Original Article

Validation of immunoexpression of tenascin-C in oral precancerous and cancerous tissues using ImageJ analysis with novel immunohistochemistry profiler plugin: An immunohistochemical quantitative analysis

Deepa Rajesh Mane, Alka D Kale, Chetan Belaldavar

Department of Oral Pathology and Microbiology, KLE University, KLE VK Institute of Dental Sciences, Belgaum, Karnataka, India

Abstract

Background: Immunohistochemistry (IHC) is a molecular technique that has grown tremendously over the years. However, the assessment is only qualitative which is subjective and causes errors. Due to this limitation, several excellent markers have not gained importance and reached clinical trials. Hence, we aimed to quantify IHC by ImageJ analysis with a novel IHC profiler plugin. ImageJ has not been tried in oral precancerous tissues with minimal attempt for matrix markers.

Aim: This study aimed to validate the quantification of immunoexpression of tenascin-C (TN-C) in oral precancerous tissues and oral squamous cell carcinoma (OSCC) using ImageJ software with IHC profiler plugin.

Materials and Methods: After IHC staining for TN-C and image acquisition, ImageJ analysis was performed as per the standard recommended algorithm. Assessment was done by two observers by blinding the histopathological diagnosis. The immunoscore was assessed for interobserver variability using Kohen's kappa statistics.

Results: All our cases were in agreement and found to be statistically significant with $P < 0.005$. Moderate agreement was for mild dysplasia, moderate dysplasia and oral lichen planus. Substantial agreement was for oral submucous fibrosis and OSCC and almost perfect agreement noted for cases of severe dysplasia.

Conclusion: IHC can now be quantified using freely downloadable software ImageJ analysis in oral precancerous tissues and OSCC. This software with good threshold control can quantify matrix marker such as TN-C. Hence, herewith, we propose that IHC markers should be quantified using ImageJ by our entire oral pathology fraternity so as to have a standard immunoscore for all markers.

Keywords: ImageJ, immunohistochemistry, immunohistochemistry profiler plugin, tenascin-C

Address for correspondence: Dr. Deepa Rajesh Mane, Department of Oral Pathology and Microbiology, KLE University, KLE VK Institute of Dental Sciences, Nehru Nagar, Belgaum - 590 010, Karnataka, India. E-mail: deeparajeshmane@gmail.com

Received: 02.03.2017, **Accepted:** 07.03.2017

INTRODUCTION

The immunohistochemical (IHC) technique is widely performed in formalin-fixed tissues. The applications of IHC on human tissues are the key determinants in various clinical and medical researches. Since the discovery of several biomarkers, IHC has taken over as the "Brown Revolution"

Access this article online

Quick Response Code:

Website: www.jomfp.in

DOI: 10.4103/jomfp.JOMFP_234_16

How to cite this article: Mane DR, Kale AD, Belaldavar C. Validation of immunoexpression of tenascin-C in oral precancerous and cancerous tissues using ImageJ analysis with novel immunohistochemistry profiler plugin: An immunohistochemical quantitative analysis. J Oral Maxillofac Pathol 2017;21:211-7.

from the past four decades. This revolution has lead to the development of several epithelial and connective markers, detected in nuclear, cytoplasm, membranous, or matrix markers. Some of the markers had contributed as diagnostic and some as prognostic determinants. The interpretation of IHC is based on overall staining intensity (0, +, ++, +++) and proportion of neoplastic tissue stained (0%–25%, 26%–75% and >75%).[1] It was first developed by McCarty *et al*.,[2] which was called H score and later modification of this was developed by Harvey *et al*. under the name Allred or quick score.[3] Nevertheless, IHC is considered as a special stain to detect particular marker, but "how much the stain is there?" relies only on qualitative analysis by an experienced pathologist. However, IHC analysis is now considered as a "semiquantitative" analysis, depicting some features of "quantitative" like semiprecious interpreted for not precious, but still remains inappropriate.[4]

Manual assessment of IHC markers with naked eye using light microscopy illustrates high variability in the assessment of staining quality and prone to error. The advancement of IHC has led to tissue microarrays, advantage of assessment of many markers simultaneously on one microarray. However, there still remains bias in estimation and it remains subjective, time consuming and expensive. To meet the larger cancer hospitals, these manual methods continue to be a nonstandard mode of interpretation.[5]

