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Application of Computational Method for Quantitative Analysis of Protein Expression of P16ink4a in Cervical Biopsies through Immunohistochemistry

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ABSTRACT : *Introduction: The purpose of this study was to standardize the quantification of protein expression of p16INK4a in cervical biopsies using computational method, and to compare it with methods used previously, as well as with HPV viral subtypes, aiming to establish an methodology for cervical biopsies. Material and Methods: Fifty-eight cases of Pap slides and cervical biopsies were selected. Then, immunohistochemistry for p16INK4a in cervical biopsies and quantitative analysis using the software Image J were performed. The interpretation was performed using the Richart system, German Scoring, and a qualitative method. A genotyping for HPV was performed. Results: A significant association between quantification by Image J software and Richart system, German Scoring System, and qualitative method ($p < 0.05$, $p < 0.001$) was observed. When comparing the results obtained with high and low risk viral subtypes, and negative samples, a proportional and significant association ($p<0.001$) was also observed; when comparing the results with the viral subtypes separately, a significant correlation between genotypes 16, 18, 33, 39, 52, 58, and 73 ($p<0.05$, $p<0.001$) was reported. Discussion: A proportional correlation of p16INK4a quantification and Richart, German Scoring, and qualitative method scores, as well as with different viral genotypes was observed. Currently, the evaluation systems show deficiencies due to diagnostic inter- or intrapersonal variations. We suggest that the quantification of p16INK4a in cervical biopsies by Image J software shows significant results for improving diagnostic techniques of cervical cancer, which may help in effectively choosing a clinical treatment without inter- or intrapersonal variations.*

KEYWORDS: *Human papillomavirus, diagnosis, neoplasms, histology, p16 genes.*

I. INTRODUCTION

Currently, we have several methods for measuring protein expression. A widely used method is immunohistochemistry (1), whose purpose is to evaluate protein expression by the binding of antibodies specific of the protein studied. The analysis occurs by visualization of differential staining on slides with tissue sections fixed and stained proportionally to the intensity of protein expression (2). Immunohistochemistry is a tool used for investigating biomarkers, in especial for studying their relationship with cancer, such as cervical cancer (3). Recently, the American Society of Clinical Pathology found, with relevant scientific evidences, new screening methods for detecting cervical cancer, among the definitions of the Guideline, is the quantification of expression of proteins involved in HPV carcinogenesis (1). Several biomarkers for the detection of cervical diseases have been identified, many of them involved in cell cycle regulation, signal transduction cascades, DNA replication, and cell proliferation (2, 3). Some studies have shown that the expression of p16INK4 is markedly influenced in carcinomas of the cervix due to functional inactivation of pRb by HPV's oncoprotein E7 (4, 5).Therefore, it is important to standardize the evaluation methods of biomarker expression such as the p16INK4; however, there is considerable diversity in measurements due to the great variability of methods used to grade the staining observed on immunohistochemistry slides (6). Different authors randomly establish cutoff points for ranking the cases, which makes negative cases by some authors be considered positive by others and vice versa (7, 8).

The methods used so far in the literature can be summed up in two groups. The first group includes the simple binary method, which ranks the cases either as positive or negative. The second group includes semiquantitative methods, which use scales and seek to group cases among those classified as positive. Some designations used are negative/weak marking/strong marking; negative/sporadic marking/focal marking/diffuse marking; cross scale and number scale, ranging from 0 to 3 (6, 9). These methods have been shown to be subjective and to have high intra- and interobserver variability.

Despite these undesirable characteristics, semi quantitative methods seek to differentiate subgroups within those cases classified as "positive" (10). In an attempt to decrease the variation found in the literature, we propose in this work to standardize the quantification of protein expression in cervix biopsies via computational method, and thus compare them with methods used previously, in order to establish a methodology with sensitivity and specificity sufficiently suitable for cervix biopsies. In addition, we aimed to correlate protein quantification by the proposed method with viral and histopathological classifications in order to detect associations corroborating data published in the literature and so determine the quality of the proposed method.

