Human Papillomavirus-Related Cellular Changes Measured by Cytometric Analysis of DNA Ploidy and Chromatin Texture

Michael E. Scheurer,1 Martial Guillaud,2 Guillermo Tortolero-Luna,3,4 Calum McAulay,2 Michele Follen,4,5 and Karen Adler-Storthz6

1Department of Epidemiology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas
2Department of Cancer Imaging, BC Cancer Research Centre, The British Columbia Cancer Agency, Vancouver, British Columbia, Canada
3Division of Occupational and Environmental Health, The University of Texas School of Public Health, Houston, Texas
4Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas
5Biomedical Engineering Center, The University of Texas M. D. Anderson Cancer Center, Houston, Texas
6Department of Diagnostic Sciences, The University of Texas Dental Branch, Houston, Texas

Background: Image cytometry has provided two highly sensitive markers for the identification of the malignant potential of squamous lesions. Aneuploidy and chromatin texture have been investigated as quantitative measures of nuclear damage in premalignant lesions and carcinoma. Real-time PCR methods have evolved to yield highly specific measurements of mRNA expression in very sparse cellular samples.

Methods: Human papillomavirus (HPV) 16 and 18 E7 mRNA expression was measured using quantitative RT-PCR. DNA index and chromatin measures were taken from image cytology samples. The chromatin features, through discriminant analysis, were aggregated into a score, and both measurements were related to mRNA expression.

Results: mRNA level and DNA index show an increasing trend over increasing histological grades. However, DNA index and chromatin score were not correlated to mRNA levels in these samples. Chromatin score differed by mRNA type found with HPV 18 infected samples having a higher score than those with HPV 16. Samples infected with HPV 16 and HPV 18 had even higher chromatin scores.

Conclusions: DNA index and chromatin score were not directly correlated with mRNA levels. However both mRNA and DNA index were related to histological grade, and chromatin score was associated with HPV type. Therefore, DNA index and mRNA levels could be independent predictors of cervical dysplasia, and chromatin score could be related to the viral integration process in cells infected with HPV 18 or dual infections.

Key terms: human papillomavirus; image cytometry; aneuploidy; chromatin; viral RNA; quantitative PCR

Human papillomavirus (HPV) is recognized as a necessary causal agent of cervical carcinoma; however, HPV infection alone does not guarantee progression to cancer. While well-executed screening programs utilizing the Pap smear have reduced the incidence of and mortality from cervical cancer by ~85% (1), the sensitivity of classical cytology to detect high-grade lesions ranges from 20% to 76% (2). This makes the early detection of the malignant potential of borderline lesions difficult. Of the few women who do develop low-grade squamous intraepithelial lesions (LSIL), 11% will progress to high-grade squamous intraepithelial lesions (HSIL), and an equal fraction of those high-grade lesions will progress to invasive carcinoma (3). The adoption of liquid-based cytology methods has greatly improved upon the sensitivity of classical cytology, showing a sensitivity of 84% (2). However, several adjunct diagnostic methods, including DNA image cytometry, show great promise for increased accuracy and reproducibility of results (reviewed in Bocking and Nguyen) (4).

Grant sponsor: National Cancer Institute; Grant numbers: P01 CA82710, R25 CA57730.
*Correspondence to: Karen Adler-Storthz, Department of Diagnostic Sciences, UT Dental Branch at Houston, 6516 MD Anderson Blvd, DBB 4.133, Houston, TX 77030, USA.
E-mail: karen.a.storthz@uth.tmc.edu
Received 19 July 2006; Revision 11 October 2006; Accepted 17 November 2006
Published online 4 January 2007 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.b.20173

© 2007 Clinical Cytometry Society
Chromosomal aneuploidy (aberrations in number from normal diploid) is a key event in carcinogenesis and is found in most cervical cancer cases and some precancerous lesions (5,6). Quantitative analysis of DNA aneuploidy, a measurement of chromosomal aneuploidy, can serve as a marker of the cytogenetic alterations resulting from genetic instability (7). In addition to quantifying DNA ploidy, cytometric measures of chromatin texture also provide useful information concerning the malignant potential of a cell independent of nuclear atypia (8).

