Detection of Human Papillomavirus Type 16/18 DNA in Cervicovaginal Cells by Fluorescence Based In Situ Hybridization and Automated Image Cytometry


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Received for publication May 18, 1993; accepted August 20, 1993

Automatic fluorescence image cytometry (AFIC) is a fast, sensitive, and reliable approach for screening slide-based clinical specimens. In this study, we applied AFIC to identify cancer-associated human papillomavirus (HPV) genotypes 16 and 18 in individual cells of cervical smears using a sensitive fluorescence based in situ hybridization (FISH) assay. HPV sequences were labeled by FISH and the cells imaged using an epi-fluorescence microscope coupled to a low-light color CCD camera. Before application to clinical specimens, AFIC was assessed using fluorescent calibration beads and cervical cancer cell lines containing known numbers of integrated HPV genomes per nucleus. Assessment showed that our AFIC had a linear response, was quantitatively accurate, and had the sensitivity to detect one HPV genome per nucleus. After acquisition of images, computer algorithms identified every cell nucleus (via a fluorescent DNA counterstain) and quantified the FISH signal per nucleus. AFIC was employed to screen 27 patient specimens for HPV 16/18, of which 12 were positive. The HPV status of the specimens positively correlated with the pathological diagnosis, and since AFIC automatically and correctly located every cell, it was possible to directly compare morphology and HPV status in the same cell. In conclusion, the combination of FISH and AFIC is a sensitive and quantitative method to detect high risk HPV sequences in cervical smears. © 1994 Wiley-Liss, Inc.

Key terms: Fluorescent in situ hybridization, cervical smears, human papillomavirus, automated fluorescence image cytometry

Cervical dysplasia and carcinoma are common among women throughout the world. Approximately 500,000 women worldwide are diagnosed annually as having cervical cancer (24) and the mortality rate associated with this malignancy is second to breast cancer (20). In the United States, it is estimated that 13,000 women develop invasive cervical cancer annually (25).

Substantial evidence has accumulated associating specific human papillomaviruses (HPVs) with human anogenital disorders, most notably cervical cancer (3,11,13,35). To date, over 60 different types of HPV have been described, of which about 20 have been associated with anogenital lesions (8). These 20 HPVs have been divided into "high risk" and "low risk" groups based on their association with benign vs. ma-

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*Supported by grants from NIH (CA51323) and the Office of Research on Women's Health, the Whitaker Foundation, and a contract with American Innovision, Inc.

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lignant lesions. Furthermore, the majority of malignant lesions are infected with the “high risk” genotypes HPV-16 or HPV-18 (26,32,34).

The primary screening test used for detecting cervical abnormalities is the Papanicolaou (PAP) smear. However, it has been shown that conventional PAP smears only detect between 50 to 80% of abnormalities subsequently found by histological examination of biopsy specimens (19,28). Therefore, extension of the PAP smear test to include the detection of additional early “risk factors” for progression to cervical cancer, such as the presence of HPV genotypes 16/18, should greatly enhance the diagnostic accuracy of this test.

The most sensitive and reliable assays for detecting HPV are based on nucleic acid hybridization (2,29). Antibodies can be used to detect HPV infection, but they are not genotype specific and detection relies on the presence of viral capsid proteins which are made only at the late stage of HPV replication. Hybridization assays, however, are based on detection of nucleic acids that are present in all stages of HPV replication. Non-radioactive non-fluorescent in situ hybridization (ISH) assays have been reported for HPV detection in smeared cervicovaginal cells (4,6,15,22), but lack the ability to quantitate HPV copy number at the single cell level. Furthermore, detection of HPV infection using these approaches relies on the observer microscopically viewing the entire slide, because automatic screening of specimens labeled in this way has not yet proven possible.