II. MATERIALS AND METHODS

The sample size was defined by statistical calculation, and was set to a security level of 95% and a sampling error of 5%. Furthermore, the sample size was verified in studies with patients diagnosed with cervical cancer (11-13). Based on the above, we selected 58 slides of cervical biopsies, embedded in paraffin and stained with HE method. The samples were separated into three groups according to the classification proposed by Richart and Barron in 1968(17), which provides criteria for identifying cervical intraepithelial neoplasia (CIN) according to the degree of histological lesion of the cervical epithelium, while the samples of this study were divided into control group, a group comprising patients with HPV/NIC1, and group comprising patients with NIC2/NIC3. The immunohistochemistry process by EnVision ® system was performed by using the Cintec® p16INK4 histology kit (Dako Cytomation, Glostrup, Denmark), according to the manufacturer's instructions, with primary p16INK4 mouse anti-human antibody, clone E6H4. The 3-μm cuts performed on silanized slides were counterstained with Mayer's hematoxylin.Each case was reviewed separately by two pathologists, who classified the samples of the present study according to different methods. After classification by both professionals, the data was checked and a mean of the determinations was established.

The samples were classified according to the semiquantitative method with the cross scale of the German semiquantitative scoring system, which correlates with the expression levels from 0 to 3 (0, 1+, 2+, 3+) (7, 10, 14), with rating "0" being used in cases without immunostaining for p16INK4, "1+" for weak immunostaining, "2+" for moderate immunostaining, and "3+" for strong immunostaining. From the biopsies collected, genotyping for HPV was performed by using the polymerase-chain-reaction (PCR) method to identify the viral subtype. The scanned images were transferred to a computer, and the intensity of staining was determined using the NIH ImageJ 1.36b software (National Institutes of Health, Maryland, USA).Image J is a public-domain image-analyzing software on Java platform inspired by NIH Image software for Apple's Macintosh. Therefore, it can be run in different operating environments provided they have a suitable Java virtual machine. The repertoire of the software's functions can be extended by different off-the-shelf plug-ins, available on the Internet(18).

The NIH ImageJ 1.36b software was downloaded from NIH's website (http://rsb.info.nih.gov/ij); color selection and classification of positively stained points was done by using a distribution diagram for the colors red, green and blue (RGB), which shows the changes in intensity and color saturation. This distribution provides information on pixel quantity of the analyzed image (18). This was obtained through the threshold_colour plug-in, in which the color range of interest was defined. The software turns white those areas that meet this standard and black the remaining areas (Figure 1) (15, 16).To verify the effectiveness of protein expression quantification by using the NIH Image J 1.36b software, we quantified the immunostaining of cervical biopsies and associate these with different types of semiquantitative methods previously established in the literature (6, 17).For statistical comparison, we used one-way ANOVA test for nonparametric data and Tukey's post-test, with $p < 0.05$ being considered significant.

III. RESULTS

To evaluate the effectiveness of quantification by using the Image J software, the values obtained with the pathological classification of lesion types described by Richart's (1990) standardized system were compared (21). A significant relationship can be observed between the immunostaining values obtained for p16INK4 and classification as NIC1, CIN2, CIN3, and negative, with and statistically significant differences having been evidenced when comparing negative samples and samples classified as CIN3 ($p<0.001$) and samples classified as NIC1 and negative samples ($p<0.05$) (Figure 2a).When comparing the immunostaining quantification and the German semiquantitative score, which correlates the expression levels with a scale between 0 and 3 (0, 1+, 2+, 3+), the results obtained were also proportional with regard to the German score, showing significant differences among all the groups ($p<0.001$) (Figure 2b).In order to get more evaluative parameters, we compared quantified immunostaining with subjective rating as "negative", "weakly positive" (FRA+), and "strongly positive" (FOR+). As a result, a significant relationship ($p<0.001$) between immunostaining and

immunohistochemistry classification was observed (Figure 2c).To explore associations between p16INK4a levels and HPV viral subtypes, we analyzed expression levels by Image J software and compared them with viral subtypes identified in samples (Figure 2e). We also segmented the subtypes into two groups, one high-risk group (HPV AR) (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and one group of subtypes with potentially high-risk genotypes (HPV PAR) (HPV 26, 53, 66, 67, 68, 70, 73, and 82), and HPV-negative samples (22) (Figure 2d).Significant associations ($p<0.001$) between the p16INK4 expression levels and high-risk subtypes were observed, as already evidenced in the literature (Figure 2d) (23). When we consider the rate of protein expression and relate it with the different viral subtypes observed in the sample, we can analyze the immunostaining of p16INK4a in subtypes 16, 18, 33, 52, 58, and 73 ($p<0.001$) (Figure 2e), corroborating previous data found in the literature (18, 19).

