV18.

Following development of good mAbs to E6, *in situ* detection of the E6 protein was the logical next step. Nuclear and membrane localization for HPV18 E6, with mAb C1P5, was performed using insect cells (Grossman et al., 1989) and a recombinant baculovirus expression system that produced HPV proteins with similar biological properties to native proteins (Park et al., 1993). Further studies involving a mAb against the HPV16 E6 open reading frame, but with the precise epitope unknown, showed cytoplasmic staining via immunofluorescence (Kim et al., 1994). A mAb against the 23 amino acid, C-terminal polypeptide of the HPV18 E6 open reading frame was also used to demonstrate nuclear localization for HPV16 and 18 (Scheffner et al., 1990). Due to the lack of a reliable detection method, such inconclusive results were typical of HPV E6 localization studies in the 1990s.

After these early studies, a need was clearly identified for generating and characterizing highly reactive and specific monoclonal antibodies to study HPV16 E6 functionality *in situ* (Wlazlo et al., 2001). Previous HPV16 E6 localization studies within human carcinoma cells had produced contradictory results, likely due to low endogenous E6 protein levels and poor reactivity of the polyclonal antisera used. In the early 2000s, new mAbs

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against the recombinant HPV16 E6 protein were produced to determine the precise intracellular localization of E6 in transformed cells and whether the cellular distribution of E6 correlated with p53-independant functions (Masson et al., 2003). The purpose was to identify regions of E6 that could be targeted for functional inhibition: they narrowed their search to a region of 10 residues at the N-terminus of HPV16 E6. In CaSki cells (which contain about 600 copies per cell of integrated HPV16 and some HPV18 sequences), they found strong inhibition of p53 proteolysis using these antibodies, specifically mAb 6F4 (Giovane et al., 1999). The 4C6 antibody was produced similarly, yielding specificity to the N-terminus region of HPV16 E6. Immunoblotting with antibodies 1F4, 1F1, and 6F4 yielded positive results and indirect immunofluorescence microscopy showed nuclear localization in transfected cells (Choulier et al., 2002). However, this group was unable to confidently detect endogenous E6 protein in CaSki and SiHa, resorting to immunogold staining with electron microscopy (Masson et al., 2003).

More recently, this group generated monoclonal antibodies against the second zinc-binding domain of HPV16 E6 (16ZD2) and used them with the previous 6F4 mAb (N-terminus specific) to test their inhibition against E6 with E6AP and degradation of p53 (Lagrange et al., 2005). The three new antibodies made were 1F5, 3B8, and 3F8; these were validated using western blots and immunofluorescence of transfected cells and CaSki. Finally, they used delivery of 4C6, 6F4, and 3F8 with small interfering RNA to SiHa and CaSki to suppress growth by preventing p53 degradation (Courtête et al., 2007). However, in our hands none of the aforementioned methods yielded reproducible staining.

Overall, a lack of effective, commercially available mAbs for HPV16 E6 has beleaguered researchers. The purpose of the current study was to evaluate antibody-mediated detection of the HPV16 E6 oncoprotein for subcellular localization and expression analysis. We used the 4C6 Strasbourg clone, based on preliminary results suggesting it was the best primary antibody candidate, to establish an optimized technique for colorimetric immunocytochemistry that could reliably localize E6 oncoprotein *in situ*. We then established a quantitative analysis protocol for determining protein-level HPV16 E6 expression.

Results

HPV16 E6 oncoprotein was reliably detected in the nuclei of established cervical carcinoma cell lines via immunocytochemistry

