Computer Assisted Immunohistochemical Score Prediction Via Simplified Image Acquisition Technique

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ABSTRACT

Background: techniques of image analysis have been used extensively to minimize interobserver variation of immunohistochemical scoring, yet; image acquisition procedures are often demanding, expensive and laborious. This study aims to assess the validity of image analysis to predict human observer's score with a simplified image acquisition technique.

Materials and methods: formalin fixed- paraffin embedded tissue sections for ameloblastomas and basal cell carcinomas were immunohistochemically stained with monoclonal antibodies to MMP-2 and MMP-9. The extent of antibody positivity was quantified using Imagej® based application on low power photomicrographs obtained with a conventional camera. Results of the software were employed to predict human visual scoring results with stepwise multiple regression analysis.

Results: the overall prediction of epithelial score depicted as r square value was 0.26 ($p<0.001$) which was obviously higher than that of stromal score (0.10; $p<0.01$). Epithelial and stromal MMP-2 score prediction was generally higher than that of MMP-9. Collectively, ameloblastomas had a more efficient score prediction compared to basal cell carcinomas.

Conclusion: there is a considerable variability in the prediction capacity of the technique with respect to different antibodies, different tumors and cellular versus stromal score.

Keywords: immunohistochemistry, image analysis **(J Bagh Coll Dentistry 2017; 29(1):83-88)**

INTRODUCTION

Quantification of staining results is a crucial step within the methodology of thousands of immunohistochemical study designs; where attempts are always made to reach objective, concise and reproducible results. Semiquantitative scoring is the most common approach that involves one or more trained observer to convert what he/she sees into numeric or logical values[(1)].

Advancements in computer hardware capabilities through the last few decades facilitated the use of image processing programs to collect data from captured images[(1, 2, 3)]. One of the commonly employed multiplatform programs is "Imagej"; which is an open source software developed by NHS in 1997 and was involved in many microscopic research designs. The software had many built in or optional "add on" functions in the form of combined analytic steps merged into single click; they are called "plugins" and "macros" [(4)]. Varghese et al. (2014) developed an Imagej® compatible macro that would separate and quantify diaminobenzidine(DAB) and Hematoxylin colors according to pixel intensity within images of immunohistochemically stained tissue sections[(5)]. This study aims to evaluate the validity of this specific macro function with respect to human observer's percentage scoring of two epithelial tumors (Ameloblastoma & Basal cell carcinoma) stained with two monoclonal antibodies (MMP-2, MMP-9) with a simplified image acquisition technique.

MATERIALS AND METHODS

Formalin fixed, paraffin embedded tissue specimens of ameloblastomas (AB) and basal cell carcinoma (BCC) were retrieved from the archives of oral pathology laboratory at the college of dentistry/Baghdad university and medical city laboratories. Five um thick sections were mounted on positively charged slides and stained immunohistochemically with monoclonal antibodies for MMP-2 (AB: n=32; BCC: n=26; ab2462,1:200) and MMP-9 (AB: n=33; BCC: n=29; ab76003,1:100) using EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436) . Stained tissue sections were evaluated by two observers with at least 5 high power fields (X40) for cellular and stromal positivity as a percentage of positive cells to the total high power field (HPF) then, a mean of the fields was calculated. Visual scoring values of 2 experienced observers were correlated by Pearson correlation coefficient, the values were accepted when R between the observers' readings exceeds (0.80),

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otherwise; the sections were re-evaluated to reach a consensus.

At least, 3 photomicrographs for the DAB and hematoxylin stained tissue sections were captured with a 10X objective using an ordinary digital camera fixed to the eyepiece (Samsung-GT-N7100). With Imagej's region of interest (ROI) selection options; multiple cellular areas were surrounded with selection polygons followed by IHC macro application (Fig. 1). For any given case, a mean value of each IHC profiler parameters result was calculated (Pixel count, percentage of high positive, percentage of positive, percentage of low positive, percentage of negative). The same preceding steps (selection and macro application) were applied to stromal areas only.

Stepwise and multiple regression analysis for human score prediction was employed for statistical analysis using SPSS.22 software, adopting the Imagej's output data as independent variables (predictors).

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RESULTS

Tumor epithelial and stromal expression was found in tissue sections of ameloblastoma and basal cell carcinoma for MMP-2 and MMP-9 to different extents.

Combined visual and computer assisted immunohistochmical scoring characteristics of the two antibodies are summarized in tables 1 and 2. The predictive ability of the software algorithm, reflected by the (R square) varied across different tumors, antibodies and the evaluated compartments (epithelial or stromal). Tumor cellular evaluation with the software showed the highest R square values for the multiple stepwise regression in which the percentage of negative contribution (N%) and the pixel count (PC) were the most common independent variables that predict the human visual score.