Contrary to this, many of the laboratory investigations performed are of quantitative assays, which can be easily correlated to clinical signs and symptoms such as blood sugar, hemoglobin estimation, calcium, hormone and vitamins levels. Most of these assays are considered to be gold standard, and clinical interventions are purely based on these laboratory results. Due to limitations in the interpretation of IHC, the available excellent markers are not able to assess as prognostic determinants or could reach the stage of clinical trials. To standardize the IHC interpretation, several computer-assisted staining estimations of IHC biomarkers in formalin-fixed tissues using software programs have been developed. The scoring calculations of these programs are based on modern cellular imaging systems. Some of the widely used software used for IHC analysis are TMARKER,[5] Multiplex IHC and Multispectral Image Analysis,[6] Spin Context Localization Method,[7] Micrometastasis Detection System (MDS),[8] AQU Analysis,[9] Fiji,[10] CMYK model,[11] VORSTAIN software (BC Cancer research center Vancover, Canada),[12] and many more.

However, many of these software systems are expensive or require hardware attachments for image capture and analysis. The quantification analysis by these methods depends on threshold adjustment, but this itself leads to variability and can become subjective. Moreover, many of these software are helpful in quantifying either nuclear or cytoplasmic immunoexpression. Till date, evaluation of connective tissue/matrix markers has not been attempted using software. Hence, to overcome these limitations, we explored quantifying immunoexpression of matrix molecule tenascin-C (TN-C) using free open software ImageJ (ACTREC, Navi Mumbai, India) analysis with a novel plugin IHC profiler.

MATERIALS AND METHODS

A total of 100 cases of oral precancerous and cancerous tissues were included in the present study. It includes twenty cases each of mild dysplasia, moderate dysplasia, oral submucous fibrosis and oral lichen planus and ten cases each of severe dysplasia and oral squamous cell carcinoma (OSCC). The present study is a part of a major project to evaluate the role of TN-C among 550 cases of oral precancerous and cancerous tissues after appropriate sample size calculation. The present sample includes only validating the ImageJ analysis for IHC profiler plugin.

Immunohistochemistry protocol

The sections were taken on gel-coated slides and were stained with IHC using PolyExcel/horseradish peroxidase/diaminobenzidine (DAB) Detection System (Cat# No.: PEH2-6 ml, Genepulse Scientific, Bengaluru, India) and rabbit monoclonal anti-TN-C antibody (EPR4219, ab108930, Abcam) at 1:100 dilution with phosphate-buffered saline. Slides were deparaffinized, dehydrated through graded alcohols and rinsed with distilled water. For every step of IHC, tris-phosphate buffer was used as the wash buffer (Genepulse Cat# No.: PS006). Excess wash buffer was removed by blotting with tissue paper and care was taken to prevent drying of the sections. Endogenous peroxidase activity was blocked by incubating the slides with 3% H_2O_2 for 10 min. Antigen retrieval was performed by heat-induced epitope retrieval using citrate buffer (Genepulse, Cat# No.: PS007) at pH 6.0, at 96°C for three cycles in an EZ Retrieval Microwave (BioGenex, Hyderabad, India). Sections were removed from the citrate buffer, cooled to room temperature and then incubated for 1 h with primary antibody. Sections were then incubated with the biotinylated secondary antibody with polymerase chain background for 30 min in a humidified chamber. To visualize the reaction, slides were incubated with DAB for 10 min, and then counterstained with Harris hematoxylin for 30 s, followed by bluing in running tap water. Finally, the slides were dehydrated, dipped in xylene and mounted.

Image acquisition

Images were captured using binocular Leica research light microscope (Leica™ DM2500) at bright field. Images were captured at ×10 magnification using CCD color video camera (Leica DFC320) attached to a computer system. The field was selected with a good contrast of DAB chromogen and hematoxylin which is considered region of interest. All the images were acquired using Leica application software version 3.5.0 (Germany) which was installed within the computer. Before capturing the images, the color density and white balance were standardized for all images. All the acquired images were saved as JPEG format.