Fluorescent stains enable the screening to be automated, because they allow the reliable and accurate detection of all cells in a specimen using computerized image analysis (30). This is achieved by labeling the cells with spectrally-distinct fluorescent stains, one specific to total DNA and the others specific to the molecular disease markers of interest (7). Since all nucleated cells in PAP smears contain abundant DNA, high contrast images of the stained nuclei can be obtained using epi-fluorescence microscopy. Automatic image analysis algorithms can then correctly detect all the nuclei on each slide from the epi-fluorescence images (17). After detection of nuclei, disease markers present in each nucleus can be automatically quantified from their epi-fluorescence images. This is done by calculating the total fluorescence signal in each region corresponding to a nucleus. Quantification is accurate because the intensity of the emitted fluorescence is directly proportional to the stain concentration in the specimen and because automatic image analysis is highly consistent in defining the boundaries of nuclei (16). Furthermore, automatic image analysis requires minimal manual effort and low levels of labeled markers can be detected using a low-light camera (31), making fluorescent stains at least as sensitive as light-absorbing (colorimetric) stains.

In this study, we report the development of a sensitive FISH assay for detecting HPV 16/18 in cell monolayers prepared from cervical specimens. The advantage of this FISH technique is that it combines the high sensitivity of alkaline phosphatase with the fluorescent precipitated product of alkaline phosphatase red substrate (21). After FISH, the specimens were analyzed using our automatic fluorescence image cytometer (AFIC) (17). The sensitivity and quantitative accuracy of the AFIC was independently assessed using fluorescent calibration beads, and then the AFIC, in combination with FISH, was assessed using cervical carcinoma cell lines containing known numbers of the integrated HPV genome per cell. In addition, using the approach described here, cervical smears could be stained with PAP stains after FISH and relocated using automatic cell relocating algorithm allowing direct correlation of HPV status and morphology at the single cell level.

MATERIALS AND METHODS

Automatic Fluorescence Image Cytometer

The AFIC is shown schematically in Figure 1. It consisted of an inverted microscope (Axiovert 10, Zeiss, Thornwood, NY) equipped for epi-fluorescence and transmitted illumination. The epi-fluorescence light source was a 75 W mercury-xenon arc lamp (Hamamatsu Corp., Middlesex, NY). Hoechst (excitation [ex] = 365 nm, dichroic mirror [dm] at 390 nm and emission [em] = 420 nm long pass), fluorescein (ex = 450–490 nm, dm at 510 nm and em = 520 nm long pass), and rhodamine (ex = 546 nm, dm at 580 nm and em = 590 nm long pass) filter cubes (Omega Optical, Inc., Brattleboro, VT) were used. The objective lenses were an Achroplan with 40× magnification, 1.3 numerical aperture (NA), oil immersion (44 02 55, Zeiss), and a 40× Plan-Neofluar (0.75 NA, dry, 40 03 50, Zeiss). Image acquisition was performed with the Omnichrome 9 imaging system (American Innovation, Inc., San Diego, CA), using a cooled, color, light-integrating charge-coupled device (CCD) camera as the detector. The color camera generated images which were visually similar to the microscopic scene directly seen through the eye pieces. Although use of a color camera reduced the detection efficiency, this could be compensated for by accumulating (integrating) the signal for greater periods of time on the faceplate of the camera. This also proved more precise than using a high gain intensifier tube (31). The output from the camera (red, green, and blue analog signals), was first converted to hue, saturation, and intensity (H, S, and I) and then by use of an analog-to-digital convertor (A in Fig. 1, to digital images of 640×480 pixels. Each pixel corresponded to a 0.3×0.3 μm square on the specimen. The H, S, and I were represented in each pixel by 3 eight-bit numbers. The images were temporarily stored in an IBM compatible 486 AT personal computer which also controlled the image acquisition as well as the stage translation and focus drive of the microscope via a serial port. After acquisition, images were transferred via a local area network to a SPARC-1 workstation.
Figure 1. Schematic diagram of the AFIC. The images from the microscope were recorded by the color camera, but could also be directly viewed on the color monitor. The analog images were digitized by the analog-to-digital converter, A, and were then stored in the frame buffer memory, F. (The I board was for image processing, but was not used in this study.) Physically, the F, I, and A circuit boards are housed in the back plane of the host PC/AT 486 computer. The PC also controlled the stepper motors for stage translation (S1 and S2) and for focusing (S3) via the XYZ controller. Recorded images were transferred via ethernet to the SPARC-1 workstation for analysis and archiving on optical disks.