Accurate and reproducible detection of the HPV16 E6 oncoprotein *in situ* is useful as a marker for HPV16 presence and expression of its functional oncoproteins. Specifically, detection of E6 provides evidence of protein-level expression and localization within a cell. Studies which aim to modulate E6 expression and function require reliable detection of their target to validate the functional relationship of any experimental response. Typically, preliminary studies are performed using monolayer cell culture as a basic biological model. Immunocytochemistry is a commonly used technique for observing protein expression and localization. A protocol exists for the detection of HPV16 E6 oncoprotein (courtesy of Arbor Vita Corporation (Fig. 1)), but it required optimization in our hands. The UltraVision LP kit (Thermo Scientific, Catalog no. TA-125-PH), which was used in the original protocol, includes a separate antibody enhancer and secondary HRP-conjugated polymer. This kit is not available in Canada. Instead, the closest possible substitution, the UltraVision ONE kit (Thermo Scientific, Catalog no. TL-060-HLJ) was initially used as an alternative in the Zehbe laboratory. SiHa and CaSki cell lines, both of which contain HPV16, were used as positive controls, and C33A, an HPV16 negative carcinoma cell line, was used as a negative control. Both SiHa and CaSki cells yielded strong positive staining, but so did C33A cells albeit to a lesser extent (Fig. 2A). The non-specific staining in our negative control C33A cells could be resolved with agitated washing steps (up and down slide dips). However, this decreased the specific staining substantially (Fig. 2B) and could not be compensated by prolonging the incubation time of primary antibody and subsequent detection steps (data not shown). Overnight ($\sim$16 h) antibody incubation at 4 °C did not appear to have a significant improvement to reduce unspecific staining and lower antibody concentrations (1–10 μg/mL, compared to 20 μg/mL) decreased the staining intensity for all cells (data not shown). This prompted us to try the recently launched Novocastra Bond Polymer Refine Detection kit (Leica Microsystems, Catalog no. DS9800) which offers greater sensitivity than the UltraVision ONE kit due to a two-step post-primary step with multifunctional linkers. The new kit enabled positive staining in SiHa and CaSki cells, predominately localized to the nucleus, but yielded no staining in C33A cells (Fig. 3A). E6 staining in SiHa and CaSki was not seen in all cells, and did not appear to have uniform intensity. SiHa had fewer and less intensely positive cells compared to CaSki. Omission of the primary antibody gave no staining in any cells, indicating that positive staining is due to the primary antibody (Fig. 3B). Consistent results were observed in two independent laboratories (the Arbor Vita and the Zehbe lab), with testing done by separate individuals, yielding a refined detection protocol (Fig. 4). Two other Strasbourg clones, 3F8 and 6F4, the latter of which works well in Western blots (Zehbe et al., 2009), were also tried with our optimized protocol in the Zehbe lab. However, these clones yielded non-specific cytoplasmic staining in HPV positive and negative cells alike, which was not improved by changing fixation to paraformaldehyde, methanol or acetone (data not shown).

Quantitative analysis of immunocytochemistry for unbiased detection of HPV16 E6 oncoprotein expression

To shed the constraints of subjectivity, as offered by qualitative results, a quantitative analysis protocol was developed using the CellProfiler software (Carpenter et al., 2006; Kamentsky et al., 2011). CellProfiler allows the complex and automatic analysis of biological images. The user designs "pipelines" by specifying individual functions to be performed in sequential order; reproducible and customizable analysis of large image-sets is possible. For our purposes, images were processed by first identifying individual cells based on DAB and haematoxylin stain followed by isolating, measuring, and averaging cell DAB intensities (Fig. 5). This process was performed iteratively for 25 images each of SiHa, CaSki, and C33A. An objective analysis was thus employed to determine if there were significant differences between positive and negative controls.

Mean intensities derived from CellProfiler analysis of 400$\times$ images were determined to be non-parametric and were statistically tested using a Kruskal–Wallis rank sum test (non-parametric ANOVA equivalent) followed by pairwise Wilcoxon rank sum post-hoc analysis. CaSki and SiHa both had significantly higher mean intensities than negative control C33A and all no primary Ab controls ($^{***}P<0.001$, $n=25$) (Fig. 6). CaSki had $\sim$5.8 fold greater mean intensity than SiHa ($^{***}P<0.001$, $n=25$). There was no significant difference between C33A and no primary Ab control cells.

Since the cervical carcinoma cell line CaSki has approximately 200 times more viral DNA copies than SiHa, transcript and protein-level fold change was calculated to determine if this magnitude of difference was also reflected throughout transcription and translation. Relative transcript-level differences, as determined by standard

1. Prepare samples by adding ThinPrep PreservCyt Solution at a cell suspension to fixative ratio of 1:20 (v/v)
2. Permeabilization with 0.1% Triton X-100 in 1X TBS for 5 min (100 µL per slide)
3. Wash with 3 times 1X TBS with 0.01% Tween 20 (v/v) for 3 min each in container with fresh wash buffer, replacing after each wash step
4. Apply 100 µL per slide Endogenous Peroxidase Blocking reagent to cells for 10 min
5. Washing (as in step 4)
6. Apply 100 µL per slide Protein Blocking Solution for 5 min
7. Washing (as in step 4)
8. Apply 100 µL per slide 5% goat serum and 2% dry milk in 1X TBS for 10 min
9. Washing (as in step 4)
10. Incubate cells for 30 min using anti-HPV16 E6 mAb 4C6 (20 µg/mL in 1X TBS containing 1% BSA)
11. Washing (as in step 4)
12. Apply 100 µL per slide for Antibody Enhancer for 10 min
13. Washing (as in step 4)
14. Incubate cells for 15 min with secondary antibody conjugated polymer
15. Washing (as in step 4)
16. Apply 100 µL per slide DAB solution for 5 min
17. Washing (as in step 4, but with distilled water)
18. Mount by covering slides with 1 to 2 drops of mounting medium
19. Inspect cells by microscopy and secure data via electronic photography