On the other hand, evidently lower R square values were found upon applying the software to the stromal elements, with more versatile predicting variables between different antibodies and different tumors.

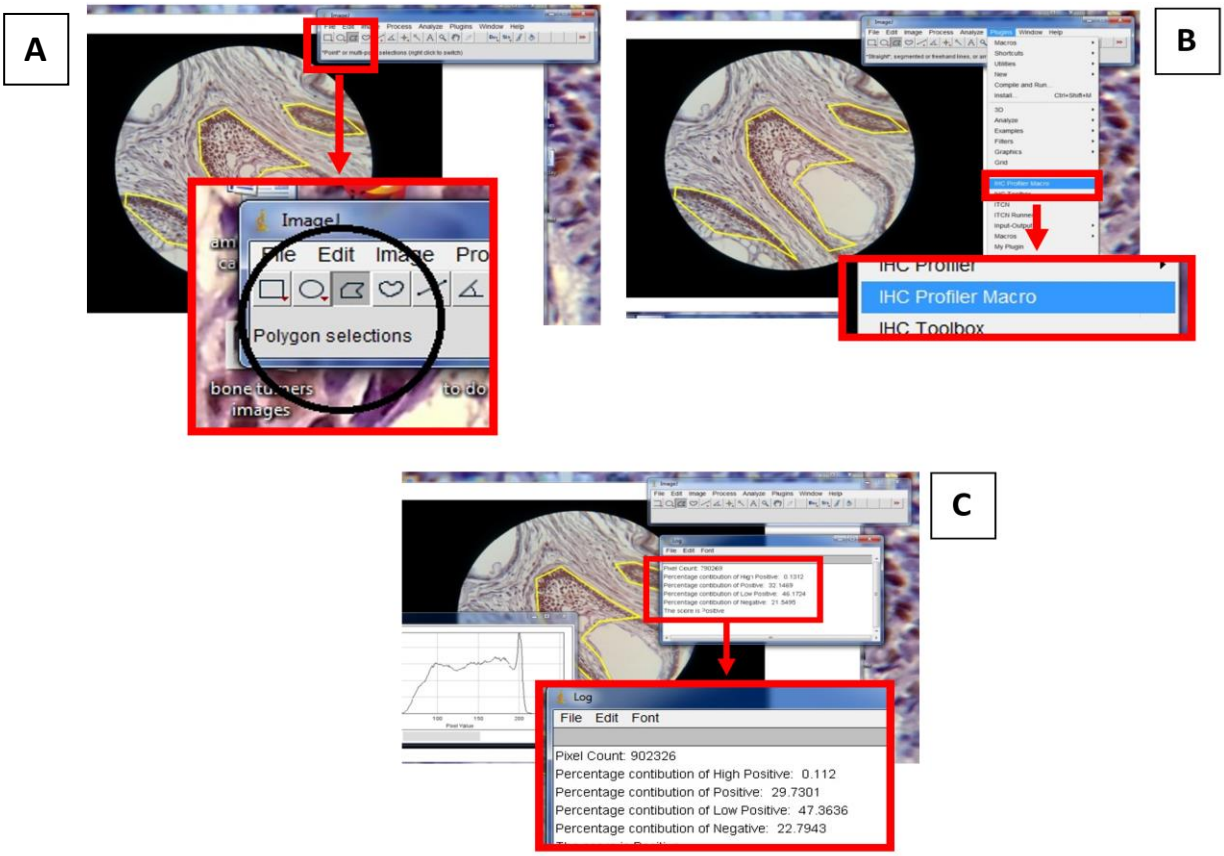

Figure 1: (A) Applying multiple polygon selection to regions of interest (ROI) of an image. (B) Choosing "IHC profiler macro" from the Imagej's plugins drop down menu. (C) Software's Results window

DISCUSSION

Reproducibility is a major issue in interpretation of immunohistochemical results quantification, both intra-observer and inter-observer agreements are a pre-requisite for any research to be valid [6]. Several attempts have been made to neutralize human observer's possible bias through image based computer assisted analytic approaches with variable success [1, 3, 7, 8].

A research group at the Molecular Functional Imaging Laboratory in India developed a macro (a single computer instruction that expands automatically into a set of instructions to perform a particular task) named "IHC profiler" ; compatible with Imagej®, the macro would separate images of DAB stained immunohistochemical sections to 3 fractions of varied brown positivity and one more for blue negativity depending on individual pixel color values and display the results as a percentage contribution of high positive, positive, low positive and negative[5]. In this study; the macro was applied to low power micrographs of immunohistochemically stained tissue sections of AB and BCC and the resulting output in addition to the number of pixels analyzed were plotted to predict the corresponding human observer's results according to standard scoring methods. In order to be more time effective and simple for the non-experienced observer, this study had major design differences from the original work by Varghese et al. [5]; the images were acquired with a conventional digital camera rather than a slide scanner in an attempt for method simplification to be accessible for any low budget laboratory, moreover; the study used a low magnification power for photomicrographs for a generalized view that would minimize the number of photographs needed for analysis. Ameloblastoma and basal cell carcinoma were selected in this study as prototypical examples for benign and malignant epithelial tumors that bear a morphological mimicry.