ImageJ analysis

The ImageJ is a free software, downloaded from the internet of the recent version of ImageJ 1.48 version (NIH, Bethesda, Maryland) (Java 1.8.9_66). After IHC staining for TN-C by a standard recommended protocol and image acquisition, ImageJ analysis was performed. Validation of ImageJ analysis for matrix protein TN-C was performed by two observers who are experienced pathologists. Quantification of immunoscore of all the images was blinded for histopathological diagnosis. Prior to start up with the analysis, both the observers gained knowledge in the use of ImageJ with IHC profiler plugin and the controlling of threshold level was skilled and maintained without much adjustments by both the observers. After being skilled in the use of software, standard recommended protocol as per Varghese *et al.* was followed.[13]

The installed ImageJ software was opened; saved image was dragged and inserted in the software. In the tool bar, "plugins" was opened and clicked for IHC profiler plugin [Figure 1]. Then, a dialog box opens with the options of cytoplasmic-stained image or nuclear-stained image. These are two operation modes which are newly installed in IHC profiler plugin. The cytoplasmic mode selects only DAB cytoplasmic stain and it does not have any option for selecting the area of stained part of the image. The nuclear mode not only selects the nuclear-stained DAB, but it also selects any part of the image with DAB immunoreaction. All our images are of matrix immunoexpression of TN-C, either expressed predominantly at epithelial connective tissue interface in precancerous tissues or around the tumor islands. Hence, we worked with nuclear mode which has a selection option. After clicking the nuclear mode, the dialog box for color deconvolution opens with the vector H DAB, and then the image installed gets deconvoluted into three red, green, blue channels of images with separation of only hematoxylin, only DAB immunoreaction image and other image with only highlighted DAB immunoreaction. The threshold was maintained standard, without any adjustment. In the edit tool, the option "create selection" was clicked; the highlighted DAB immunoreaction got selected [Figure 2]. In the plugin option, IHC macro was pressed to get the quantification of immunoreaction as a log score of high positive, positive, low positive, negative, final core and also with histogram [Figure 3]. All the deconvoluted images, histogram and log score were saved as JPEG images in a separate folder [Figures 4-7]. The quantified immunoscore was entered into an Excel spreadsheet. The validation of immunoscore of both the observers was done using SPSS software version 22 (Standard statistical analysis software) by implementing Kohen's kappa statistics (slight agreement: 0–0.2, fare agreement: 0.21–0.4, moderate agreement: 0.41–0.6, substantial agreement: 0.61–0.8 and almost perfect agreement: 0.81–1), with $P < 0.005$ was considered statistically significant.

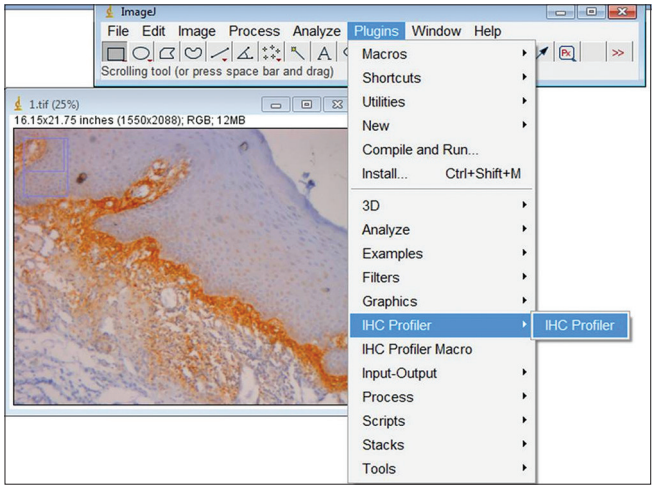

Figure 1: Photomicrograph of the ImageJ software showing immunohistochemistry profiler plugin