The HPV oncogenes E6 and E7 are responsible for the ability of the virus to transform cells. Uncontrolled expression of these two genes results from the integration of the viral genome into the host cellular DNA. The E7 oncogene, in particular, exerts its transforming function by interrupting cell differentiation and inducing DNA synthesis (9). It accomplishes this by interacting with the cellular tumor suppressor gene product, retinoblastoma protein (pRB) (10,11). It can also induce abnormal centrosome duplication (12,13) and chromatin condensation (14) possibly leading to chromosome instability.

In this study, real-time PCR was used to quantitate mRNA of the E7 oncogene from HPV 16 and HPV 18 isolated from cervical samples of women with abnormal pap smears exhibiting normal histology and women with intraepithelial lesions. Quantitative cytology was performed on pap smears from the same women. DNA ploidy and chromatin texture were correlated with mRNA expression to determine the relationship between viral oncogene expression and cytogenetic effects in the cell.

**MATERIALS AND METHODS**

**Study Population**

The present study drew from a larger study designed to evaluate emerging optical technologies for cervical neoplasia. The study population of the parent study consisted of women with abnormal pap test results attending the colposcopy clinics at The University of Texas M. D. Anderson Cancer Center, Lyndon B. Johnson General Hospital, and Memorial Hermann Hospital in Houston, Texas and the British Columbia Cancer Agency in Vancouver, British Columbia, Canada between October 2000 and July 2003. IRB approval was received from each institution involved in the study, and participants gave written informed consent prior to being enrolled in the study.

For the present study, all study participants were identified as positive for HPV16 and/or HPV18 DNA by PCR. Cases were women classified by biopsy as having squamous intraepithelial lesions (SIL). Those with CIN I or HPV-associated changes (HAC) were classified as LSIL, those with CIN II or CIN III were classified as HSIL. Noncases were women classified with a normal biopsy or as having atypical squamous cells of undetermined significance (ASCUS).

**Data and Specimen Collection**

A demographic and epidemiologic risk factor questionnaire, administered at study enrollment, was used here to describe the study population. Study physicians collected clinical specimens using an endocervical cytobrush for a variety of laboratory procedures including quantitative cyto- and histopathology, HPV typing, and HPV DNA and mRNA analyses. A biopsy specimen was also taken from each participant for histopathologic confirmation of disease. DNA specimens were placed in 250 μL of PBS with 0.02% sodium azide immediately following cytologic sampling and stored at ~80°C until extracted, ~1 month after collection. RNA specimens were placed in 250 μL of lysis solution (Ambion, Austin, TX) immediately following cytologic sampling and stored at ~80°C until extracted; length of storage varied from 5 months to 3 years after collection.

**Nucleic Acid Isolation and Quantitation**

Details of HPV DNA and HPV E7 mRNA quantitation have been reported elsewhere (15). Briefly, viral DNA was extracted from cervical cytobrush specimens using a commercially available kit (Qiagen DNA Mini Kit, Qiagen, Valencia, CA) and analyzed for HPV consensus by PCR for HPV 16 and HPV 18 type-specific probes by 32P hybridization. Total mRNA was extracted from cervical cytobrush specimens using a commercially available kit (RNAqueous, Ambion, Austin, TX) and reverse transcribed into cDNA (RETROscript, Ambion, Austin, TX). An equal quantity of cDNA (20 ng) from each sample, measured by spectrophotometry, was analyzed by real-time PCR for quantitation of HPV 16 and HPV 18 E7 oncogene expression according to the type-specific DNA results. For example, if the sample was positive for HPV16 DNA and negative for HPV 18 DNA, RT-PCR was done using only the 16 primers. Absolute quantitative real-time PCR with the SYBR Green I (Molecular Probes, Eugene, OR) fluorescent intercalation dye was used to quantitate HPV E7 using a set of plasmids carrying the E7 gene for calculation of a standard curve (16).