(Sun Microsystems, Mountain View, CA) for analysis and archiving on optical disks.

Image Acquisition and Analysis

Slides containing a monolayer smear of cervicovaginal cells were placed on the stage of the microscope, the appropriate filter cube and objective lens were selected, and the scene manually focused. The scene was then imaged using a fixed camera gain and black level, but with variable camera faceplate integration times. The output from the camera was filtered through real-time look-up tables, which converted any pixel intensities exceeding the permitted range for each color (0 to 255) to black. This enabled the operator to conveniently see image saturation, if present, and reduce the integration time. For each acquired image, the stage coordinates relative to a fixed origin on the slide were also recorded automatically (see below).

Images were background subtracted and analyzed using algorithms which detect all cell nuclei from images of the DNA counterstain (17). This was done by automatically calculating threshold intensities, and defining regions of the images above the threshold as nuclei and regions below the threshold as the background. The algorithms correctly detected virtually 100% of isolated nuclei (16). Regions classified as nuclei in each image were then used as a mask and mapped over the FISH image of the same microscopic scene, to quantify the corresponding fluorescent HPV signal from each nucleus.

Characterization of the Automatic Fluorescence Image Cytometer

The AFIC was assessed using fluorescent calibration beads to determine its quantitative precision, linearity, and limiting sensitivity.

Quantitative precision. A slide-based monolayer of fluorescent beads (DNA Check, 10 μm in diameter, coefficient of variation [CV] for fluorescence intensity of 1.62% [Coulter Corp., Hialeah, FL]) was prepared as described in Lockett et al., 1992 (18). The beads did not significantly photobleach. Light microscopic scenes of
this specimen, each containing a single isolated bead, were imaged using the 40× Plan-Neofluar dry lens, the fluorescein filter set, and an integration time of nine frames. In order to correct for non-uniformity, a uniform source of fluorescence (Wratten gelatin film, no. 8, CAT 149 5498, Kodak, Rochester, NY) was imaged. Background images for both the beads and gelatin film were recorded using the same integration time as the original bead and film images. Background images were subtracted from the images of the beads and the uniform source, and bead images were corrected for non-uniformity by dividing with the image of the uniform source and multiplying by a constant to keep pixel intensities approximately the same as before. Beads were automatically detected and quantified from these corrected images using the image analysis algorithms on the workstation. The CV, defined here as the ratio of the sample standard deviation (SSD) and mean of the bead intensities, was used as a measure of quantitative precision.

Limiting sensitivity and linearity. The linear response of the AFIC was assessed by imaging the gelatin film. Five successive images of the gelatin film were recorded under the same conditions, except a different neutral density (ND) filter was placed in the excitation light path. The ND filters transmitted 0, 3, 12, 50, and 100% of the light and measurements were made using the Hoechst and rhodamine filter sets. Background images were subtracted from the images of the gelatin and the total fluorescence intensity determined. Linearity was determined by calculating the correlation coefficient (CC) of the best fit straight line through the points on a graph of the percent transmittance of the neutral density filter vs. total bead intensity.

The linearity was assessed in a similar study to the above, except the intensity of the excitation light was kept constant and the integration time of the camera was changed.

Both the linearity and limiting sensitivity of the AFIC were also assessed using Quantum 26 beads (Flow Cytometry Standards Corp., Research Triangle Park, NC; 7.3 μm diameter). These contain five populations of beads each having either 0, 1.1 × 10⁴, 3.5 × 10⁴, 2.3 × 10⁵, or 5.5 × 10⁵ molecules of equivalent soluble fluorescein (MESH₅) on their surface. A separate slide consisting of a monolayer of beads in aqueous medium interposed between a slide and coverslip was made for each population. For each bead population, a microscopic scene containing at least 10 beads was imaged using the fluorescein cube and the Achrostmaph 40× oil lens. The two brightest populations were imaged for an integration time of 0.83 s; the population of 3.5 × 10⁴ MESH was imaged for 0.83, 1.67, and 3.33 s, and the two weakest populations were imaged for 3.33 s. Background images were recorded for each integration time and were subtracted from the respective bead images. Total intensities from the beads were determined by interactively placing a circular mask, slightly larger than the area of the beads, over the location of the beads and adding up the pixel intensities within this mask. The automatic algorithms were not used, because they could not detect the weakest beads. In this study, the linearity was determined using both the CC and the Chi-squared (χ²) test. The limiting sensitivity of the AFIC was taken as the MESH/μm² of the weakest detectable beads.