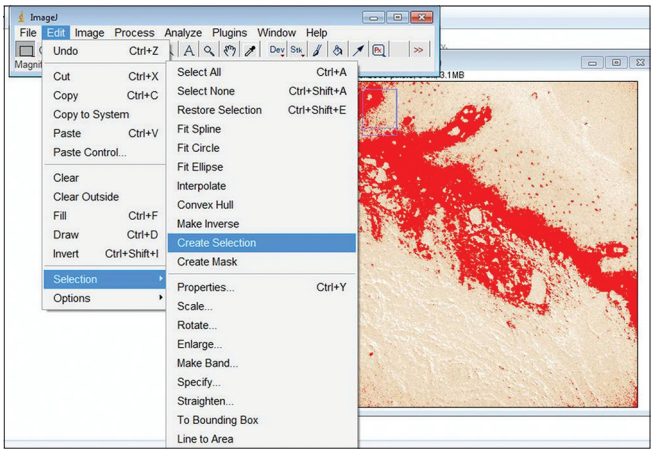

Figure 2: Photomicrograph of the ImageJ software showing the selection of "create selection" option

RESULTS

On observing the interobserver variability using kappa values of quantified immunoscore of TN-C, we observed that all our cases were in agreement and found to be statistically significant with $P < 0.005$. Moderate agreement was for mild dysplasia, moderate dysplasia and oral lichen planus. Substantial agreement was for oral submucous fibrosis and OSCC and almost perfect agreement was noted for cases of severe dysplasia. Hence, from this inference, we could substantiate that immunoscore of matrix protein TN-C can be estimated using ImageJ analysis with IHC profiler plugin [Figures 4-7 and Table 1].

DISCUSSION

ImageJ software is a freely available program which is based on Java public domain image processing system which was first developed at the National Institute of Health Rasband *et al*.[13] This software is considered to be the standard software for quantification of markers. ImageJ has been modified, and several plugins have been incorporated to aid in wider applicability. One such plugin is IHC profi ler with IHC profiler macro which helps in quantifying the immunoexpression of markers.[13] TN-C is one of the large extracellular matrix proteins, which plays a very important role in cancer progression, neoangiogenesis and metastasis. TN-C has also reached the stage of clinical trials in other systemic malignancy, but it has been not much explored in oral precancer and OSCC.[14,15] As ImageJ is widely used for cancerous tissues, moreover it has not been used for connective tissue markers, we aimed to validate TN-C by ImageJ in oral precancer and cancer tissues.

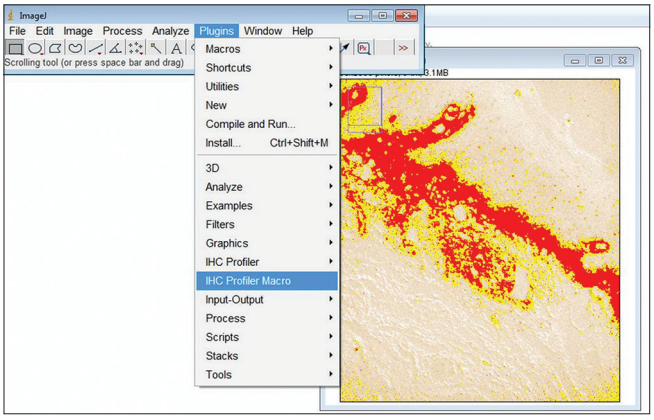

Figure 3: Photomicrograph of the ImageJ software showing the selection of immunohistochemistry profiler macro option

It has been suggested that in the context of effective disease management, the value of clinical test is very important. Similarly, by detecting the amount of estrogen receptor in breast cancer can help identify patients who can be beneficial with the hormonal therapy. Due to controversial facts in

Table 1: The Validation of Immunoexpression of TN-C in Oral Pre Cancer and Cancer using Image J Analysis with IHC Profiler Plugin

Cases	Number of cases	Kappa Value	P value	Agreement
Mild Dysplasia	20	0.502	< 0.001	Moderate
Moderate Dysplasia	20	0.583	<0.009	Moderate
Severe Dysplasia	20	1	<0.001	Almost Perfect
OSMF	20	0.667	<0.001	Substantial
OLP	20	0.414	0.003	Moderate
OSCC	20	0.615	0.035	Substantial

