Original Article

Digital Image Analysis versus Visual Scoring for Bcl-2 and P53 Protein Expression in Glioma

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Summary:

Background: Traditionally, evaluation of the results of immunohistochemistry was done by visual quantification.

Materials and methods: for reliable evaluation, more time-efficient and user friendly method we used simple computer program with image analysis options as independent parameters for reading positive results. To test the validity of visually scored results, we compare and correlate the results of Digital image analysis (DIA) variables with the visual scores of 280 pictures taken from entire stained glioma tumor sections for Bcl-2 and P53 oncoproteins in different glioma tumor grades.

Results: In this study, rates expression of both oncoproteins was evaluated visually in glioma tumor samples (Bcl-2=72.41% and P53=82.76%), no statistical significant differences were observed according to pathologic grades. Similarly, these results were also explained by data obtained by DIA variables that has been closely correlated with visual scores. More importantly, the DIA variables explained little discrepancy in the visual scores of both oncoproteins.

Conclusions: the quantitative DIA measurements of the immunohistochemically stained sections made the results more objective and supported pathomorphological diagnosis.

Keyword: Digital image analysis, immunohistochemistry staining.

Introduction:

Apoptosis, or programmed cell death, plays an important role in cell proliferation control in physiological and pathological conditions (1). Such role has special interest for brain tumor development and progression. The apoptosis process activated and regulated by a number of genes and proteins, such as P53 and Bcl-2. P53, a cell proliferation regulating gene and a pro-apoptotic which is altered in as much as half of all astrocytomas, have a controversial role in glioblastoma prognosis (2, 3). Bcl-2 and the blc-2 protein family are mostly considered anti-apoptotic, providing cells with a longer time of survival by preventing cell death. Bcl-2 expression in some tumors has been related to their own prognosis (4).

In recent years, abundances of antigens have so far relied primarily on visual scoring and to a lesser extent computer-assisted image processing techniques of cellular proteins detected by immunohistochemistry (IHC) (5). A major advantage of computer processing is the avoidance of inter-observer variability in interpreting subtle antigen level changes (6). Most computer-based techniques for IHC image analysis have so far had limited applicability due to several drawbacks including a need for specific software systems, often with considerable need for user input (7-10). These image analyses have commonly been performed on the single assessment of 3,3'-diaminobenzidine (DAB) labeling for a variety of cytoplasmic and nuclear markers. Alternatively, the recent application of spectral imaging offers an optimal method to capture and analyze images at multiple wavelengths (11). In the aims of this study were to design and evaluate a user-friendly, quantitative, Computer-assisted method of analysis of Bcl-2 and p53 oncoproteins in immunohistochemically stained sections from patients with glioma. Image analysis methods were compared in the IHC staining scores of both Bcl-2 and p53 protein. Following, the performance of a high throughput automated different image records were compared to observer-dependent methods, including count of positive color, surface area and its percentage of positive color.

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Materials and Methods:

Tumor Specimens: Twenty-nine paraffin-embedded glioma specimens were reviewed by a pathologist and classified according to the WHO classification criteria (12). Histopathological examination revealed 2 grades I astrocytomas, 2 grade II oligodendrogliomas, 10 grade III anaplastic astrocytomas, 3 grade III anaplastic mixed gliomas, and 12 grade IV glioblastoma multiforme.

Bcl-2 and p53 immunohistochemical staining:

Immunohistochemistry. Five μm sections were cut from formalin-fixed, paraffin-embedded tissue blocks and placed on polylysine-coated glass slides. After overnight packing at 65ºC, tissue sections were deparaffinized in xylene and rehydrated in ascending grades of alcohol. Endogenous peroxidase activity was exhausted by incubation of tissue sections in 0.3% H_2O_2 for 10 minutes at room temperature. 20% rabbit serum in Tris Buffered Saline (TBS) was used for blocking. For each slide 2 sections were selected (each section for primary Ab). the primary monoclonal mouse anti-human Bcl-2 antibody (124 Dako, Carpinteria, Calif.) and primary monoclonal mouse anti-human p53 (DO7, Dako, Carpinteria, Calif.). They were incubated with samples at 37 ºC for 1 hr. After rinsing with washing buffer, slides

were incubated with a secondary biotin linked anti-mouse antibody for 50 minutes at room temperature; and with the streptoavidin-peroxidase complex for 50 minutes, undertaking a washing buffer rinsing between each step. The antigen-antibody complex was visualized with the chromogen DAB and counterstained with hematoxylin. All tissue sections were stained under similar conditions to ensure equal staining quality. A negative control was performed in all cases by omitting the primary antibody, which in all instances resulted in negative immunoreactivity.