Automatic Cell Relocation Algorithm

A computer algorithm was written that stored the coordinates of the recorded images (cells) relative to the slide. This enabled the slide to be removed from the stage, stained, and replaced on the stage, thus allowing the same cells that were previously imaged to be relocated. The relocation procedure was accurate to within 0.33 μm.

Collection and Preparation of Cervical Specimens

The collection and use of patient specimens described herein was approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina. Each patient signed a consent form to participate in the study.

Cervicovaginal cells were collected by cytobrush in the dysplasia clinic at the University of North Carolina Hospitals and immediately placed in transport media (Enzo Diagnostics, Inc., Syosset, NY). Cells were first centrifuged at low speed to remove mucus and cell debris and then re-suspended in 1 × phosphate buffered saline (PBS). Monolayers of cells were prepared on 12 mm single well slides (Medical Packaging Corporation, Camarillo, CA), that had been pretreated with 2% 3-aminopropyltriethoxysilane/acetone (Sigma Chemical Co., St. Louis, MO) and dried at 37°C. Cells were subsequently fixed in ethanol/methanol solution (3:1 V/V) for 10 min and stored at −20°C.

Cell Culture

The human cervical carcinoma cell lines, HeLa, SiHa, and C33-A were grown in Eagle minimum essential medium supplemented with non-essential amino acids and 10% fetal calf serum (FCS) (Gibco BRL, Gaithersburg, MD). Ca Ski cells were grown in RPMI medium with 10% FCS (Gibco)(1,10,12,23). Ca Ski cells contain approximately 500 integrated copies of HPV-16 in their genome, whereas HeLa cells have 20–50 copies of integrated HPV-18 and SiHa cells have 1–2 copies of integrated HPV 16 (18). C33-A cells are HPV negative. All cells were washed with 1 × PBS followed by incubation with 10 ml, 0.25% trypsin (Gibco) at 37°C for 10 min. Cells were then removed from the flasks, washed with 1 × PBS and approximately 3 × 10⁵ cells were cyotspun (Cytospin 2, Shandon, Pittsburgh, PA) onto slides, fixed and stored as previously described.
Preparation and Labeling of HPV DNA Probes

*Escherichia coli* (E. coli) containing Bluescribe (Vector Cloning System, San Diego, CA) with HPV 16 and 18 were used to isolate full length HPV 16 and 18 inserts (8.0 kb) (a generous gift of Dr. M. Durst). Plasmid DNA was prepared using the QiaGen Plasmid Kit according to the manufacturer's protocol (QiaGen, Chatsworth, CA). The HPV 16 and 18 specific inserts were then isolated by restriction enzyme digestion, followed by low melting point gel electrophoresis (27). The inserts were cut from the gel, further purified by Pharmacia Sephadex Band Prep Kit (according to the manufacturer's protocol, Pharmacia, Uppsala, Sweden), labeled with digoxigenin 11-UTP by random priming, and purified by ethanol precipitation. The digoxigenin-d-UTP incorporation was quantified according to the manufacturer's protocol (Boehringer Mannheim Corp., Indianapolis, IN). PBR 328 plasmid (Boehringer Mannheim) and human placental DNA (Sigma), which served, respectively, as negative and positive controls, were also labeled with digoxigenin, purified and quantified as mentioned above.