**Control Cell Lines**

Positive controls consisted of the cervical cancer cell lines HeLa (HPV 18) and SiHa (HPV 16) purchased from American Type Culture Collection (Manassas, VA). Both cell lines were grown in Eagle’s minimal essential medium (EMEM) with 10% fetal bovine serum and penicillin–streptomycin, incubated at 37°C in a 5% CO2 atmosphere, and harvested directly in lysis buffer (Ambion, Austin, TX). Messenger RNA was isolated, and cDNA was generated in a manner similar to the cervical specimens, described above.

**Pathology Review and Diagnosis**

Details of the pathology review for the parent study have been reported elsewhere (17). Briefly, a first pathology review is done for each biopsy specimen by one of the gynecologic pathologists on clinical duty at each institution. A second blinded review is done by one of the study pathologists. Where discrepancies arise between the two readings, the slide is evaluated a third...
time by the study pathologist to arrive at the final consensus diagnosis. Liquid-based cytology was also reviewed by the cytopathologist on clinical duty and the study pathologist at each institution. Discrepancies were resolved by a third review. For the purpose of the current analysis, in cases where the cytological diagnosis did not agree with the histological diagnosis, the worse of the two was taken as the consensus. For instance, if the cytological diagnosis was ASCUS and the histological diagnosis was normal, then the participant’s consensus diagnosis was ASCUS.

**Quantitative Cytology**

Two cytologic specimens were collected and stored temporarily at 4°C in vials containing liquid-based fixative. The first sample, a Papanicolaou smear, was used to make a clinical diagnosis. The remaining sample was used for a liquid-based preparation (ThinPrep) of up to four slides. The first of the ThinPrep slides was stained using a Feulgen-based preparation, which is stoichiometric for DNA. This slide was used for quantitative analysis, while the remaining unstained slides were stored for future studies.

Digital images of the Feulgen-stained nuclei were collected using the fully-automated Cyto-Savant imaging system (Cancer Imaging, Vancouver, BC, Canada). This system includes a 12-bit double-corrected sampling Micro-Imager 1400 digital camera (pixels, 6.8 μm²). Feulgen-stained nuclei were measured with a monochromatic light at a wavelength of 600 nm using a 20 × 0.75 numerical aperture plan Apo objective lens. All cytometric measures were performed in a central laboratory in Vancouver, BC, Canada. Each slide is automatically loaded onto the microscope stage in batches of 50 slides and exhaustively scanned for abnormal (nondiploid) cells. The number of objects collected ranges from 2,000 to 20,000 depending on the cellularity of the specimen, and a maximum of 2,000 diploid cells per slide is collected.

The digital images of the objects were sorted using a multistep classification system. The first level of cell classification occurred automatically during the image acquisition process. Binary decision trees, based on training datasets from previous studies, were used to sort cell objects into normal, abnormal, and “junk” groups mostly based on DNA content, shape, and size features. The normal cells were used as the diploid standard for normalization, which is also performed automatically during scanning. “Junk” (debris, overlapping cells, etc.) was separated from cells, and cells with a DNA index greater than 1.5 (nondiploid) were placed into the abnormal category. DNA index is the normalized DNA amount of each cell; a value of 1 indicates a diploid specimen.

After scanning, the images were reviewed and further classified in a three-step process. The first two steps, performed by a cytotechnologist, involved verifying the normalization, removing remaining “junk” from the diploid cell group, and microscopically reviewing the nondiploid cell group to remove any obvious “junk”. The final step was performed by a cytopathologist who reviewed the nondiploid cells microscopically and used visual criteria to further classify them. The resulting subcategories of “nondiploid” were “true abnormal”, “normal nondiploid cells”, and reactive cells. To date, the majority of the “nondiploid” cell group consists of reactive cells, which are characterized by large round nuclei with moderately coarse chromatin texture. While they are generally increased in size, the overall nuclear/cytoplasmic ratio is maintained, and they are often found in sheets.