Stepwise regression analysis is a special variant of multiple regression that enters the independent variables (macro results in this study) in the regression model one variable at a time with omission of the effect of other variables to specify the most significant variables that predict the value of the dependent variable (human scoring in this study)[9].

At first glance, the predictive ability of IHC profiler in the cellular compartment was mostly associated with (N) variable which actually represents all the three macro variables (H, P and L) subtracted from 100%, however; pixel count had an effect in predicting AB human results more than BCC which is most probably resulting from the relatively less cellular islands selected for AB as compared to the BCC that leads to a lower pixel count. MMP-2 showed relatively high predictive results where the model explained a minimum of 40% of human scoring variability, a slightly higher R square value was noted in BCC in contrast to MMP-9 predictions which had notably lower values overall and a higher R square for AB. Regarding stromal expression, though generally less than cellular expression; R square values showed more predictive power in MMP-2 than MMP-9. Again, predictive values of MMP-2 human expression were higher in AB with conversely higher values for MMP-9 in BCC

Those results suggest that the model is definitely better predicting epithelial MMP-2 scores than MMP-9 regardless of the tumor type, moreover; it was more predictive concerning MMP-9 in AB and MMP-2 in BCC. However, overall results of the study approach indicated marked variability of the software's prediction capacity with respect to different antibodies and tumors. Keeping a minimally laborious and complicated image acquisition technique probably mandates the employment of more sophisticated computer analytic procedures to approach human visual perception.

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الخلاصة

الخلفية: استعملت تقنيات التحليل الصوري باستخدام الكومبيوتر بشكل مسهب في عدة دراسات سابقة لغرض تجنب التباين في التفسير البصري لنتائج الصبغات المناعية عند تعدد الفاحصين, مع ذلك؛ امتازت الطرق المختلفة بكونها تتطلب عمليات معقدة ومكلفة لالتقاط الصور المعدة للتحليل. تهدف هذة الدراسة لفحص امكانية توقع نتائج الفحص البصري بناءا على التحليل الصوري لصور شرائح مجهرية ملتقطة بطريقة مبسطة.

مواد وطرق العمل: تضمنت الدراسة عينات مثبتة بالفورمالين ومطمورة بشمع البارافين للوَرَمٌ الأَرُوميٌّ الميَنائيّ(اميلوبلاستوما) و سرطان الخلية القاعدية صبغت شرائحها باجسام مضادة احادية النسل موجهة الى MMP-2 و MMP-9 بطريقة التصبيغ الكيميائي المناعي. تم تقييم نتائج الاصطباغ المناعي بالطرق البصرية المعتادة و بوساطة التحليل الصوري لبرنامج Imagej® والتي اجريت على صور مجهرية واطئة التكبير ملتقطة باستخدام كاميرا اعتيادية من ثم قورنت نتائج التحليل الصوري مع نتائج الفحص البصري المجهري عن طريق احتساب صحة توقع التحليل الصوري باسلوب الانحدار الخطي المتسلسل.

النتائج: ظهرت نتائج اعلى للتوقع في اجزاء الاورام الظهارية R^2 ($p<0.001$) 0.26=مقارنة بالاجزاء السدوية منها ;($R^2=0.10$ ($p<0.01$, وبشكل عام؛ كانت نتائج التوقع للاجزاء الظهارية والسدوية لمؤشر MMP-2 اعلى من نتائج مؤشر MMP-9, كذلك؛ كانت قيمتها اعلى في ورم (الاميلوبلاستوما) مقارنة بسرطان الخلية القاعدية.

الاستنتاجات:تتباين نتائج التوقع التحليلي للطريقة المستخدمة بشكل ملموس بين المؤشرات والاورام المختلفة والمناطق المتعددة داخل الاورام.