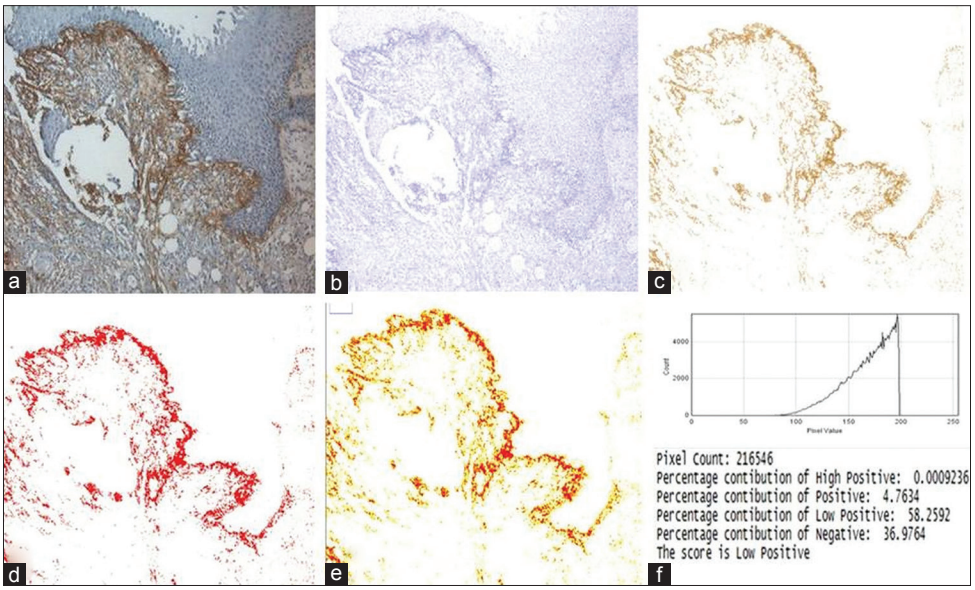

Figure 4: Photomicrograph of immunohistochemistry image of tenascin-C (a), deconvoluted images (b-d), highlighted and selected immunoexpression (e), histogram and log of quantified immunoexpression of tenascin-C in oral epithelial dysplasia (f)

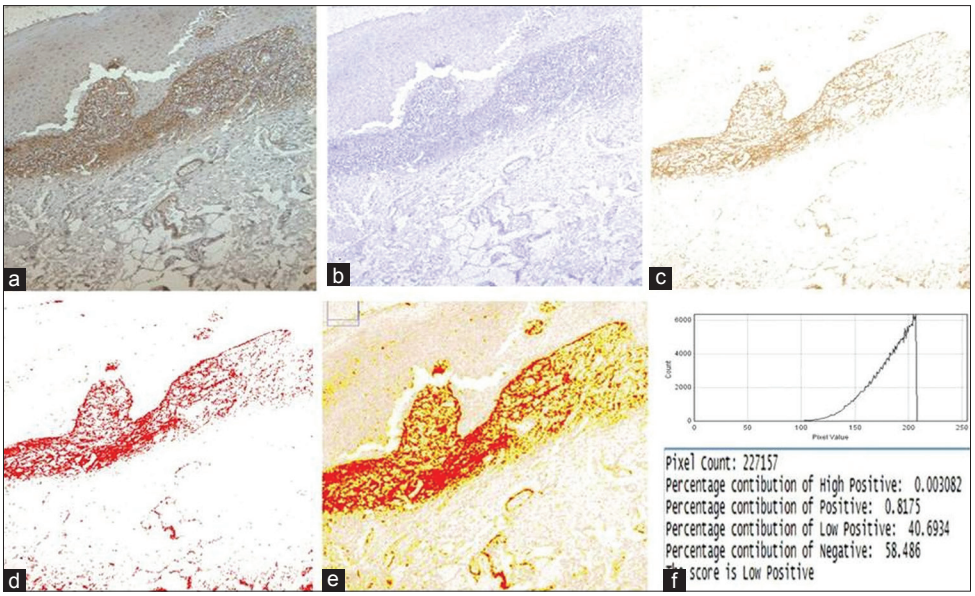

Figure 5: Photomicrograph of immunohistochemistry image of tenascin-C (a), deconvoluted images (b-d), highlighted and selected immunoexpression (e), histogram and log of quantified immunoexpression of tenascin-C in oral lichen planus (f)