In Situ Hybridization

All incubations were done at room temperature, unless otherwise stated. Cells were placed in 0.2N HCl for 20 min and washed with 1 x SSC (150 mM NaCl, 150 mM Na-citrate, pH 7.0) for 5 min. Slides were then treated with 5 µg/ml proteinase K (Boehringer Mannheim) in proteinase K buffer (10 mM Tris, 2 mM CaCl₂, pH = 8.0) (Sigma) for 15 min at 37°C. After a 5 min wash with 0.5% glycine (Sigma), slides were washed with 1 x SSC. Cells were then placed in 0.1 M triethanolamine and 0.25% acetic anhydride for 15 min and washed with 1 x SSC for 5 min. Pre-hybridization solution was prepared with 4 x SSC, 45% formamide (Fluka, Ronkonkoma, NY), 1 x Denhardt's solution (Sigma) and 100 µg/ml denatured salmon sperm DNA (Sigma). One hundred microliters of pre-hybridization solution was placed on each slide and incubated in an in situ incubator (Triangle Biomedical Sciences, Durham, NC) for 45 min at 42°C. The hybridization solution was similar to the pre-hybridization solution, except that it contained 5% polyethylene glycol (Sigma) and either labeled HPV-16/18 probe, PBR328, or human placental DNA at a final concentration of 50 ng/ml. Three slides from each sample were treated separately with hybridization solution (50 µl) containing one of the three probes. A cover slip was placed over the drop of hybridization solution. Denaturation of the target and probe DNA was carried out simultaneously by placing the slides on a heating block (Baxter, McGaw Park, IL) (95°C, 5 min), followed by placing the slides on ice and covering the edges of the cover slips with rubber cement. Slides were then incubated for 2 h at 42°C. Subsequently, the rubber cement was removed and the slides were placed in a solution of 2 x SSC and 45% formamide (42°C, 20 min) to remove the cover slips. Following consecutive washes in 1 x and 0.1 x SSC solution (50°C, 20 min), cells were blocked using a solution of 100 mM Tris-HCl, 150 mM NaCl, 2% normal sheep serum (Sigma), and 0.3% Triton-X100 (Sigma) at pH = 7.5 for 20 min. Alkaline phosphate conjugated sheep anti-digoxigenin (Fab Fragment) (Boehringer Mannheim) was diluted 1:200 in the above blocking solution. One hundred microliters of this antibody solution was placed on each slide, covered with parafilm, and incubated in a humid chamber (30 min). Slides were then washed sequentially in 100 mM Tris-HCl, 150 mM NaCl (pH = 8.0, 20 min), 100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂ (pH = 9.5, 20 min), and 100 mM Tris-HCl (pH = 8.5) containing 1 mM levamisole (Sigma) for 10 min. Slides were subsequently placed in alkaline phosphatase substrate solution (red substrate kit, Vector Laboratories, Burlingame, CA), prepared according to the manufacturer's instruction, and incubated in the dark (20 min). This particular alkaline phosphatase substrate was chosen because the quantity of its reaction product in the cell nuclei could be controlled by the incubation time, it was visible under both brightfield and fluorescence illumination, it did not photobleach significantly, and the signal remained stable for several months. After several washes in 1 x PBS, 10 µl of mounting media containing Hoechst 33342 (Molecular Probes, Eugene, OR) was added, cover slips placed over cells and sealed to the slides with nail enamel.

Detection and Quantification of HPV 16/18 DNA in Human Cervical Cell Lines

Following FISH, slides of the four cervical carcinoma cell lines were imaged (using the 40 x oil immersion objective and rhodamine filter set) and the resultant images automatically analyzed using the AFIC as described above. The camera integration time was 0.17 s for CaSki cells and 1.0 s for the other cell lines. Consequently, the measurements of the FISH signals from the CaSki cells were multiplied by 5 to compensate for this difference in integration times. Background signals were determined from non-fluorescent (i.e., non-nuclear) regions in the same slide. The precision of the FISH/AFIC combination with respect to quantifying the HPV copy number per nucleus was determined using the same method as for the fluorescent beads.

Application of FISH/AFIC for Quantifying HPV DNA in Cervical Specimens

Labeled cervical cells were automatically analyzed as described above. A specimen was considered HPV 16/18 positive if at least one cell displayed a positive FISH signal above background after a camera integration time of 0.67 s. The HPV status of each specimen
was compared to its cytological or histological diagnosis done by an independent pathologist. For patients where we had pathological diagnoses from more than one specimen, we used the worst diagnosis. The comparison was illustrated using a confusion matrix. In addition, HPV positivity was compared to morphology at the single cell level by utilizing the automatic cell relocation algorithm described above.

