International Journal of Medical Science and Clinical Invention 7(12): 5162-5168, 2020
DOI:10.18535/ijmsci/v7i12.06 **ICV: 77.2**
e-ISSN:2348-991X, p-ISSN: 2454-9576

Research Article

Digital Image Analysis of Immunohistochemistry CD103 Using QuPath Software in Acral Melanoma

Fauzan Ali Zainal Abidin[1*], Hermin Aminah Usman[1], Sri Suryanti[1], Bethy Suryawathy Hernowo[1]

[1]Department of Anatomical Pathology, Faculty of Medicine, Universitas Padjadjaran/Hasan Sadikin General Hospital, Bandung, Indonesia

Abstract

Background: Nowadays, pathology services are more developed for quantitative diagnostic evaluation. The quantitative diagnostic evaluation requires detailed accuracy and can be done using digital image analysis (DIA). Assessment of the CD103 in acral melanoma needs to be done quantitatively. A visual microscopy evaluation of CD103 has difficulties in counting CD103 positive lymphocytes. These difficulties are due to the expression of CD103, which is very little, whereas the field of view of the tumor mass is considered quite exhaustive when viewed under a microscope. The evaluation of CD103 could be done digitally with the DIA technique to overcome these difficulties. The DIA technique is carried out automatically by counting the lymphocytes that express CD103 with bioimage analysis software. QuPath is one of the bioimage analysis software, has characteristics of cross-platform, intended for bioimage analysis and digital pathology. This study aims to compare the CD103 count by visual microscopy evaluation and DIA in acral melanoma.

Methods: This study was a cross-sectional study. CD103 immunostained slides of 40 cases of acral melanoma were eyeballing assessed by two pathologists and automated digital images analyzed by one pathologist using QuPath.

Results: Wilcoxon signed ranks test showed no significant difference between the visual microscopy evaluation and DIA of CD103 count (p = 0,580).

Conclusions: Digital image analysis using QuPath can be used to count the CD103 automatically.

Keywords: CD103, digital image analysis, melanoma, QuPath.

Introduction

The development of pathology is high-speed. Previously pathology services prioritized qualitative diagnostic based on holistic pathological judgment and relatively limited clinical information. At present, pathology services are more developed for diagnostics that are semi-quantitative and quantitative evaluations of pathological conditions and biomarker expression.[1] In this case, anatomical pathology becomes more quantitative or analytical. Therefore, the diagnostic process needs more accuracy.[2, 3] There are many imaging analysis softwares developed for better diagnostic accuracy to carry out various types of quantitative pathology diagnostic tasks and research.[1, 4]

One of the quantitative evaluation of the expression of the biological markers is to assess tumor-infiltrating lymphocytes. Tumor-infiltrating lymphocytes of solid malignant tumors are an important prognostic factor.[5] This can be applied in solid tumors such as breast cancer[6], colorectal cancer[7], and melanoma.[8] Melanoma is a malignancy originating from the skin's melanocyte system. This tumor is the most aggressive of all skin malignancies.[9] Acral melanoma is one of the rare and the most aggressive subtypes of melanoma. Many prognostic factors for acral melanoma are known, and numerous prognostic models have been developed in an attempt to predict which patients ultimately will develop advanced disease.[10] One of the prognostic factors is the immunoexpression of CD103 in the tumor-infiltrating lymphocytes.[11]

CD103 is an immunological factor in the tumor microenvironment that plays a role in controlling

melanoma progression.[11] Evaluation of CD103 immunoexpression is carried out quantitatively by counting the lymphocytes that express CD103 in tumor-infiltrating lymphocytes.[11, 12] There are two evaluation techniques, visual microscopy evaluation and digital image analysis. Because of the critical role of CD103 as a useful prognostic factor, the reproducibility of the evaluation of CD103 is fundamental.[11, 13] Kim et al.[13], in their research, showed that the reproducibility of CD103 using visual microscopy evaluation was not optimal because of the high interobserver variability. Thus, it is necessary to evaluate the CD103 with digital image analysis with bioimage analysis softwares.