Fig. 1. Flowchart of original immunocytochemical protocol for detection of HPV16 E6 oncoprotein. The original protocol provided by Arbor Vita Corporation using the UltraVision LP kit (Thermo Scientific, Catalog no. TA-125-PH). Cells were stored in fixative at 4 °C. Unless otherwise stated, all subsequent steps were performed at room temperature.

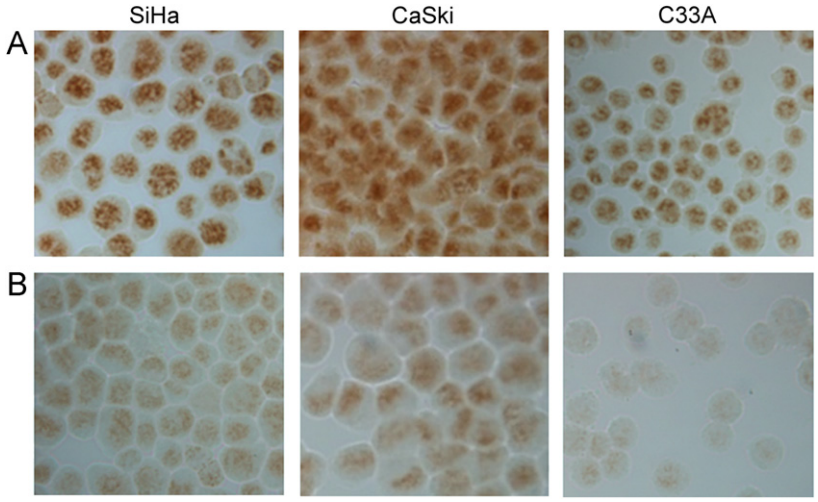

Fig. 2. Immunocytochemical detection of HPV16 E6 oncoprotein using the original protocol. Micrographs of immunocytochemistry for HPV16 E6 oncoprotein using the original protocol with (A) mAb 4C6 primary antibody and the UltraVision ONE kit and (B) same procedure, but with agitated washing steps. Distinct brown color, caused by oxidation of DAB by HRP, represents positive staining. Images are cropped from 400× magnified captures. Linear adjustments to brightness and contrast were applied consistently for all images.