A two-level quality control procedure has been implemented for automatic quantitative cytology screenings. First, every batch of slides that is Feulgen stained includes a quality assurance (QA) slide containing cultured human tumor cells (HL60). One of these slides was scanned with every batch of cervical slides as a quality control of the staining process. To verify the scanning process, a specially manufactured reference slide was used which contains multiple objects with the same known optical density and size. This slide was scanned three times and results in three files, each containing 200 objects from the same area. This process was performed at the beginning and at the end of every batch of slides scanned and allows strict control on the scanning process consistency.

Nuclear feature measurements were performed on digital cell images according to computations, as described in detail elsewhere (18). One-hundred six features were measured for each cell image (Table 1). These may be divided into three categories:

1. Morphological features: These features describe the nuclear size, shape, and boundary irregularities. They serve to characterize increases in size of nuclei, as well as the increasingly severe distortions in nuclear shape that are associated with aneuploid progression of SIL. These changes may take the form of increasingly asymmetrical nuclear shapes. Abrupt angular variations in the nuclear contour, as well as any appearance of prominences or raggedness of nuclear borders, may be observed and characterized.

2. DNA content: The photometric features estimate the absolute intensity, optical density levels of the nucleus, and the intensity distribution characteristics. The Feulgen–Thionin stain is stoichiometric for DNA and behaves according to the Beer–Lambert law of absorption so that the integrated optical density (IOD), integrated over the area of the cell, is proportional to the DNA content of the cell. The DNA index for each cell is defined as the IOD divided by a normalization constant, the IOD of internal diploid controls. DNA index is used to determine the ploidy status of the specimen. The IOD of the diploid control population is used for discrete texture measurements in order to compensate for differences in stain intensity.

3. Texture features: Texture features describe the variations in optical intensity over the nuclear image and present an objective and quantitative method for charac-
terization of changes in chromatin appearance. Discrete texture features are based on threshold segmentation of the object into regions of low, medium, and high optical density. The thresholds are scaled to the sample staining intensity as represented by the IODnorm value determined from the reference population. Markovian texture features characterize grey level correlation between adjacent pixels in the image. Non-Markovian texture features describe the texture in terms of local maxima and minima of grey level differences in the object. Fractal texture features describe the texture using local differences integrated over the object at multiple resolutions. This measures the complexity and contrast within specific chromatin patterns. Run-length texture features describe chromatin distribution in terms of the length of consecutive pixels with the same compressed grey level value along different orientations (0°, 45°, 90°, 135°). This measure characterizes the extent and distribution of regions of homogeneous chromatin condensation. In order to make run-length features rotationally invariant, for each run-length feature we only use the mean and standard deviation over the four directions.

We were most interested in measuring two features: DNA index and chromatin texture. The DNA index was calculated over the entire cell population on each slide. The chromatin texture feature was calculated as a multi-component score for only the normal diploid cell population on each slide. The chromatin texture feature was calculated as a multi-component score for only the normal diploid cell population. We defined integrated HPV-associated changes (iHAC) as changes in chromatin features induced by expression of the E7 protein. Under the hypothesis that textural features measured by cytometry can discriminate those cells with E7 expression, we generated an individual cell score, which was a phenotypic measure of the degree and intensity of deviation of an epithelial cell with integrated HPV DNA. On the two extremes of this cellular scale, we first defined a group of normal diploid cells (DNA index between 0.9–1.1) selected from cervical specimens from the HPV DNA-positive and RNA-negative group of women and then a group of cells from cervical specimens from HPV DNA-positive and RNA-positive group. By training the system on these two sets of phenotypically identical cells, we can be certain that the changes we detect are not due to some underlying process, in this case HPV infection. A stepwise linear discriminant analysis was performed between these two groups to select the most relevant cytometric features. These features (Table 1) included: fractal_dimen, contrast, lowDNAcomp, fractal1_area, cl_prommence, avg_short_runs, low vs med_DNA, hiDNAamnt, den_dark_spot, and correlation. Correct classification by the discriminant analysis was 59%. This is similar to our findings for other samples and tissue types (lung and other cervix samples). A canonical score, or iHAC score, based on the discriminant analysis was then calculated for each individual cell in each specimen. The threshold that maximized the discrimination of the training set was used as the cutoff value for an iHAC-positive cell in the test set. The chromatin changes were then reported as percentage of cells for which the iHAC score was below the threshold. All statistical analyses for the generation of the iHAC score were performed with the STATISTICA package (StatSoft, Tulsa, OK).