Some softwares for analysis of digital imaging Ki67 are commercially available. However, they require considerable costs to obtain the software. Generally, they can only be used on computers with certain specifications.[2, 4] In addition to commercial software, researchers have developed software that can be used freely (freeware) for digital imaging analysis, one of which is QuPath.[14] QuPath is a cross-platform, open-source software developed at the University of Edinburgh, aimed at biological imaging analysis and quantitative digital pathology.[14] QuPath supports all types of computer operating systems. This software was developed as an application that can be used on Windows-based computers, Mac OS X, and Linux to support many applications and various imaging files for pathology and bioscience analysis. The QuPath open-source software platform[14] was used to analyze the CD103 immunoexpression in this study. The QuPath software can be downloaded at https://qupath.github.io/.[14]

This study aims to determine the differences between visual microscopy evaluation and digital image analysis of CD103 counting using QuPath software in acral melanoma.

Material and Method

The Ethics Committee of the Hasan Sadikin General Hospital, Bandung, approved this study's ethical clearance (LB.02.01/X.6.5/123/2020).

The samples are CD103 stained preparations from patients diagnosed histopathologically with acral melanoma in Hasan Sadikin General Hospital, Bandung, Indonesia. A total of 40 CD103 immunostained slide samples were consecutively selected from January 2014 until April 2020.

CD103 staining

The acral melanoma tissue in paraffin blocks was consecutively selected from the Anatomical Pathology department Hasan Sadikin Hospital, Bandung, Indonesia. The tissue was cut with a thickness of 4µm using a microtome, then depleted with xylol, rehydrated with decreased ethanol concentration, then immersed in PBS in 15 minutes (3x5 minutes). The tissue pieces were then incubated in Dako Antigen Retrieval Buffer (Dako, Santa Clara, California, USA) in microwave 94°C for 20 minutes and followed by cooling at room temperature for 20 minutes and washed with PBS for 15 minutes (3x5 minutes), then incubated in Block Peroxidase for 10 minutes, PBS for 10 minutes and followed by incubation in CD103 rabbit monoclonal antibody (clone EP206, Cell Marque, Burlington, USA, at 1:50 dilution) at 4 ° C. After incubation with CD103, the preparations were then re-incubated with secondary antibodies Labeled Polymer HRP (Dako, Santa Clara, California, USA) for 60 minutes at room temperature. Lastly, the counterstain phase was stained with Hematoxylin Meyer, then dehydrated with increased ethanol concentration, purification in xylol then the slide was covered with a cover glass.

Imaging process

The imaging process used the whole slide scan technique with dotSlide Ver.2.0 software (Olympus, Tokyo, Japan). Imaging was carried out using a slide scanner with microscope Olympus BX51 model equipped with an Olympus XC10-IR color camera connected directly to a computer with a 32-bit Microsoft Windows XP SP 2 operating system. The processor used is Intel Pentium 4 and 1,016 GB of random access memory (RAM). A total of 40 CD103 virtual slides were obtained and stored on the computer. The CD103 virtual slides were then analyzed using QuPath software on a computer with macOS operating systems. The processor used is 2.9 GHz Intel Core i5 and 8Gb of RAM.

Visual microscopy evaluation

CD103 immunoexpression was assessed qualitatively. Evaluation of CD103 immunoexpression was carried out by counting the number of CD103 positive tumor-infiltrating lymphocytes under a light microscope. The CD103 positive lymphocytes have brown color on the cell membrane. Counts were carried out in 5

large visual fields (400x) that were randomly selected in areas with large numbers of tumor-infiltrating lymphocytes.