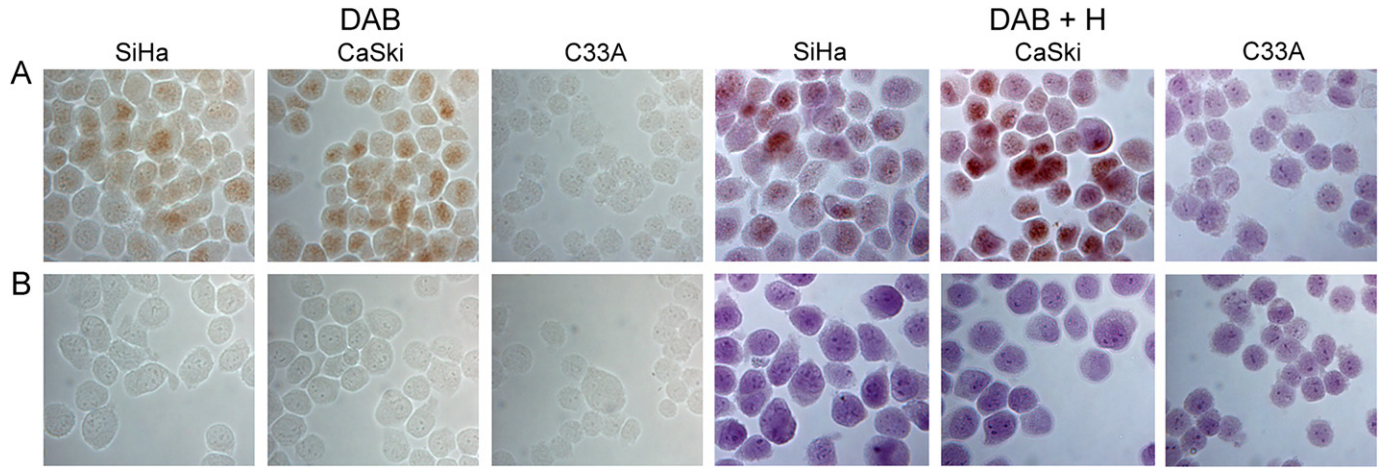

Fig. 3. Optimized immunocytochemical detection of HPV16 E6 oncoprotein. Micrographs of immunocytochemistry for HPV16 E6 oncoprotein using the modified protocol with (A) mAb 4C6 primary antibody and (B) with no primary antibody. Distinct brown color, caused by oxidation of DAB by HRP, represents positive staining. Haematoxylin was used as a counterstain. Images are cropped from 400 × magnified captures. Linear adjustments to brightness and contrast were applied consistently for all images.

1. Fix suspended cells at 1:20 (v/v) in ThinPrep PreservCyt Solution, store at 4 C, remove fixative and resuspend in PBS
2. Cytospin 3: add 100 µL (1 x 10^5 cells) to cytofunnel, spin at 1000 RPM for 5 min, draw wax circles
3. Permeabilization with 100 µL of 0.1% Triton X-100 in 1X TBS with 0.05% sodium azide for 5 min at room temperature in an aluminium foil-covered humidified tray (carryout all incubation steps under these conditions)
4. Wash 3 times 3 min with 1X TBS-T (0.05%) in a slide rack; lift slides up-down 10 times for each wash
5. Apply 3% hydrogen peroxide for 10 min
6. Washing (as in step 4)
7. Apply anti-HPV16 E6 mAb 4C6 (20 µg/mL, diluted with 1% BSA in 1X TBS with 0.05% sodium azide) for 30 min
8. Washing (as in step 4)
9. Apply Bond Polymer Refine Detection post primary reagent for 10 min
10. Washing (as in step 4)
11. Apply secondary antibody polymer for 15 min
12. Washing (as in step 4)
13. Prepare DAB solution, apply quickly, and closely monitor (~10 min)
14. Washing (as in step 4, but with distilled water)
15. Counterstain with haematoxylin if desired
16. Mount by covering slides with 1 to 2 drops of mounting medium
17. Inspect staining by brightfield microscopy and digitally capture images from multiple fields of view
18. Use CellProfiler image analysis software to objectively quantify the intensity of DAB staining for each sample

Fig. 4. Flowchart of modified immunocytochemical protocol for detection of HPV16 E6 oncoprotein. Our modified protocol for the detection of HPV16 E6 oncoprotein using the Novocastra Bond Polymer Refine Detection kit (Leica Microsystems, Catalog no. DS9800). Cells were stored in fixative at 4 °C. Unless otherwise stated, all subsequent steps were performed at room temperature.

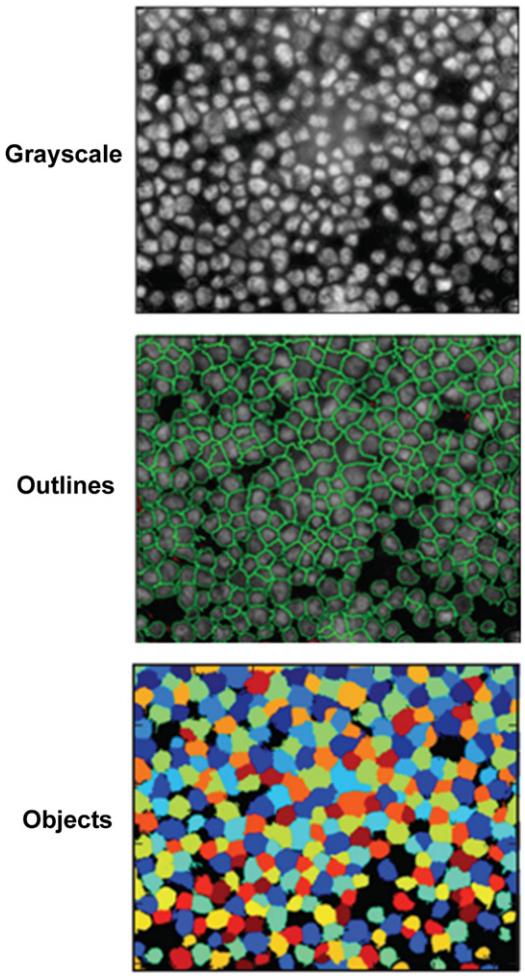

Fig. 5. CellProfiler was used to objectively measure DAB intensity from immunocytochemistry images. Using CellProfiler software, images were converted to greyscale haematoxylin and DAB. The "IdentifyPrimaryObjects" function was used to outline individual cells from the background, followed by object assignment. Mean DAB intensity was measured automatically for each image by calculating and averaging the greyscale DAB intensity for every object.