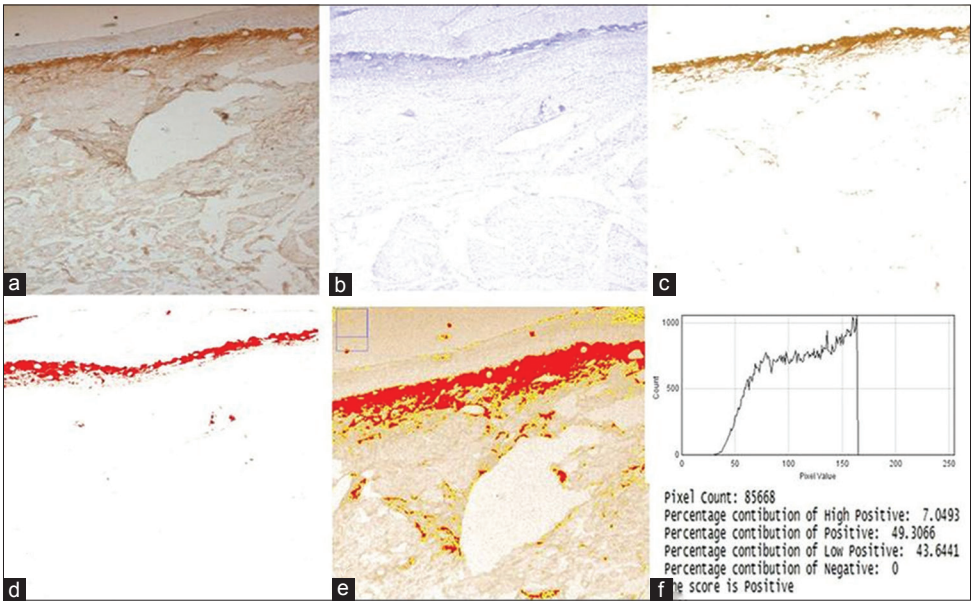

Figure 6: Photomicrograph of immunohistochemistry image of tenascin-C (a), deconvoluted images (b-d), highlighted and selected immunoexpression (e), histogram and log of quantified immunoexpression of tenascin-C in oral submucous fibrosis (f)

manual interpretation of IHC markers, hormonal therapy has not been successful in all patients.[16] Moreover, the standardization of IHC analysis is the need of biomedical research. In a very short period of time within a year, novel automated unsupervised algorithm was tried on estrogen and progesterone receptors' expression in the breast cancer tissue based on MatLab 7 (Mathworks, Apple Hill Drive, MA, USA). The authors were successful in assessing larger cohort breast cancer patients and proposed that this algorithm could achieve new prognostic and predictive values for hormonal therapy. Hence, this digital novel approach has proposed for the future as a personalized medicine.[17]

Digital scoring systems can be standardized and implemented for uniform immunoscore with minimal interobserver variability. Ellis *et al*. analyzed human epidermal growth factor receptor 2 status in breast cancer by standard HercepTest, fluorescence *in situ* hybridization (FISH) and digital scoring by MDS™. They found that MDS system is reliable and accurate compared to visual scoring and FISH scoring. Fuhrich *et al*. compared the digital histological score (D-HSCORE) using ImageJ with manual HSCORE for endometrial β3 integrin expression by IHC. They observed that even the less experienced researcher could efficiently