Table 1: Epithelial visual and computer assisted immunohistochemical scoring results

EPI		HU% (SD)	COM					MR		SR	
	T(N=)		PC (SD)	H% (SD)	P% (SD)	L% (SD)	N% (SD)	R^2	R	R^2 (predictors)	R
MMP-2	AM(32)	49.69 (26.73)	157354.28 (139176.80)	1.68 (2.08)	22.03 (14.41)	43.05 (9.31)	33.24 (14.37)	0.403**	0.635	0.401** (N%,PC%)	0.633
	BCC(26)	29.23 (25.91)	222826.57 (110227.33)	0.21 (0.73)	6.17 (10.17)	52.37 (13.25)	41.25 (18.70)	0.513**	0.717	0.432** (P%)	0.658
	TOTAL(58)	40.52 (28.08)	186703.92 (130163.06)	1.02 (1.77)	14.92 (14.89)	47.23 (12.09)	36.83 (16.79)	0.460**	0.678	0.460** (N%,L%,PC%)	0.678
MMP-9	AM(33)	33.18 (33.04)	115325.78 (76825.35)	0.99 (2.28)	15.07 (13.91)	46.49 (14.27)	37.44 (20.35)	0.396**	0.630	0.165* (H%)	0.406
	BCC(29)	50.17 (29.69)	192840.09 (114912.70)	0.52 (1.65)	8.96 (15.00)	51.98 (14.17)	38.53 (19.12)	0.108	0.329	-	-
	TOTAL(62)	41.13 (32.41)	151582.47 (103333.77)	0.77 (2.01)	12.21 (14.64)	49.06 (14.37)	37.95 (19.63)	0.268**	0.518	0.222** (N%,PC%)	0.471
MMP-2 & MMP-9	AM(65)	41.31 (31.00)	136016.73 (113057.03)	1.33 (2.20)	18.50 (14.48)	44.80 (12.11)	35.37 (17.65)	0.368**	0.607	0.368** (H%,N%,PC%)	0.607
	BCC(55)	40.27 (29.65)	207015.52 (112695.65)	0.38 (1.30)	7.64 (12.91)	52.17 (13.62)	39.82 (18.80)	0.175*	0.418	0.138** (N%)	0.371
	TOTAL(120)	40.83 (30.27)	168557.84 (117895.85)	0.89 (1.89)	13.52 (14.76)	48.17 (13.29)	37.41 (18.24)	0.265**	0.514	0.263** (H%,N%,PC%)	0.513

* *p* value less than 0.05

** *p* value less than 0.01

Table 2: Stromal visual and computer assisted immunohistochemical scoring results

ST		HU% (SD)	COM					MR		SR	
	T (N=)		PC (SD)	H% (SD)	P% (SD)	L% (SD)	N% (SD)	R^2	R	R^2 (predictors)	R
MMP-2	AM(32)	33.75 (24.59)	209169.66 (129089.16)	0.46 (0.60)	6.28 (6.24)	38.79 (14.41)	54.47 (19.68)	0.136	0.369	-	-
	BCC(26)	16.15 (23.34)	160510.38 (86234.71)	0.07 (0.23)	2.01 (2.66)	23.91 (12.35)	74.01 (14.77)	0.307	0.554	0.196* (N%)	0.443
	TOTAL(58)	25.86 (25.41)	187356.88 (113667.69)	0.28 (0.51)	4.36 (5.38)	32.12 (15.35)	63.23 (20.06)	0.234**	0.483	0.208** (N%)	0.456
MMP-9	AM(33)	33.33 (29.98)	137776.38 (68084.49)	0.60 (1.27)	7.35 (8.60)	47.48 (16.45)	44.57 (22.16)	0.313*	0.560	0.224** (H%)	0.473
	BCC(29)	46.38 (23.45)	158181.20 (79239.71)	0.35 (0.75)	6.36 (10.17)	36.25 (15.07)	57.03 (22.59)	0.07	0.264	-	-
	TOTAL(62)	39.44 (27.13)	147320.57 (73615.38)	0.49 (1.06)	6.89 (9.31)	42.23 (16.68)	50.40 (23.05)	0.137	0.370	0.092* (H%)	0.304
MMP-2 & MMP-9	AM(65)	33.54 (26.70)	172923.84 (108089.00)	0.53 (0.99)	6.82 (7.50)	43.20 (15.97)	49.44 (21.40)	0.201**	0.448	0.172** (H%)	0.415
	BCC(55)	32.09 (27.73)	159282.26 (81852.97)	0.22 (0.58)	4.30 (7.86)	30.41 (15.07)	65.06 (20.95)	0.199*	0.446	0.194** (L%)	0.440
	TOTAL(120)	32.88 (27.07)	166671.45 (96800.32)	0.39 (0.84)	5.67 (7.74)	37.34 (16.77)	56.60 (22.51)	0.116**	0.340	0.105** (H%)	0.324

* *p* value less than 0.05
** *p* value less than 0.01

EPI: Epithelium, ST: Stroma, T: Tumor, AB: Ameloblastoma, BCC: Basal cell carcinoma, HU%: Human visual score, COM: Computer score, PC: pixel count, H%: percentage contribution of high positive pixels, P%: percentage contribution of positive pixels, L%: percentage contribution of low positive pixels, N%: percentage contribution of negative pixels, MR: Multiple regression, SR: Stepwise regression