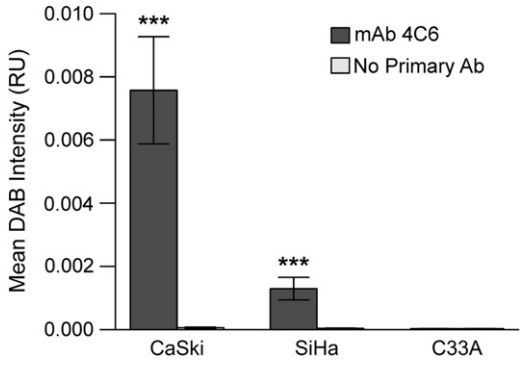

Fig. 6. Quantitative analysis demonstrates significant signal intensity differences between CaSki, SiHa, and C33A. Mean intensities (relative units) derived from CellProfiler analysis were analyzed using a Kruskal–Wallis rank sum test (non-parametric ANOVA equivalent) followed by pairwise Wilcoxon rank sum post-hoc tests. CaSki and SiHa both had significantly higher mean intensity than negative control C33A and all no primary Ab controls ($^{***}P<0.001$, $n=25$). CaSki had ~5.8 fold greater mean intensity than SiHa ($^{***}P<0.001$, $n=25$). There was no significant difference between C33A and no primary Ab control cells. Means are presented as mean ± SEM.

RT-qPCR analysis, matched that of our protein quantification (Fig. 7). Welch's two-sample *t*-test for unequal variance was used for statistical comparison. CaSki had ~5.7 fold greater E6 mRNA

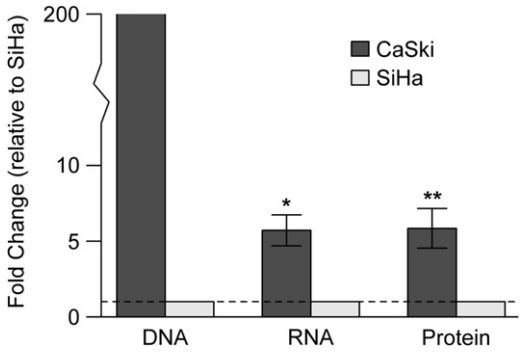

Fig. 7. DNA, mRNA, and protein-level fold change between CaSki (high-copy) and SiHa (low-copy) cervical carcinoma cell lines. Caski had ~200 fold greater viral copies, ~5.7 fold greater E6 transcript expression ($^{*}P<0.05$, $n=3$), and ~5.8 fold greater E6 protein expression ($^{**}P<0.01$, $n=25$) compared to SiHa. Welch's two-sample *t*-test for unequal variances was used for comparing fold change data. SiHa has fold change of 1 for baseline. Means are presented as mean ± SEM.

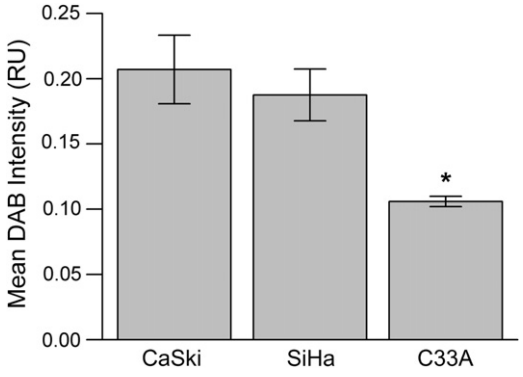

Fig. 8. Quantitative analysis can demonstrate significant signal intensity differences between HPV16 E6 positive and negative cells with high background. Mean intensities (relative units) derived from CellProfiler analysis were determined to be parametric and were tested using a one-way fixed effects ANOVA followed by Tukey's HSD post-hoc analysis. CaSki and SiHa both had significantly higher mean DAB intensity than the negative cells ($^{*}P<0.05$, $n=5$) but were not significantly different from each other. There was no significant difference between negative cells. Means are presented as mean ± SEM.

expression than SiHa ($^{*}P<0.05$, $n=3$), approximately equal to protein expression fold change of ~5.8 ($^{**}P<0.01$, $n=25$), but much lower than the 200 fold change in viral copy number.