Visual scoring of immunohistochemistry: Stained sections were visualized with a standard light microscope (OLYMPUS) at 400X power. At least 5 images captured by a digital camera size: 3072 X 2304, Bit Depth: 32 and saved in a JPG format were representing entire tumor area.

Visual scoring defined semiquantitatively and performed by two evaluators as the follow:

Table 1: Scoring system for Pcl2 and P53 proteins expression.

	P53	Bcl-2
	Nuclear	Cytoplasmic
Negative	>5%	>5%
I	5-25%	5-25%
II	25-50%	25-50%
III	50-75%	50-75%
VI	75-100%	75-100%
Reference	Pollack et al., 2002	McDonald et al., 2002.

Thus, the mean of image scores was used as the final score for individual marker in each case. Image analysis was performed in areas designated by whole tissue core masks since anti-Bcl-2 and anti-P53 oncoprotein demonstrated high specificity for cancer cells.

Digital Image Analysis of Tissue Sections: Morphometrical measurements of DAB chromogen were displayed from representative IHC staining with Adope Photoshop CS4 extended Version11 program. Each single image of the entire tumor area automatically calculates the stained area, representing single cells or sheets of cells within the tumor.

The technique of selection of similar features on a digitized immunohistochemical image has previously been described in detail (Lehr et al. 1997). The selection of a specific color on a digitized image by selection of a specific color, the cursor of the Magic Wand tool is clicked on any object on the image displaying brown color. The selected area is automatically highlighted. To specify how broad a range of color the Magic Wand tool should include in the selection. Using the Select Similar command, all pixels on the image are highlighted that fall within the selected color range and are not touching the initial selection. Because the selected area is automatically highlighted on the screen, the selection process can be controlled at every step and necessary corrective measures can be taken. For example, the Select Grow command expands the color range to expand the selection to neighboring pixels. The selection continues to grow as often as this command is repeated (13, 14). Once the DAB chromogen is selected an important spatial information can be obtained for the specific chromogen, number of all selected features were quantified, the selected surface area on the entire image were also calculated and can be expressed as percentage of the entire image.

Statistical analysis:

Data were recorded as mean values and non-parametric data were presented as frequency and percentage, chi square test for comparison of categorical data; while one way ANOVA was used to compare the mean counts among different study groups and p-value <0.05 was considered significant. Pearson correlation was used to find possible correlation between values.

Results:

Bcl-2 and p53 Expression: The positive staining for Bcl-2 protein expressed as brown granules were mainly located in cytoplasm of tumor cells (Figure 1: B). the positive staining of P53 protein expressed as brown granules, was distributed mainly in cell nuclei (Figure 1: A). The positive expression rates of Bcl-2 and P53 are shown in table 1. No significant difference ($p>0.05$) were found among pathologic grades. The abnormal Bcl-2 expression rate was detected in 21 of 29 (72.41%) tumors, (2 of 2 grade I, 5 of 6 grade II, 9 of 12 grade III, and 5 of 9 grade IV). 24 of 29 (82.76%) tumors stained positively for p53 (2 of 2 grade I, 3 of 6 grade II, 10 of 12 grade III, and 9 of 9 grade IV).For both markers according to the pathologic grades, digital count of positive color, area and percentage of area covered by the positive color were not statistically significant difference (table: 2). By another mean, the result of Pearson correlation showed that there were no correlation with tumor grades (table: 3). These results make obvious that the data obtained through image analysis was no characteristic for tumor grade and thus they are not able to distinguish as by visual scores (table: 1).

Table 2: Descriptive statistics of Bcl-2 and P53 frequencies and percentage of different grades of glioma tumors.