**Statistical Analyses**

Demographic characteristics were summarized, and differences by disease status were determined by the \( \chi^2 \) test for categorical variables or by analysis of variance (ANOVA) for continuous variables. Copy number of mRNA for each HPV type was transformed using the natural logarithm to minimize positive skewness. ANOVA
was used to discern differences in the mean DNA index and chromatin features (chromatin score) among the types of mRNA detected in the samples. The Kruskal–Wallis nonparametric ANOVA method was used to discern differences in the mean mRNA levels, DNA index, and iHAC score among the categories of dysplasia (normal, LSIL, HSIL). Cuzick’s nonparametric test for trend was used to assess the presence of a trend in mRNA levels, DNA index, and iHAC score across the levels of dysplasia. Dysplasia was scored for ranking as 1, 2, and 3 for normal, LSIL, and HSIL, respectively. All statistical analyses were performed using Intercooled STATA 8.2 (Stata Corp., College Station, TX).

RESULTS

When the current study began, there were 1,477 cervical specimens available for mRNA analysis. Of these, 940 (64%) were negative for dysplasia, 281 (19%) had LSIL, 228 (15%) had HSIL, and 28 (2%) were missing a definitive histologic diagnosis. DNA analysis by PCR was completed for 870 participants; of those, 378 (43%) were positive for HPV 16 and/or HPV 18 DNA. Thirty samples were lost for various reasons during the mRNA extraction process. There were no differences by histology of these samples (data not presented). Of the 348 samples remaining for RNA quantification, two were excluded because the patients were identified to have squamous cell carcinoma. Therefore, 346 participants were identified for mRNA quantitation. Of those, 46% were positive for HPV 16 DNA alone, 29% were positive for HPV 18 DNA alone, and 29% were positive for both HPV 16 and HPV 18 DNA (data not presented). Of these, six were excluded from analysis due to missing histology data and six were excluded for missing DNA index, leaving 334 for analysis of mRNA level and cytometric features by histological grade.

Histological analysis of biopsies from each patient revealed 217 (65%) subjects were negative for dysplasia, 74 (22%) had LSIL, and 43 (13%) had HSIL. Demographic characteristics did not differ significantly by the type of HPV mRNA isolated (Table 2). Women with HPV 16 mRNA were slightly younger (mean age 39 years) compared to other groups. There were no significant differences between the groups based on race, smoking status, education level, marital status, annual income, or study site.

Of the 334 patients included in this analysis, 103 (31%) yielded a positive result for either HPV 16 or HPV 18 mRNA (Table 3). The prevalence by histological grade varied slightly, 27% for normals, 41% for LSIL, and 35% for HSIL. However, these differences were not statistically significant ($P = 0.070$). Mean mRNA levels differed significantly between histological categories; this significance appears to be due to higher levels among the HPV 18-positive samples. DNA index showed a significant upward trend ($P < 0.001$) from 1.03 to 1.05 to 1.16 for normal, LSIL, and HSIL, respectively. There was a significant difference in DNA index by histological grade for both HPV 16 and HPV 18; however, the trend was not significant for HPV 18.