RESULTS

Characterization of the AFIC

Quantitative precision. Figure 2a) shows the mean pixel intensities (after background subtraction) of eight DNA check beads measured at different horizontal positions in the image. Clearly, the sensitivity of the AFIC across the imaging area was uneven, but after non-uniformity correction, the uniformity significantly improved (Fig. 2, b). Before correction, the CV of the bead intensities was 4.3%, and decreased to 2.1% after correction. This study was repeated three more times with similar results obtained on each occasion. Since the quoted CV of the beads was 1.62%, it was thus estimated that the error introduced by the AFIC was 1.3%. We conclude from these results that the AFIC has a high degree of precision. The CV for the MESSF of the quantum 26 beads was not quoted by the manufacturer, therefore the beads could not be used to assess quantitative precision.

Linearity and limiting sensitivity. Figure 3A shows the linearity of the AFIC system response measured using the gelatin film. Line "a" is the mean pixel intensity of the image (using the Hoechst cube) plotted against the percent transmission of the ND filter in the excitation light path. Line "b" is the same as line "a," except a rhodamine filter set was used. Line "c" is a plot of intensities vs. camera integration time. These results demonstrate that the AFIC displays a linear response as a function of both percent transmission of the ND filter and camera integration time. Non-linearity was observed when pixel intensities were below 5, due to the black level setting of the camera.

Figure 3B demonstrates the linearity of the AFIC system, measured using the quantum 26 beads. The CC of the best fit straight line was 0.998, and the probability of fit for this line was between 10 and 20% by the $\chi^2$ test ($\chi^2 = 6.5; \nu = 4$). This deviation from true linearity was similar in size to the deviation measured using different transmission filters and the Hoechst cube (Fig. 3A, line a). Beads with $3.5 \times 10^4$ MESSF produced a linear response when measured at different integration times (CC = 0.998 and the probability of fit was between 90 and 95% by the $\chi^2$ test; $\chi^2 = 0.72; \nu =$
Detection and Quantification of HPV 16/18 DNA in Human Cervical Cell Lines

FISH in combination with AIFC was used to detect and quantify HPV 16/18 sequences in CaSkI, HeLa, and SiHa cells. C33-A cells served as a negative control. Figure 4 illustrates the signals generated in these cell lines after hybridization of specific HPV probes to the HPV genome (left column); Hoechst counterstain is shown in the right column. Punctate fluorescent signals were observed in all three cell lines containing HPV DNA, but no signal was observed in the C33-A cells. The FISH signal from the CaSkI nuclei after a 0.17 s integration (Fig. 4A) was comparable to HeLa and SiHa signals (Figs. 4B,C) after 1 s integration. Interestingly, a more punctate signal was observed in HeLa nuclei compared to SiHa nuclei, which represent the larger HPV copy number in HeLa cells vs. SiHa. Only 60% of SiHa cells showed a positive signal, which was probably due to differences in fixation and accessibility of the nuclei to the probe.

The images depicted in Figure 4, together with images of other cells, were analyzed using the AIFC. Figure 5A shows the regions (black) automatically detected as being cell nuclei from the image shown in Figure 4E. These regions were mapped over the images shown in Figure 4A in order to quantify the total FISH signal per nucleus. Figure 5B shows the estimated HPV copy number for each cell line, plotted against the mean FISH signal quantified from the nuclei of each cell line (this experiment was repeated twice and the same result was obtained each time). The best fit straight line through the data points corresponding to the CaSkI, HeLa, and SiHa cells was calculated using least squares regression analysis. The probability of fit for this line was between 20 and 80% by the χ² test (χ² = 1.3, υ = 2), which indicated a linear relationship between HPV copy number and FISH signal per nucleus. It is unclear why the point corresponding to C33-A cells did not lie on this line. However, since the detected signals from SiHa nuclei were significantly greater than the signals from C33-A nuclei, we believe that our FISH/AIFC combination has the sensitivity to detect one HPV genomic copy per nucleus.