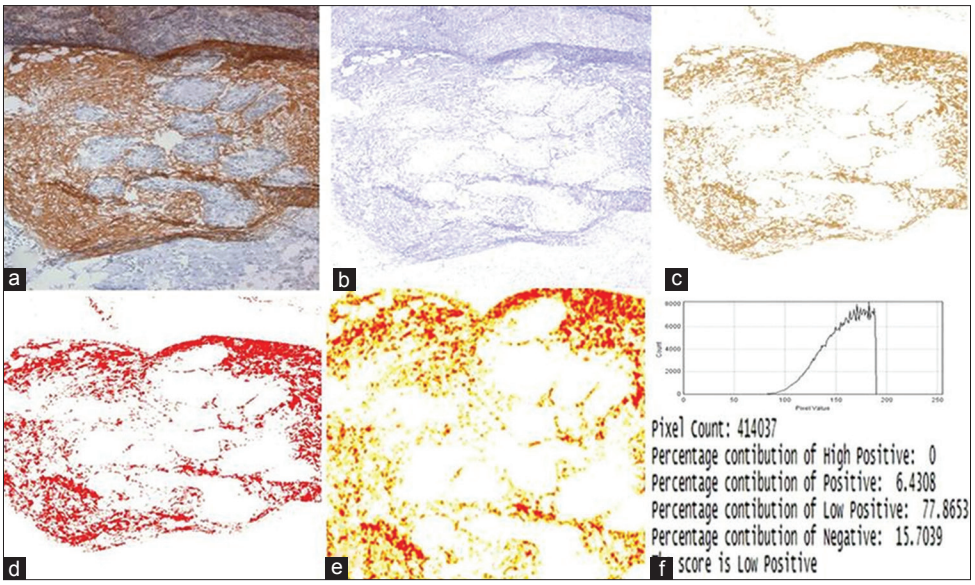

Figure 7: Photomicrograph of immunohistochemistry image of tenascin-C (a), deconvoluted images (b-d), highlighted and selected immunoexpression (e), histogram and log of quantified immunoexpression of tenascin-C in oral squamous cell carcinoma (f)

score D-SCORE using ImageJ with 0% interobserver variation, whereas 50% variability was found in manual HSCORE.[18] The present research is in accordance with the literature with agreement between both the pathologists from moderate, substantial to almost perfect agreement with statistical significance. We also observed that ImageJ can also be used for precancerous tissues and also for matrix markers such as TN-C. We also observed that the intensity of immunoexpression of TN-C can be quantified even if the score is low positive in precancerous and cancerous tissues, but the score was found to be in higher denomination in OSCC [Figures 4f, 5f, 6f and 7f]. Discrepancies observed by manual scoring can be improvised by digital scoring for intensity of expression of all IHC markers.

Currently, there are several platforms for digital scoring systems as mentioned earlier, but appropriate control of threshold is very important. Helmy and Azim in their research to determine the efficacy of ImageJ in the assessment of apoptosis suggested that ImageJ is one of the standard useful tools and contributes a lot in spite of being free software.[19] IHC profiler plugin which is compatible to ImageJ software showed an accuracy of 88.6% compared to manual scoring. It has also been suggested that, in the view of biological variations in the human tissue samples with slight discrepancies in the staining protocol, still this percentage is excellent in applicability in research. As most of the available software have been tried with only single marker, this IHC profiler plugin has been tried with multiple markers with multiple malignancies.[20] Moreover, the threshold can be controlled with this novel plugin so as to minimize interobserver variability. Nevertheless, ImageJ can be used efficiently for all nuclear, cytoplasmic and also for matrix markers, but it has only limitation in its use for membrane-immunoexpressed markers, and the percentage of expression of markers cannot be assessed.

CONCLUSION

Our research is the first of its kind in the implementation and validation of ImageJ with IHC profiler plugin which has been till now not tried in precancerous tissues and also for assessment of matrix markers. Hence, by our present research, we propose that:

- ImageJ analysis with IHC profiler plugin can be used even in precancerous tissues and also can be implemented in the assessment of matrix markers
- Nevertheless, TN-C, the molecule which is a multifunctional and mysterious protein, which can be assessed by its functional role and prompt its application in clinical trials, can now be uniformly assessed throughout the world by ImageJ analysis with IHC profiler plugin
- There are tremendous research projects which are based on IHC in the field of dentistry, especially in the specialization of oral pathology. Hence, we would like to propose that ImageJ with IHC profiler plugin can be widely used in our entire oral pathology fraternity so as to have a standard scoring system with very minimal interobserver variability in analyzing IHC markers on formalin-fixed tissues
- IHC markers can now be uniformly assessed for intensity by ImageJ with IHC profiler plugin for all

kinds of nuclear, cytoplasmic and promising matrix markers such as TN-C.

Acknowledgment

We would like to acknowledge Dr. Praveen Arany, Professor at University of Buffalo, for recommending the usage of ImageJ analysis and providing the study material and software to initiate our study. We also sincerely thank Amirali Bhukhari, Abhijit De and Chetan Ahire from Advanced Centre for Training, Research and Education in Cancer, Navi Mumbai, who have developed IHC profiler plugin and also we thank them for training the authors for the use of this software.