To further demonstrate the utility of this quantitative analysis, mean intensities were also determined from staining with high background in positive and negative cell lines (as seen in Fig. 2). Mean intensities derived from CellProfiler analysis of 400× mAb 4C6 images were determined to be parametric and were statistically tested using a one-way fixed effects ANOVA, followed by Tukey's HSD post-hoc analysis. CaSki and SiHa both had significantly higher mean DAB intensity than C33A cells ($^{*}P<0.05$, $n=5$), but were not significantly different from each other (Fig. 8).

Discussion

In this paper, we present the results of rigorous testing, resulting in a protocol for reliable detection of the HPV16 E6 oncoprotein via immunocytochemistry followed by a refined quantitative analysis. Specifically, using this optimized detection method we have demonstrated that E6 oncoprotein is mainly located in the nucleus and consistent with relative mRNA

expression levels. Furthermore, we can objectively determine a detection threshold for samples of unknown E6 status; this would be of great importance for diagnostics and research purposes alike.

The complexity in detecting the *in situ* HPV16 E6 protein is due to a combination of several factors: antibody sensitivity, antigen availability, and technique-specific handling. Prior to our study, antibody sensitivity and specificity had been a necessary focus of research. Although we now have effective anti-HPV16 E6 antibodies, such as mAb 4C6, we still must be concerned about the availability of E6 protein as an antigen (Masson et al., 2003; Lagrange et al., 2005). HPV16 E6 is quite promiscuous; the two zinc-binding domains and C-terminal PDZ-binding domain allow interaction with a variety of cellular proteins, with many that act in the nucleus at a transcriptional level (Mantovani and Banks, 2001; Kumar et al., 2002). Our results confirm a primarily nuclear localization of E6 oncoprotein, as suspected due to its binding activities, and as previously evidenced by electron microscopy coupled with immunogold staining (Masson et al., 2003). An interesting finding is the lack of E6-positive staining in some cells. This staining pattern could be explained by differential expression of E6 in unsynchronized cell cultures which is paralleled by the fact that p53 is sporadically present (Jackson et al., unpublished data). There is also a potential with higher passage and confluent cells to have E6 expression drop off, possibly due to an accumultation of mutations making E6 superfluous while activating different pathways to allow continued proliferation.

Experimenter-specific handling and bias is an issue with most immunocytochemical techniques. To limit subjectivity, our quantitative analysis allows an objective method of assaying samples. One potential problem is inter-experimental staining variability associated with imaging properties (brightness, DAB staining, microscope and image capture parameters, etc.). Despite this potential drawback, automated analysis is easily implemented in CellProfiler, while relative staining intensities between positive and negative biological samples should remain distinguishable (Carpenter et al., 2006; Kamentsky et al., 2011). The consistency of relative E6 mRNA and protein levels between CaSki and SiHa is another supportive finding, lending credence to the reliability of our technique. Although transcript-level differences in general do not necessary equal protein differences *in situ*, this consistency is interesting in light of the much higher difference in viral copy number between these two cell lines. Although a recent study has aimed to clarify these differences (Roberts et al., 2008), it appears that the amount of viral DNA does not correspond linearly to E6 transcription and translation. For HPV16 researchers, subcellular co-localization and quantification with functionally important molecules (such as p53) can be easily implemented. To enhance subcellular features, cells may be prepared and stained directly on chamber slides rather than by gravitation. Finally, immunocytochemistry coupled with quantitative analyses may also be applied to clinical samples to offer an objective and potentially automated test.

The HPV16 E6 oncoprotein remains an important potential diagnostic biomarker for invasive cervical cancer and precancerous lesions (Sellors et al., 2011). The systematic study presented here serves as a foundation for *in situ* antibody-mediated detection of the HPV16 E6 oncoprotein. We confirmed nuclear localization and relative expression levels of E6 in cervical carcinoma cells CaSki and SiHa. Most importantly, we have demonstrated an objective quantitative method for *in situ* protein-level detection of E6. The optimization of standard immunological techniques for HPV16 E6 detection will prove useful in a variety of scenarios, both research and clinical. Our protocol can be implemented in clinical labs, given the routine use of compatible and automated detection systems. Additionally, software could be developed that enables objective and automatic quantification of HPV16 E6 protein in clinical samples.