IV. DISCUSSION

The quantitative or semiquantitative assessment of immunohistochemistry slides varies inter- and intrapersonally 26; the study of Galloway et. al. (2011) confirmed this interpretation variation among histopathologists and trainees from the same area; it was considered that these professionals are not free from error, and that they tend to overestimate the quantification of immunohistochemistry (26). Thus, it is crucial to develop methods that aim to decrease this variation in order to increase the accuracy and exactness of immunohistochemistry. Other authors have demonstrated the application of computational methods in the final interpretation of biopsy slides of cancerous tissue ((20, 21)); Image J software has been used by some authors (22, 23); however, to this day, there are no studies that evolve using the Image J software in biopsies of cervical cancer precursor lesions. In the present study, we compared the quantification of slides of cervical biopsies immunostained for p16INK4 by using Image J software, and compared the results with the main quantification and semiquantification methods established in the literature. The first method analyzed was the classification by the Richart et. al. system (1990) (21), which is well established in clinical practice and has high significance levels (31). Despite its high applicability, Raab et al. (2006) showed that there is an error rate of 9.52% between the correlation of cytological and histological analysis (32), thus indicating the need for improving the technique. With the computational method used in this study, a correlation between the data obtained and the classification by the Richart et al. system (Figure 2a) was observed, indicating that the higher the Richart et al. score the higher the expression of p16INK4a ($p<0.05$, $p<0.001$), corroborating data found in the literature (33).

To verify the effectiveness of the proposed method, we compared the data obtained with the German score, in which grading of the immunostaining is given by number of crosses; in the presence of intense immunostaining, a greater number of crosses is assigned (0, 1+, 2+, 3+). In this analysis, an association between the levels obtained from a quantitative technique and the semiquantitative technique can be clearly verified ($p<0,05$, $p<0,001$) (Figure 2b), showing once again that the results in the present study corroborate data previously published(24), and demonstrating for the first time the utilization of a quantitative technique with great effectiveness when compared to other methods.To increase the significance of the method proposed in the present study, we compared results obtained with the qualitative classification of immunohistochemistry used by some authors (35); an association between the techniques can also be observed ($p<0.001$) (Figure 2c), indicating that the quantification proposed has a correlation with different standardized techniques in the literature .Several studies show a correlation between expression levels of p16INK4a and different viral subtypes of HPV (25, 26); to investigate if this association is also present when u sing the proposed quantitative method, we compared data obtained with HPV AR (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and HPV PAR (HPV 26, 53, 66, 67, 68, 70, 73, and 82) (27); the expression was significantly higher in the HPV AR group when compared to biopsies negative for HPV ($p<0,001$) (Figure 2d), thus corroborating previously published data (28, 29); the same was observed in Figure 2e, which shows p16INK4a expression segmented by viral type.From the data obtained in this study, we conclude that quantification of p16INK4a in cervical biopsies by using Image J software has significant results for improving diagnostic techniques of cervical cancer and therefore might help determining an effective clinical treatment without inter- or intra-personal variations. Correlation of the quantification by the proposed technique with viral subtypes indicates its practical and reliable applicability.

More studies are needed to confirm specificity and sensitivity levels of the proposed method, which would provide the basis for further studies.

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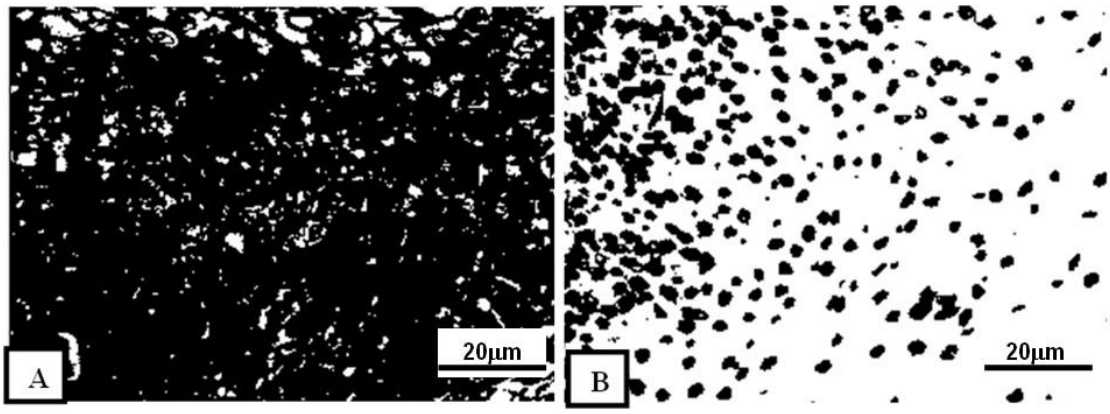

