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Evaluation of p53 immunohistochemical expression using open-source software for digital image analysis: A tissue microarray study of penile squamous cell carcinomas

ABSTRACT

The addition of molecular biomarkers is needed to increase the accuracy of pathologic factors as prognosticators of outcome in penile squamous cell carcinomas (SCC). Evaluation of these biomarkers is usually carried out by immunohistochemistry. Herein we assess p53 immunoexpression using freely-available, open-source software packages for digital image analysis. We also compared the results of digital analysis with standard visual estimation. A tissue microarray (TMA) was built using 39 cases of penile SCC. Percentages of p53 positive cells were higher by visual estimation than by digital analysis. However, correlation was high between both methods. In summary, evaluation of p53 immunoexpression is feasible using open-source software packages for digital image analysis. Although our analysis was limited to penile SCC, the rationale should also hold for other tumor types in which evaluation of p53 immunoexpression is required. This approach would reduce interobserver variability, and would provide a standardized method for reporting the results of immunohistochemical stains. As these diagnostic tools are freely-available over the Internet, researchers and practicing pathologists could incorporate them in their daily practice without increasing diagnostic costs.

Keywords: penile carcinoma, p53, immunohistochemistry, digital image analysis

INTRODUCTION

Most penile tumors are squamous cell carcinomas (SCC) arising at the distal mucosa covering glans, coronal sulcus, or foreskin [1]. Several pathologic features of the primary tumor, including histologic grade, tumor extent, and perineural invasion, have been established as prognosticators of outcome [2]. However, additional markers are needed to increase the accuracy of these pathologic factors. With this goal, immunohistochemical expression of cell cycle-related markers has been used to estimate prognosis of penile SCC [3]. Nevertheless, differences in evaluation criteria hinder the comparison of series, thwarting the standardization of clinically-useful thresholds of immunohistochemical expression. Aiming to overcome these difficulties, digital image analysis using proprietary software has been proposed, either for research or clinical practice. However, the high costs of these diagnostic tools preclude their routine implementation. Herein we evaluate open-source software packages, freely available over the Internet, to analyze the immunoexpression levels of p53 in penile SCC. We provide universal resource locators (URLs) for downloading these packages, and a basic protocol for digital image analysis. Finally, we also compare the results of digital analysis with standard visual estimation of p53 immunoexpression.

MATERIAL AND METHODS

Tissue Microarray Building and Immunohistochemistry

Thirty-nine cases of formalin-fixed, paraffin-embedded penile SCC were used to build a tissue microarray (TMA) at the Johns Hopkins TMA Lab Core (Baltimore, MD). Each case was randomly sampled 3–9 times, depending on tumor size, yielding a total of 156 tissue cores of 1 mm of diameter. Pathologic evaluation was done using the criteria proposed in the AFIP *Atlas of Tumor Pathology* [1]. Immunohistochemistry for p53 (antibody against p53, clone-BP53-11, Ventana Medical

Systems, Inc. Tucson, AZ) was performed on automated systems from Ventana XT (Ventana Medical Systems, Inc. Tucson, AZ). The reaction was developed using streptavidin-HRP detection I-View kit (Ventana Medical Systems, Inc. Tucson, AZ). All sections were then counterstained with hematoxylin, dehydrated, and cover-slipped.

Evaluation of p53 Immunoexpression

Each TMA spot was scanned using the APERIO system (Aperio Technologies, Inc., Vista, CA) and uploaded to TMAJ, an open-source platform for online evaluation of TMA images (available at http://tmaj.pathology.jhmi.edu). Images were scanned at a 20x resolution, yielding an image scale of 2.65 microns/mm. Percentage of p53 positive cells was then established using visual and digital analysis. For this purpose, images were downloaded from the TMAJ database to a local computer. For visual analysis, percentages of p53 positive nuclei were estimated by naked eye on a computer screen, without the use of any specialized software. For digital analysis, the open-source software ImageJ version 1.44 (available at http://rsb.info.nih.gov/ij) was used along with the FIJI package (available at http://fiji.sc/Fiji) and the immunoratio plug-in (available at http://imtmicroscope.uta.fi/immunoratio).