### Table 2

Demographic Characteristics of Study Population by Type of mRNA Found

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (n = 334)</th>
<th>None (n = 231)</th>
<th>HPV16 (n = 53)</th>
<th>HPV18 (n = 45)</th>
<th>HPV16/18 (n = 5)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Race White</td>
<td>204 (62)</td>
<td>139 (61)</td>
<td>36 (68)</td>
<td>26 (58)</td>
<td>3 (60)</td>
<td>0.747</td>
</tr>
<tr>
<td>Black</td>
<td>45 (14)</td>
<td>31 (14)</td>
<td>5 (9)</td>
<td>8 (18)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>57 (17)</td>
<td>37 (16)</td>
<td>11 (21)</td>
<td>8 (18)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>25 (7)</td>
<td>21 (9)</td>
<td>1 (2)</td>
<td>3 (7)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Age [Mean (SD)]</td>
<td>42 (13.1)</td>
<td>42 (13.0)</td>
<td>39 (12.5)</td>
<td>43 (14.2)</td>
<td>45 (14.2)</td>
<td>0.423</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.862</td>
</tr>
<tr>
<td>Ever</td>
<td>130 (39)</td>
<td>92 (40)</td>
<td>18 (34)</td>
<td>18 (40)</td>
<td>2 (40)</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>201 (61)</td>
<td>136 (60)</td>
<td>35 (66)</td>
<td>27 (60)</td>
<td>3 (60)</td>
<td></td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.779</td>
</tr>
<tr>
<td>&lt;HS</td>
<td>14 (4)</td>
<td>11 (5)</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>HS/some college</td>
<td>177 (53)</td>
<td>124 (54)</td>
<td>27 (51)</td>
<td>23 (51)</td>
<td>3 (60)</td>
<td></td>
</tr>
<tr>
<td>College degree</td>
<td>87 (26)</td>
<td>57 (25)</td>
<td>18 (34)</td>
<td>11 (24)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>Advanced degree</td>
<td>38 (11)</td>
<td>26 (11)</td>
<td>5 (9)</td>
<td>7 (16)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Refuse/don’t know</td>
<td>15 (5)</td>
<td>10 (4)</td>
<td>2 (4)</td>
<td>3 (7)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.275</td>
</tr>
<tr>
<td>Married</td>
<td>186 (56)</td>
<td>137 (59)</td>
<td>44 (45)</td>
<td>21 (47)</td>
<td>4 (80)</td>
<td></td>
</tr>
<tr>
<td>Not married</td>
<td>145 (44)</td>
<td>91 (39)</td>
<td>29 (55)</td>
<td>24 (53)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>Annual income $</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.554</td>
</tr>
<tr>
<td>$&lt;20,000–49,999</td>
<td>110 (33)</td>
<td>72 (31)</td>
<td>17 (32)</td>
<td>18 (40%)</td>
<td>3 (60)</td>
<td></td>
</tr>
<tr>
<td>$50,000+</td>
<td>139 (42)</td>
<td>104 (46)</td>
<td>19 (36)</td>
<td>15 (33%)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>Refuse/don’t know</td>
<td>35 (11)</td>
<td>22 (10)</td>
<td>8 (15)</td>
<td>4 (9%)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>Study site</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.661</td>
</tr>
<tr>
<td>Houston</td>
<td>306 (92)</td>
<td>210 (91)</td>
<td>48 (91)</td>
<td>43 (96)</td>
<td>5 (100)</td>
<td></td>
</tr>
<tr>
<td>Vancouver</td>
<td>28 (8)</td>
<td>21 (9)</td>
<td>5 (9)</td>
<td>2 (4)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Values within parentheses indicate percentages.
DNA index did not differ across the groups for type of mRNA measured (Table 4). The mean DNA index was in the diploid range (0.9–1.1) for all groups. However, the chromatin score differed significantly according to the type of mRNA measured. The greatest change was seen in dually infected samples with a mean score of 52. The score decreased for singly infected samples, with HPV 18-infected samples having a higher mean score than those with HPV 16.

The iHAC score was not significantly different between levels of histology (Table 3); however, scores were consistently higher for LSIL specimens independent of the HPV type found. There was a significant difference (P = 0.04) in iHAC score by type of HPV RNA detected in the sample (Table 4). In addition, there was a significant trend (P = 0.01) in iHAC score with increasing severity of HPV RNA present. For instance, there was a higher iHAC score for HPV 18 (42.5) than HPV 16 (40.1) and even higher for dually infected specimens (51.8).

**DISCUSSION**

In the present study, we show that DNA index measured by image cytometry increases with increasing histologic grade. We also show that mean HPV 18 E7 RNA levels increase with increasing histologic grade. In addition, the chromatin texture score that we created (iHAC) increases with increasing severity of HPV type.