Figure 1. Image representation after definition and implementation of the positivity interval with the threshold color plugin of the Image J software. Illustration A shows the analysis of a cervical biopsy with p16INK4 staining with a value of "3+", while illustration B shows the analysis of a cervical biopsy with negative staining for
p16INK4.

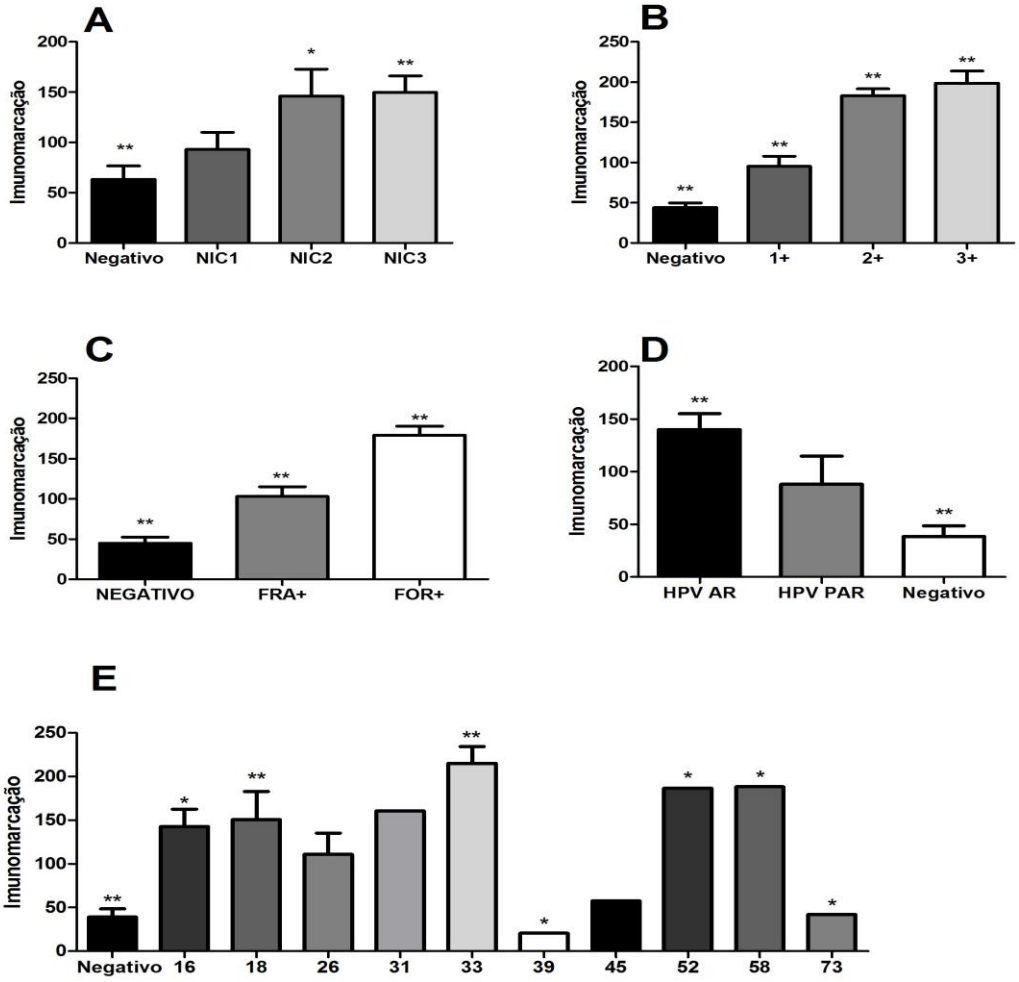

Figure 2. Comparison between quantification of p16INK4a in cervical biopsies and Richart system (A), German scoring system (B), qualitative method (C), HPV AR, HPV PAR, and negative cervical biopsies (D), HPV subtype (E). *p<0,05, **p<0,001.