Tumor grade	Bcl-2 (Positive) total	P53 (Positive) total
Grade I	(2) 2	(2) 2
Grade II	(5) 6	(3) 6
Grade III	(9) 12	(10) 12
Grade IV	(5) 9	(9) 9
Total	(21) 29	(24) 29
Percentage	72.41%	82.76%
Chi square value	9.834	10.225
Df	9	9
Sig. (2-tailed)	0.364^{NS}	0.333^{NS}

NS= No Significance difference

Table 3: results of comparison of digital image analysis of immunohistochemical variables according to tumor grade classification.

	Count of positive reaction	Surface area of reaction	Percentage of reaction
Bcl-2	P=0.350^{NS}	P=0.754^{NS}	P=0.723^{NS}
P53	P=0.088^{NS}	P=0.052^{NS}	P=0.086^{NS}

NS= No Significance difference

Table 4: results of correlation coefficient between digital image analysis of immunohistochemical variables and tumor grade.

	Count of positive reaction	Surface area of reaction	Percentage of reaction
Bcl-2	r=-0.225	r=-0.144	r=-0.088
	P=0.240^{NS}	P=0.456^{NS}	P=0.649^{NS}
P53	r=0.343	r=0.407	r=0.383
	P=0.068^{NS}	P=0.028(*)	P=0.040(*)

NS= No Significance difference
(*)= Significance difference p≤ 0.05

Table 5: results of comparison of digital image analysis of immunohistochemical variables according to visual scoring system.

	Count of positive reaction	Surface area of reaction	Percentage of reaction
Bcl-2	p≤0.001(**)	p≤0.001(**)	p≤0.001(**)
P53	p≤0.001(**)	p≤0.001(**)	p≤0.001(**)

(**)= Highly Significance difference p≤0.001.

Table 6: results of correlation coefficient between digital image analysis of immunohistochemical variables and visual scoring system.

	Count of positive reaction	Surface area of reaction	Percentage of reaction
Bcl-2	r=0.923	r=0.807	r=-0.095
	p≤0.001(**)	p≤0.001(**)	P=0.623
P53	r=0.960	r=0.894	r=0.882
	p≤0.001(**)	p≤0.001(**)	p≤0.001(**)

(**)= Highly Significance difference p≤0.001.

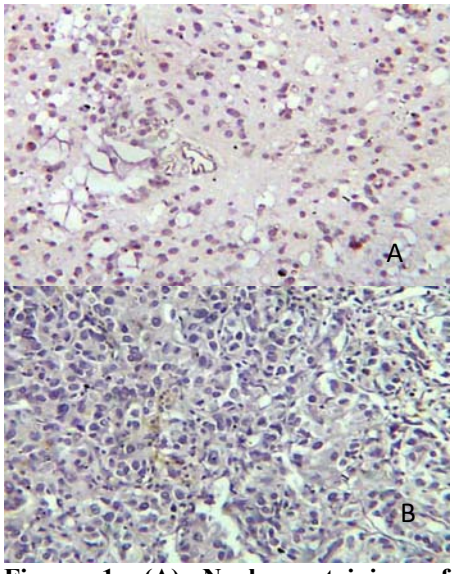

Figure 1. (A) Nuclear staining of P53 (B) cytoplasmic staining of Bcl-2 oncoproteins detected by peroxidase DAB positive brown color reaction in Oligodendroglioma cells.