The interest in using quantitative pathological markers for detecting abnormal cervical cytology stems from the desire to increase the sensitivity and specificity of classical cytology, especially for borderline and difficult to detect lesions. Palcic et al. estimated that only 8% of the 60 million Pap smears done in the USA in a year are “not negative”, meaning ASCUS or higher (19). Only 15% of these will have a repeat positive on follow-up, and of those that are positive on follow-up, 3% possibly have malignant potential. These estimates leave an excess of about 4 million false-positive women who undergo diagnostic and treatment procedures unnecessarily.

Measurements of DNA ploidy and chromatin texture have been suggested as useful and easily quantifiable markers for the progressive potential of a lesion. Skyldberg et al. showed that increasing grades of CIN are paralleled by the increasing number of centrosomes and aneuploidy (20). Bollmann et al. recently showed the existence of aneuploidy in ASCUS infected with high-risk HPV (21). Duensing et al. have shown that HPV E6 and E7 can independently induce aneuploidy by two separate pathways (13,22). By inactivating p53, E6 causes an accumulation of abnormal centrosome number that occurs in addition to nuclear atypia, and the degradation of pRb by E7 induces abnormal centrosome duplication leading to chromosome missegregation and aneuploidy. In addition, several investigators have shown that the progression from low-grade to high-grade lesions to invasive carcinoma is accompanied by aneuploidy (both gains and losses) of several chromosomes, in particular chromosomes 1, 7, and X (8,23–25).

Changes in nuclear texture (chromatin features) have been shown in carcinomas from several different tissues, including colon, breast, larynx, and cervix (26–30). These features are present not only in malignant cells, but also in adjacent histologically normal cells. Dreyer et al. showed that fractal texture features (chromatin measurements) can be used to assess malignancy in tissue sections as an alternative to DNA ploidy (31). More recently, our group showed that subvisual changes in chromatin texture can be detected by quantitative histopathology in cytologically normal as well as abnormal samples (17). Looking at prognosis of invasive carci-

<table>
<thead>
<tr>
<th>Mean mRNA, DNA Index, and iHAC Score by HPV Type and Histology</th>
<th>Total</th>
<th>Normal</th>
<th>LSIL</th>
<th>HSIL</th>
<th>P-value</th>
<th>P_trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>334</td>
<td>217</td>
<td>74</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive for mRNA</td>
<td>103</td>
<td>58</td>
<td>30</td>
<td>15</td>
<td>0.07</td>
<td>0.32</td>
</tr>
<tr>
<td>Mean ln mRNA (SD)</td>
<td>2.5 (2.6)</td>
<td>2.0 (2.1)</td>
<td>2.5 (2.6)</td>
<td>4.2 (3.7)</td>
<td>0.007</td>
<td>0.003</td>
</tr>
<tr>
<td>HPV 18</td>
<td>2.6 (3.3)</td>
<td>1.5 (2.0)</td>
<td>3.1 (3.4)</td>
<td>7.4 (5.3)</td>
<td>0.019</td>
<td>0.007</td>
</tr>
<tr>
<td>HPV 16</td>
<td>2.2 (1.6)</td>
<td>2.3 (2.0)</td>
<td>1.6 (0.9)</td>
<td>2.7 (1.1)</td>
<td>0.14</td>
<td>0.32</td>
</tr>
<tr>
<td>Mean DNA index (SD)</td>
<td>1.05 (0.11)</td>
<td>1.03 (0.08)</td>
<td>1.05 (0.10)</td>
<td>1.16 (0.18)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HPV 18</td>
<td>1.03 (0.08)</td>
<td>1.02 (0.04)</td>
<td>1.01 (0.03)</td>
<td>1.19 (0.14)</td>
<td>0.003</td>
<td>0.064</td>
</tr>
<tr>
<td>HPV 16</td>
<td>1.06 (0.15)</td>
<td>1.01 (0.01)</td>
<td>1.06 (0.13)</td>
<td>1.24 (0.25)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean iHAC score (SD)</td>
<td>39.1 (14.3)</td>
<td>38.5 (14.4)</td>
<td>41.8 (14.4)</td>
<td>37.2 (13.7)</td>
<td>0.16</td>
<td>0.49</td>
</tr>
<tr>
<td>HPV 18</td>
<td>39.7 (14.4)</td>
<td>38.5 (13.9)</td>
<td>43.5 (15.1)</td>
<td>39.4 (16.9)</td>
<td>0.52</td>
<td>0.37</td>
</tr>
<tr>
<td>HPV 16</td>
<td>38.9 (14.4)</td>
<td>38.6 (14.8)</td>
<td>40.8 (14.3)</td>
<td>37.1 (12.7)</td>
<td>0.18</td>
<td>0.48</td>
</tr>
</tbody>
</table>