The punctate FISH signals in CaSkI cells was also visible using bright field microscopy (Fig. 6A); Figure 6B confirms that the fluorescent signal was the same as that detected by bright field microscopy. Using other substrates for alkaline phosphatase such as NBT-X phosphate (Boehringer Mannheim) we have been able to detect HPV sequences in CaSkI, HeLa, and SiHa cells (data not shown). Furthermore, the specificity of our FISH was demonstrated by a lack of signal when HPV-18 and HPV-16 probes were used in CaSkI and HeLa cells, respectively (data not shown). Similarly, when HPV-16 or HPV-18 probe was used in C33A cells, no signal was observed (Fig. 4D).

Application of FISH/AIFC for Quantifying HPV DNA in Cervical Specimens

Using FISH, HPV 16/18 sequences were detected in 12 out of 27 cervicovaginal specimens collected in the cervical dysplasia clinic at the University of North Carolina Hospital (Table 1). Although specimens were considered positive for HPV 16/18 if only one labeled nucleus was seen, in all the positive specimens at least several nuclei were labeled. Figure 7 shows three typical microscopic scenes containing HPV positive nuclei. The bright-field and fluorescence images are presented, in order to show negative cells that are invisible in the fluorescence images. Three slides from each specimen were treated with the same quantity of digoxigenin labeled PBR-328 (negative control), human genomic DNA (positive control), as well as HPV-16/18 under the same in situ hybridization conditions. All specimens showed positive signal for human DNA probe (Fig. 7D,H) and no signal for PBR328 plasmid (data not shown).

The AIFC was used to quantify the FISH signal in 16 nuclei from clinical specimens and the straight line in Figure 5 was used to estimate the HPV copy number of the nuclei from their measured signals. Nuclei visually negative for HPV had less than 0.25 HPV copies per nucleus based on the straight line, whereas visually HPV positive nuclei were estimated to have, on average, 75 HPV copies per nucleus (SSD = 75 copies). The two HPV positive nuclei shown in Figure 7E and F were estimated to have approximately 150 and 250 copies, respectively.

HPV status was directly correlated with the cytology in the same cell. This was done by utilizing the automatic cell relocating algorithm (see above). After locating all cell nuclei and determination of HPV positivity, the slide was removed from the stage, stained with the Papanicolaou (PAP) stains (according to manufacturer’s protocol, Sigma), placed back on the stage, and individual cells previously recorded were automatically relocated and visually inspected. Figure 8 shows examples of HPV positive and negative cells that were initially examined using phase contrast microscopy (Fig. 8A,C, respectively), PAP-stained, relocated and re-imaged using bright-field microscopy (Fig. 8B,D). We observed that some HPV positive cells had enlarged nuclei compared to negative cells (compare the positive and negative nuclei in Fig. 7C) and some displayed kolkocytotic features. Kolkocytosis, however, was not observed in all positive cells as has been reported by other investigators (15).

Table 1 compares the HPV and pathological status of clinical specimens determined using FISH and manual cytological screening from the same patients. The severity of the dysplasia correlated positively with the presence of HPV. In particular, we note that all patients diagnosed with carcinoma intraepithelial neo-
plasia (CIN) grade 3 (a potential premalignant lesion) were HPV 16/18 positive, while only one patient with benign abnormalities was HPV 16/18 positive.

DISCUSSION

Genital HPV is believed to be a major contributing factor in the pathogenesis of cervical intraepithelial neoplasia (CIN) as well as cervical carcinoma (14,33). Therefore, a fast, sensitive, and reliable method to detect HPV in cervical specimens would be a valuable addition to the clinical diagnosis of these diseases. Although most nucleic acid hybridization assays are sensitive and reliable when detecting HPV, the ISH assay has the additional advantage that it localizes the HPV signal within morphologically preserved cells. Thus, HPV can be detected in the same specimens used for standard cytological screening and HPV infection and cell morphology can be directly compared at the individual cell level. For example in this study, we observed that some infected cells, in contrast to uninfected cells, were koilocytotic (also observed by other investigators) (15), and had enlarged nuclei. Comparisons of disease-related parameters at the single cell level such as koilocytosis, presence of high risk HPV genome, presence of HPV oncoproteins (E6, E7), and overexpression of oncogenes can provide valuable data. Comparisons of this sort should lead to an improved etiological and epidemiological understanding of cervical cancer, which should in turn lead to earlier more precise clinical diagnoses.