Materials and methods

Cell culture

All mammalian cell cultures were maintained in a 5% CO_2, 37 °C humidified incubator, with nutrient growth media replaced every second day and routine verification of *Mycoplasma* contamination, as indicated by DAPI-based testing. Both cervical carcinoma cell lines SiHa (ATCC HTB-35) (Friedl et al., 1970) and CaSki (ATCC CRL-1550) (Pattillo et al., 1977) were used as HPV16 E6 positive controls. CaSki was passaged at lower confluence (60% vs. 70% confluence for all other cells) due to suspected loss of E6 expression at high densities. C33A (ATCC HTB-31), a HPV negative cervical carcinoma cell line, was used as a negative carcinoma control (Auersperg, 1964). Cervical carcinoma cell lines SiHa, CaSki, and C33A were all maintained in DMEM (Sigma-Aldrich, Catalog no. D5796) supplemented with 10% FBS (Sigma-Aldrich, Catalog no. F6178) and 1% antibiotic/antimycotic (Invitrogen, Catalog no. 15240-062).

Antibodies

Clones 4C6, 6F4 and 3F8 (Masson et al., 2003; Lagrange et al., 2005) were used as primary monoclonal mouse anti-HPV16 E6 antibodies. Epitopes for 4C6 and 6F4 are known to reside in the N-terminus region of the E6 protein and clone 3F8 has epitopes in the C-terminus region of the E6 protein. All three mAbs were provided by Arbor Vita through communication with Dr. Johannes Schweizer. Antibody stocks were stored at −80 °C while short-term working stocks were stored at 4 °C. For immunocytochemistry, mAb mouse anti-α-tubulin (Sigma, Catalog no. T6074-200UL) diluted 1:500 (4 μg/mL) and mAb rabbit anti-human p53 (DAKO, Catalog no. M3629) at 1:100 (0.24 μg/mL) were used as system controls.

Preparation of microscope slides

Cells were trypsinized, counted, washed with PBS by centrifugation, and resuspended in PBS. Cell suspensions were fixed 1:20 (v/v) in a buffered methanol-based ThinPrep Non-Gyn PreservCyt solution (Hologic, Catalog no. 234004). Fixed suspensions were stored at 4 °C for up to 2 months. For slide preparation, fixed cells were centrifuged and resuspended in PBS at a concentration of 1.0–1.5×10^6 cells/mL. Cytospin 3 Cell Preparation System (Thermo Shandon) was used to prepare microscope slides via gravitation. 100 μL (1.0–1.5×10^5 cells) was added to each cytofunnel chamber. Slides were centrifuged at 1000 RPM for 5 min, air dried for 5 min, and immediately used for immunological detection.

Immunocytochemistry

Several variations of the following procedure were attempted and reported within the results; what follows is an optimized protocol. Hydrophobic barriers were drawn around fixed cell zones using a wax pencil or Dako pen (DAKO, Catalog no. S2002). Cells were then permeabilized for 5 min with 100 μL 0.1% (v/v) Triton X-100 (Cedarlane Labs, Catalog no. T8655) in Tris buffered saline (TBS) with 0.05% (w/v) sodium azide at room temperature in an aluminum foil-covered, humidified tray. The pH of all buffered TBS stocks was regularly monitored to be in the range of 7.4–7.5 at room temperature. All of the following reagent incubations were performed similarly. Permeabilized slides were placed in a rack

and washed 3 times for 3 min in TBS with 0.05% (v/v) Tween 20 (TBS-t) (Thermo Scientific, Catalog no. TA-125-TW) with 10 up-down slide dips for each wash. Endogenous peroxidase activity was blocked by applying 3% hydrogen peroxide for 10 min followed by washing as above. For HPV16 E6 detection, primary mouse mAb 4C6 was applied at 20 μg/mL in 1% bovine serum albumin in TBS with 0.05% sodium azide for 30 min. As a system control, α-tubulin and p53 were also detected. Non-specific secondary antibody binding was controlled by omitting the primary antibody and applying just diluent. Slides were washed as before followed by application of Novocastra Bond Polymer Refine Detection kit post-primary reagent for 10 min (Leica Microsystems, Catalog no. DS9800). Slides were washed as before and incubated with the secondary polymer for 15 min. Colorimetric detection was facilitated by application of 3,3'-diaminobenzidine (DAB+) chromogen (DAKO, Catalog no. K3468). Slides were rinsed and washed 3 times for 3 min with distilled water. If haematoxylin counterstain was desired, slides were dipped in Harris haematoxylin and blued under cold tap water to preferred intensity. Slides were finally rinsed with distilled water and cover slips were applied using 1–2 drops of mounting medium (Vector Laboratories, Catalog no. H-1000). Stained cells were inspected by bright field microscopy (Zeiss Axioskop) and images were digitally captured (QImaging Retiga 1300 RGB camera). HPV16 E6 positive cells were identified by their distinctive dark brown stain while negative cells had no staining.