**Table 4**

<table>
<thead>
<tr>
<th>Mean (SD) of DNA Index and iHAC Score by Type of mRNA Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA index</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Total (n = 334)</td>
</tr>
<tr>
<td>1.05 (0.11) 1.05 (0.10)</td>
</tr>
<tr>
<td>39.1 (14.3) 38.0 (13.6)</td>
</tr>
</tbody>
</table>

Cytometry Part B: Clinical Cytometry DOI 10.1002/cyto.b
noma, Weyn et al. contend that quantitative pathology, including measures of nuclear morphology, chromatin texture, and histology, acts as an excellent marker of prognosis (32). In the current analysis, iHAC scores were, on average, highest in the LSIL group. This could be attributed to the sequence of events in carcinogenesis in which chromatin remodeling (being represented by the iHAC score) is most likely to occur during LSIL to allow for integration of viral DNA (33). The scores also increased when only HPV 18 RNA was found and even more when both types were found in the sample. This likely reflects the more aggressive nature of HPV 18.

Image cytometry enables the measurement of both DNA ploidy and morphonuclear features, and is able to identify morphological changes in samples of few cells. Many investigators have shown the prognostic capabilities of morphonuclear features termed malignancy associated changes (MACs) (19,29,34,35), and others the association between ploidy and lesion progression (36–38); however, few have utilized the full complement of image cytometry. Nemec et al. proposed a model for progression that included ploidy, morphometrical, and chromatin texture features that boasted of high specificity and sensitivity (39).

Here, we suggest an additional measure to add to the list of potential prognostic factors, mRNA expression level. We previously showed that DNA ploidy and mRNA level are both related to histological grade, especially the mRNA levels of HPV 18 (15). This is important when attempting to predict histological grade from these types of data. We were especially interested in the relationships between these variables. Increases in the DNA index of cells were not associated with the type of mRNA detected in the specimen. However, the chromatin score was related to the type of mRNA. Even with a small number of dually infected samples, there was a significant trend (P = 0.01) seen in chromatin texture score from samples with no mRNA detected to those that had both HPV 16 and HPV 18 mRNA. Cells identified with HPV 18 alone showed more abnormal chromatin texture than those with HPV 16 alone, once again pointing to the higher transforming ability of HPV 18. Integration into host cell DNA is thought to be better achieved by HPV 18 (~100%) compared to HPV 16 (only 75%). Evidence also suggests that the size of the viral DNA integrated is also different between the two viral types (40).

It is unlikely that storage time of specimens played an important role in prevalence estimates. There was no significant correlation (Pearson’s r = 0.099) between length of storage and mRNA amount detected (data not shown). We also feel it is unlikely that storage temperature or collection media used played a significant role because both are standard protocols suggested by the manufacturer (Ambion), a leader in the collection and analysis of RNA for research purposes.

In the current study, there was a significant association between chromatin texture (iHAC score) and type of HPV RNA present in the sample. Whereas, DNA index was most related to progression from normal to LSIL to HSIL, these results support the hypothesis that DNA index, chromatin texture, and HPV mRNA level represent independent factors in the identification of precancerous cervical lesions. In addition, the use of cytological specimens, rather than biopsy specimens, for these analyses increases the likelihood of their being used for any large-scale screening purpose.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Jasenka Matisic from Vancouver for her assistance in the cytopathological review of the specimens presented in this manuscript. Michael E. Scheurer was supported by a cancer prevention fellowship.

LITERATURE CITED