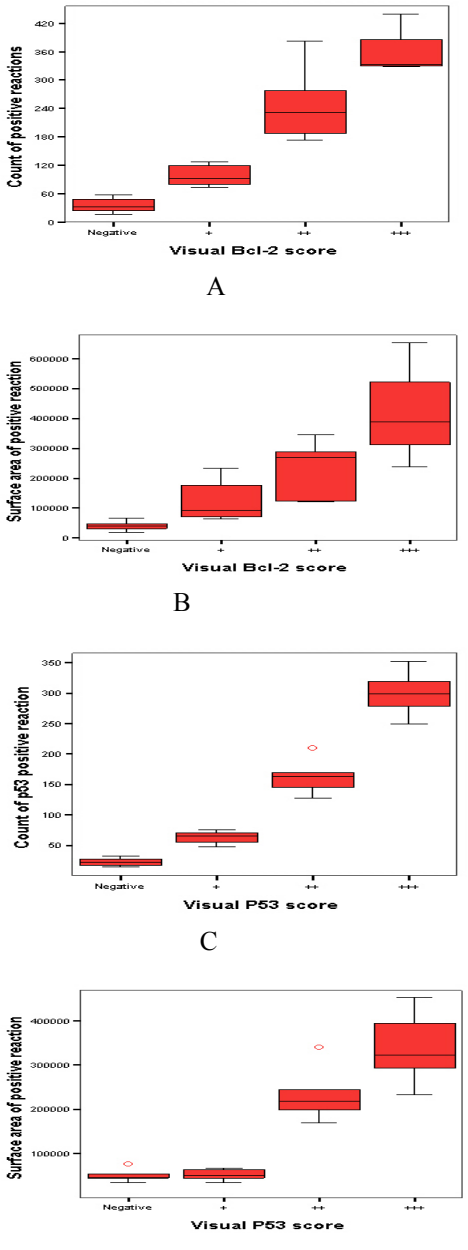

Figure 2: box and whiskers graph (median, 25% to 75% box range, min and max whiskers) representation of mean count and surface area for Bcl-2 and P53 oncoproteins, shows a direct relationship with the categorical visual scores.

Discussion:

Visual scoring remains the most commonly method in the assessment of immunohistochemistry staining for P53 and Bcl-2 oncoproteins. Which is a product of the percentage positivity and staining intensity in 5 high powered fields (15). This method of assessment is semiquantitative and remains observer dependant who is prone to error (16, 17). The selection of images for determination of Bcl-2 and P53 expression rates is very important the human eye can detect artifactual areas; however, digital selection can't separate true staining from artifactual staining. The images to be processed by digital selection must be free from artifacts. The area of

tissue in all of the images to be analyzed must be nearly the same. Results can erroneously generate a lower score for a heavily stained tissue if the amount of stained tissue in the image is small. In order to standardize our measurements, we cropped images to the same size and included the same amount of tissue in all images. Computer assisted methods of assessing immunohistochemical staining have been available for some time, however due to their expense and requirement for significant computing skills have not gained widespread usage. Most of these methods involve measuring the quantity of cells that express an antigen and do not indicate the staining intensity and are therefore semi quantitative (18) while others require an observer to identify the total nuclear area and oestrogen receptor nuclei and are therefore observer dependant (19). The method of assessing both P53 and Bcl-2 described in this study relies on a desktop personal computer and Adobe Photoshop, commercially available image analysis software. The mean IHC positive reaction for each biopsy based on a visual scoring was done manually. The more subjective method using positive color selection of bcl-2 and p53 expression was compared to results obtained using count, area and percentage of area. Since bcl-2 and p53 staining were distributed in all tumor cells, the fraction of labeled cells was of primary interest. According to visual scoring, both markers display highly statistical significant difference in the mean of count, area and percentage of area of positive color selection (table: 2). Both markers showed a direct relationship with mean count and area of positive color (Table: 3). interestingly, a higher variability characterized the lower and the upper ends of the scoring range compared to the middle of the scoring categories. This result suggests some cases at either end of the scale were misclassified and might be due to inconsistencies in the visual scores, or inclusion of non-specific staining with an automatic analysis based on whole tissue core. However, the majority of the cases from the different scoring categories were grouped within the non-overlapping top and bottom 25^{th} percentile of the values Figure: 2). Furthermore the use of a consumer digital still camera in the development of the image analysis system means that the costs of this system are within the budget of most pathology laboratories. This method of assessment is user friendly and requires minimal computing knowledge prior usage. Our data confirms an alteration in both P53 and Bcl-2 expression status with no significant correlation among tumor grades and visual scores measured manually and also with digital image analysis parameters like count of positive reactions, area and percentage positive area reaction. More importantly we report a close correlation between visual scoring system and digital image analysis parameters, suggesting the accuracy and sensitivity of digital image analysis in assessing of P53 and Bcl-2 protein expression in glioma tumors.

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