Financial support and sponsorship

The authors declare that this project is a part of funded project by the Vision Group on Science and Technology, Government of Karnataka, India. This grant was under the seed money for young scientist researcher (SMYSR) 2014, Grant Number: GRD 430.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981;29:577-80.
2. McCarty KS Jr., Szabo E, Flowers JL, Cox EB, Leight GS, Miller L, *et al*. Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. Cancer Res 1986;46 8 Suppl:4244s-8s.
3. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 1999;17:1474-81.
4. Taylor CR, Levenson RM. Quantification of immunohistochemistry – Issues concerning methods, utility and semiquantitative assessment II. Histopathology 2006;49:411-24.
5. Schüffler PJ, Fuchs TJ, Ong CS, Wild PJ, Rupp NJ, Buhmann JM. TMARKER: A free software toolkit for histopathological cell counting and staining estimation. J Pathol Inform 2013;4:S2.
6. Oguejiofor K, Hall J, Slater C, Betts G, Hall G, Slevin N, *et al*. Stromal infiltration of CD8 T cells is associated with improved clinical outcome in HPV-positive oropharyngeal squamous carcinoma. Br J Cancer 2015;113:886-93.
7. McKenna SJ, Amaral T, Akbar S, Jordan L, Thompson A. Immunohistochemical analysis of breast tissue microarray images using contextual classifiers. J Pathol Inform 2013;4:S13.
8. Ellis CM, Dyson MJ, Stephenson TJ, Maltby EL. HER2 amplification status in breast cancer: A comparison between immunohistochemical staining and fluorescence *in situ* hybridisation using manual and automated quantitative image analysis scoring techniques. J Clin Pathol 2005;58:710-4.
9. Klimowicz AC, Bose P, Nakoneshny SC, Dean M, Huang L, Chandarana S, *et al*. Basal Ki67 expression measured by digital image analysis is optimal for prognostication in oral squamous cell carcinoma. Eur J Cancer 2012;48:2166-74.
10. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, *et al*. Fiji: An open-source platform for biological-image analysis. Nat Methods 2012;9:676-82.
11. Shu J, Dolman GE, Duan J, Qiu G, Ilyas M. Statistical colour models: An automated digital image analysis method for quantification of histological biomarkers. Biomed Eng Online 2016;15:46.
12. Guillaud M, Matthews JB, Harrison A, MacAulay C, Skov K. A novel image cytometric method for quantitation of immunohistochemical staining of cytoplasmic antigens. Anal Cell Pathol 1997;14:87-99.
13. Schneider CA, Rasband WS, Eliceiri KW. NIH image to imageJ: 25 years of image analysis. Nat Methods 2012;9:671-75.
14. Midwood KS, Hussenet T, Langlois B, Orend G. Advances in tenascin-C biology. Cell Mol Life Sci 2011;68:3175-99.
15. Chiquet-Ehrismann R, Tucker RP. Connective tissues: Signalling by tenascins. Int J Biochem Cell Biol 2004;36:1085-9.
16. Nadji M. Quantitative immunohistochemistry of estrogen receptor in breast cancer: "Much ado about nothing!" Appl Immunohistochem Mol Morphol 2008;16:105-7.
17. Rexhepaj E, Brennan DJ, Holloway P, Kay EW, McCann AH, Landberg G, *et al*. Novel image analysis approach for quantifying expression of nuclear proteins assessed by immunohistochemistry: Application to measurement of oestrogen and progesterone receptor levels in breast cancer. Breast Cancer Res 2008;10:R89.
18. Fuhrich DG, Lessey BA, Savaris RF. Comparison of HSCORE assessment of endometrial beta3 integrin subunit expression with digital HSCORE using computerized image analysis (ImageJ). Anal Quant Cytopathol Histpathol 2013;35:210-6.
19. Helmy IM, Azim AM. Efficacy of ImageJ in the assessment of apoptosis. Diagn Pathol 2012;7:15.
20. Varghese F, Bukhari AB, Malhotra R, De A. IHC profiler: An open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. PLoS One 2014;9:e96801.