The immunoratio plug-in calculates the percentage of positively stained nuclear area (labeling index) by using a color deconvolution algorithm previously described by Tuominen et al [4]. This deconvolution algorithm separates the staining components (diaminobenzidine and hematoxylin) based on user-defined thresholds for positive nuclei (brown pixels) and negative nuclei (blue pixels). These thresholds were adjusted in a training set of 5 randomly selected TMA spots, until at least 95% of nuclei were identified, either as positive or negative. The same algorithm was then used to estimate in batch the percentage of positive cells. Results were exported afterward to a database containing the pathologic features of the case.

Statistical Analysis

Analyses were carried out spot by spot and using the pooled arithmetic mean of all the spots for each case. Percentages of p53 positive cells estimated by either visual or digital analyses were normalized using z-scores and compared using the Wilcoxon matched-pairs sign-rank test. The correlation between the visual and the digital methods was evaluated using Spearman's rho correlation coefficient. Spearman's rho was interpreted as follows: < 0.09, no correlation; 0.10 to 0.29, weak correlation; 0.30 to 0.49, moderate correlation; ≥ 0.50, strong correlation. Kruskal-Wallis test was used to compare percentages of p53 stratified by histologic subtype and histologic grade. A 2-tailed *P* value < 0.05 was required for statistical significant. Data were analyzed using the software STATA release 11 (StataCorp Inc., College Station, TX).

RESULTS

Table 1 shows the pathologic features of the 39 cases. Figure 1 shows scanned TMA spots of H&E and p53-stained tissue cores. Figure 2 shows the output of the digital analysis for 1 TMA spot. Percentages of p53 immunoexpression were higher with the visual method (mean 24%, SD 31%, range 0% to 95%) than with the digital method (mean 5%, SD 6%, range 0% to 26%). However, *z*-scores were similar between the 2 methods ($P = 0.49$). Correlation was high between the visual and the digital methods (Spearman's rho = 0.86, $P < 0.0001$, Figure 3). No association was found between expression levels of p53 and histologic grade or histologic subtype, either using the visual or the digital method (Table 2).

DISCUSSION

This study demonstrates the feasibility of performing digital image analysis of p53 immunoexpression using freely-available, open-source software packages. Our data suggest that visual analysis tends to overestimate the percentage of p53 positive cells. However, the high correlation between visual and digital analyses indicates that both methods are appropriate for estimating the relative levels of immunoexpression. As described in this study, digital analyses of p53 expression could offer an inexpensive and more reliable approach for evaluating the results of immunohistochemical techniques. This approach would also be less time-consuming, less prone to interobserver variability, and the printed output could be easily added to the pathology report. The use of standardized methods would also allow the direct comparison of different studies, and the selection of clinically-applicable thresholds for defining diagnosis or treatment.

In normal cells, the protein p53 plays a central role in the regulation of the cell cycle. Additionally, mutations in the tumor suppressor gene *TP53*, located on chromosome 17p13, have been identified in approximately 70% of adult solid tumors [5]. Mutation of *TP53* leads to either loss of the protein expression or, more frequently, expression of a mutant protein [3]. This mutant p53 then accumulates, resulting in an overexpression of the protein. Expression levels of p53 have been used as prognostic tools in several malignancies, including genitourinary tumors [6]. The lack of association between p53 and histologic grade or subtype we found is consistent with previous series [7]. However, several

studies have suggested that p53 levels are associated with prognosis in penile carcinomas [7–10]. In this scenario, the accurate and reproducible evaluation of p53 immunoexpression could have profound clinical implications.

In summary, evaluation of p53 immunoexpression is feasible using open-source software packages for digital image analysis. Although our analysis was limited to penile SCC, the rationale should also hold for other tumor types in which evaluation of p53 immunoexpression is required. This approach would reduce inter-observer variability, and would provide a standardized method for reporting the results of immunohistochemical stains. As these diagnostic tools are freely available over the Internet, researchers and practicing pathologists could incorporate them in their daily practice without increasing diagnostic costs.

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