Quantitative and statistical analyses of DAB staining intensity

CellProfiler, an image processing application, was used to calculate relative intensity of DAB stain in cells (Carpenter et al., 2006; Kamentsky et al., 2011). The following processing pipeline was used: "LoadImages", "ColorToGray", "ImageMath", "ImageMath", "UnmixColors", "ImageMath", "Morph", "Smooth", "IdentifyPrimaryObjects", "ExpandOrShrinkObjects", "OverlayOutlines", "SaveImages", "MeasureObjectIntensity", and "ExportToSpreadsheet". Raw DAB with haematoxylin images at 400× magnification were first loaded. Brown DAB stain was then isolated by dividing a color image into grayscale RGB channels using "ColorToGray" and dividing the red channel (with higher DAB intensity) by the blue channel (lower DAB intensity) using "ImageMath" functions. Cells were identified by first using "UnmixColors" to convert the original color image to grayscale background, DAB, and haematoxylin components. DAB and haematoxylin images were added using "ImageMath" to preserve cell details equally. To allow easier identification of clustered cells, pixels were eroded and smoothed using "Morph" and "Smooth". Individual cells in each image were identified by the following parameters of "IdentifyPrimaryObjects": typical object diameter of 35–150 pixel units with outliers discarded, Otsu Global background three-class thresholding method, weighted variance minimized, threshold correction factor of 0.5 with bounds 0.0–1.0, clumped objects distinguished and divided based on intensity, automatically calculated size of smoothing filter, and minimum allowed distance between clumped objects. Objects were expanded to reclaim eroded area using "ExpandOrShrinkObjects". To verify the accuracy of automated cell identification, outlines were overlaid onto original color images of cells. The intensity of DAB stain in each cell region was measured in relative units (RU) for each image using "MeasureObjectIntensity" and exported to a comma separated value (csv) file for statistical analysis.

Data was determined to meet parametric assumptions based on normality, homogeneity of variance, and independence. Normality was tested using histogram, Q–Q plots, and Shapiro–Wilk 's test. Homogeneity of variance was tested using a Bartlett or Levene test. If parametric assumptions were met, a one-way fixed effects ANOVA was employed with Tukey's HSD post-hoc if appropriate. Non-parametric data was subjected to the Kruskal–Wallis test followed by pairwise Wilcoxon rank-sum post-hoc. Significance level (α) was set, *a priori*, at 0.05. Data are presented as means ± SEM. Statistical analyses were performed using the statistical programming language R (version 2.15.0; R Development Core Team, 2008).

Transcript expression by RT-qPCR

RNA was extracted from cell pellets using the Arcturus PicoPure RNA Isolation kit (Applied Biosystems, Catalog no. KIT0204), with the optional DNase treatment, then reverse transcribed to complementary DNA using the High Capacity cDNA Archive kit (Applied Biosystems, Catalog no. 4322171). Reactions were made up to 60 μL with the following master mix components: 1X RT buffer, 1X primers, 1X dNTPs, multiscribe enzyme, nuclease-free dH_2O, and 30 μL RNA template. No-template controls were run with each reaction. Thermocycler (2720 Thermal Cycler, Applied Biosystems) parameters were as follows: 10 min 25 °C, 120 min 37 °C, and 5 min 85 °C followed by a 4 °C hold. E6 mRNA transcript expression was analyzed by RT-qPCR with the Applied Biosystems 7500 Real-Time PCR System. Reactions consisted of 150 ng of cDNA, 45 μL of TaqMan® Gene Expression Master Mix (Applied Biosystems, Catalog no. 4369016), 4.5 μL of TaqMan® Gene Expression Assay hydrolysis probes (Assay ID: AIAAY0O), and nuclease-free dH_2O to make up volume to 90 μL. Triplicate reaction volumes of 25 μL were loaded into a transparent 96-well plate and analyzed. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was chosen as a suitable reference gene based on previous optimization experiments (DeCarlo et al., 2008). Fold change (relative expression ratio) was calculated by the Livak method ($2^{-\Delta\Delta Ct}$, Livak and Schmittgen, 2001). Statistical analysis was performed as described in the previous section, but with a Welch's two-sample *t*-test for unequal variances.

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