**Immunohistochemical Evaluation of Cell Cycle-Related Proteins in Squamous Cell Carcinomas Using Digital Image Analysis: Validation of the ImageJ immunoratio Plugin**

Anahí M. de Chaux, George J. Netto, Alcides Chaux

BACKGROUND

Digital image analysis for immunohistochemical expression of clinically-relevant proteins is aimed at decreasing interobserver variability and thus increase objectivity in their measurements. Open-source packages may provide an inexpensive approach for this task, but to date their validity has not been tested in squamous cell carcinomas.

DESIGN

We built 4 high-density tissue microarrays (TMA) from 112 penile SCC. The following cell cycle-related proteins were evaluated: p53, Ki67, cyclin-D1 and MDM2. Percentage of positive tumor cells were estimated: (1) visually by naked eye; (2) digitally, using ImageJ (NIH, available at <https://imagej.nih.gov/ij/>), through the immunoratio plugin. Visual and digital percentages were analyzed using the Wilcoxon signed rank test and the Spearman σ correlation coefficient.

RESULTS

Visually-estimated mean percentages were significantly higher than digitally-measured mean percentages for p53 (23.9 vs 3.2%, P<0.00001), Ki67 (24.2 vs 2.1%, P<0.00001), cyclin-D1 (24.9 vs 7.9%, P<0.00001), and MDM2 (4.3 vs 0.7%, P=1.5e-4). For p53, Ki67 and cyclin-D1, mean differences between visual estimates and digital measurements were greater in grade 3 tumors, compared to grade 1-2 tumors (Table 1). The overall correlation between visual estimation and digital measurement was high for p53 and cyclin-D1, moderate for Ki67, and weak for MDM2 (Table 2). When we stratified the correlation analysis by histologic grade we found that correlation coefficients were lower for grade 1-2 tumors and higher for grade 3 tumors (see Table 2).

Table 1

|  |  |  |  |
| --- | --- | --- | --- |
| Marker | Grade 1 | Grade 2 | Grade 3 |
| p53 | 11.4 | 21.3 | 22.9 |
| Ki67 | 7.1 | 18.7 | 38.3 |
| cyclin-D1 | 5.7 | 12.7 | 23.1 |
| MDM2 | 4.9 | 5.3 | 2.2 |

Table 2

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Marker | Overall correlation | Grade 1 | Grade 2 | Grade 3 |
| p53 | σ=0.75,  P<0.00001 | σ=0.54,  P<0.00001 | σ=0.74,  P<0.00001 | σ=0.77,  P<0.00001 |
| Ki67 | σ=0.68,  P<0.00001 | σ=0.54,  P=0.00005 | σ=0.56,  P<0.00001 | σ=0.71,  P<0.00001 |
| cyclin-D1 | σ=0.72,  P<0.00001 | σ=0.62,  P<0.00001 | σ=0.63,  P<0.00001 | σ=0.76,  P<0.00001 |
| MDM2 | σ=0.38,  P=4.8e-16 | σ=0.26,  P=0.04 | σ=0.34,  P<0.00001 | σ=0.39,  P<0.00001 |

CONCLUSIONS

Percentages of positive tumor cells were significantly greater when using digital analysis compared to visual estimation by naked eye. Although the correlation was moderate to strong between both methods, the increased mean differences in the percentages for higher grades (probably related to increased nuclear pleomorphism in these tumors) and the intragrade variability in the correlation coefficients limits the usefulness and potential clinical applicability of digital measurements of positive tumor cells in squamous cell carcinomas.