The FISH technique described here, to the best of our knowledge, is the first report on detecting HPV sequences in cervical smears using a fluorescent substrate. Our use of digoxigenin labeled probes instead of biotin minimized background staining from endogenous biotin. Our technique was able to detect one HPV copy per cell (at least in 60% of SiHa cells), and thus is close to the maximal sensitivity needed for this application. In addition, our preliminary results using tailed labeled oligonucleotide probes indicates that HPV-16 genome can be detected in 100% of SiHa cells. Due to differences between clinical samples and cervical cell lines, accurate quantitative assessment of HPV copy number in cervical smears is not possible at present. However, positive control probes showed equal staining and penetration in all cells of cervical smears. In addition, cells in cervical specimens which were positive for HPV by FISH were also positive for HPV by PCR. Our FISH was also highly specific, because the HPV 16 and 18 probes only hybridized to the homologous target in the human cervical cell lines (Fig. 4).
Fig. 6 (at left). Localization of HPV-16 DNA in CaSki cells by FISH. A: bright field image. B: fluorescence image. (Magnification: ×625.)

Fig. 7 (facing page). Detection of HPV-16, 18 sequences in cervicovaginal cells using FISH. A, B, C: Scenes imaged using bright-field microscopy to visualize both the HPV positive and negative cells. The positive cells have red nuclei. D: Bright-field image of cells labeled with the positive control probe (human genomic DNA). E, F, G: The same microscopic scenes as A, B, and C, but imaged using epi-fluorescence microscopy. H: The same scene as D imaged using epi-fluorescence microscopy.

Fig. 8 (below). Relocating FISH labeled cervicovaginal cells after staining with the PAP stain. A: Phase-contrast micrograph of an HPV 16/18 positive cervical cell. B: The same cell relocated after PAP staining and imaged using bright-field microscopy. C: Phase-contrast micrograph of three HPV negative cells. D: The same cells relocated after PAP staining and imaged using bright-field microscopy. (Magnification: A, B × 925; C, D × 250.)
diagnostic information (e.g., the degree of cellular differentiation). In parallel, we intend to improve the localization of the FISH signals to sites of HPV integration into the host cell genome. This may enable us to distinguish between cells in patient specimens containing only integrated HPV, only episomal HPV, or a mixture of both types (5). We will attempt to use shorter DNA probes such as synthetic oligonucleotides or single-stranded DNA probes generated by PCR. At present, the positive control probe is complementary to total human genomic DNA. We intend to substitute this with a probe specific to an endogenous gene, that is approximately the same length as the HPV genome and is not altered by cervical carcinogenesis. This probe would help us to evaluate the sensitivity of our FISH in cervical smears. Later, we plan to extend the protocol to two color FISH so that two HPV genotypes can be simultaneously detected. Along similar lines, we will investigate the possibility of making oligonucleotide probes that will hybridize to several of the genotypes within the “high risk” group but not to low risk HPVs.

Although we have demonstrated, in principle, that the AFIC will operate entirely automatically, three additions are required to make it automatic in practice. These are the addition of a computer-controlled shutter in the excitation light path, a computer-controlled filter cube changer, and automatic focusing (9).

The goal of this study was to establish a fast, convenient, sensitive, semi-quantitative, and reliable technique for detecting HPV in intact cervicovaginal smears. To achieve this goal, we developed a sensitive FISH assay that combines the high sensitivity of alkaline phosphatase with the fluorescent product of fast red substrate. Furthermore, we combined our FISH with AFIC to accurately detect and quantify HPV signals. Quantification of the fluorescent signal in individual cervical cells can be used to determine severity and extent of HPV infection in individual patients. This, in turn, may be correlated to morphological changes of the cells, to the incidence of HPV integration into the genome, and to the progression of cervical disease.

ACKNOWLEDGMENTS

The authors thank Dr. M. Durst for the generous gift of the HPV 16 and 18 inserts, and Mrs. Sandra Atkins for typing this manuscript.

LITERATURE CITED

4. Choi YJ: Detection of human papillomavirus DNA on routine