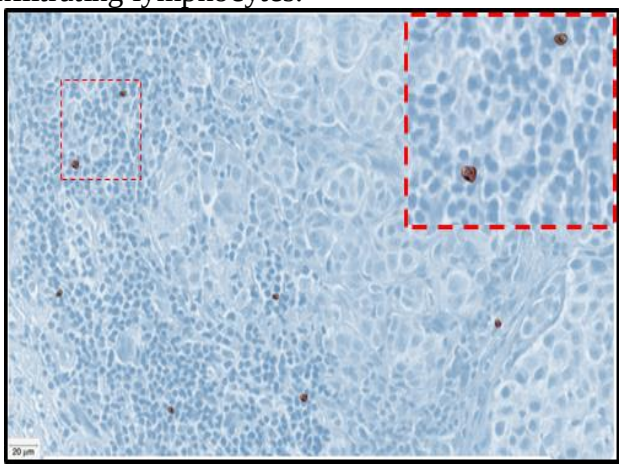

Figure 1. Visual microscopy evaluation to count CD103 positive lymphocytes in the tumor-infiltrating lymphocytes. Inset: 2 CD103 positive lymphocytes are identified.

Digital image analysis

The dotSlide Ver. 2.0 platform (Olympus, Tokyo, Japan) was used at ×20 and ×40 to digitize the slides with a pixel size of 0.2500 µm × 0.2500 µm. The QuPath open-source software platform was used to build an automated TIL scoring algorithm. We used watershed cell detection[15] to segment the cells in the image with the following settings: Detection image: hematoxylin OD; requested pixel size: 0.5 µm; background radius: 8 µm; median filter radius: 0 µm; sigma: 1.5 µm; minimum cell area: 10 µm2; maximum cell area: 400 µm2; threshold: 0.1; maximum background intensity: 2. The quality control of the cell segmentation was performed by a pathologist. In order to classify detected cells into tumor cells, immune cells (TILs), stromal cells, and others (false detections, background), we used neural network[16] as a machine-learning method with eight hidden layers (maximum iterations: 100). The last step was done by selecting the *analyze*, *cell analysis*, and *positive cell detection* buttons. Then the *cell detection parameters* dialog box appeared. The parameters used for calculating the CD103 immunoexpression in a study by Bankhead et al.[14] After the positive cell detection parameters were entered, the *run* button was clicked. QuPath will count automatically and display the number of CD103 positive lymphocytes.

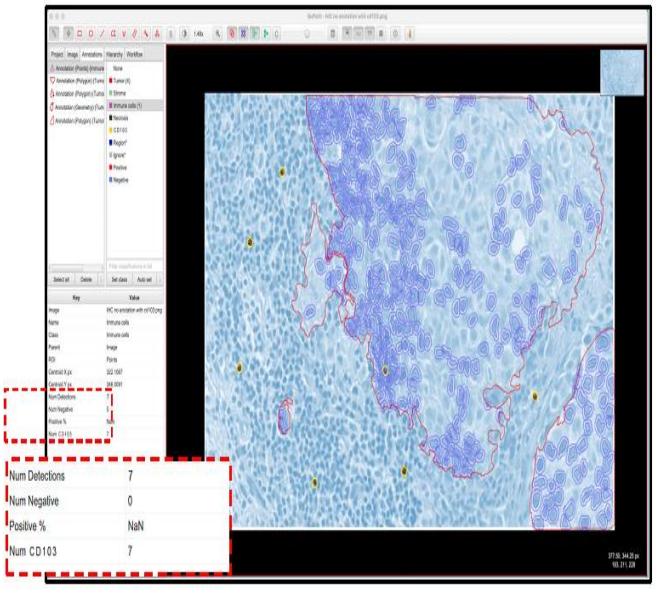

Figure 2. Digital image analysis with QuPath to count CD103 positive lymphocytes in the tumor-infiltrating lymphocytes. Inset: Total 7 CD103 positive lymphocytes are identified in this field of view.

Statistical analysis

Statistical analysis was performed using SPSS Version 23 for macOS (IBM, New York, NY, USA). Analysis of CD103 count by visual microscopy evaluation and digital image analysis were subjected to normality test. It was then subjected to a nonparametric test with Wilcoxon signed ranks test; *p*-values ≤ 0.05 was assumed to be statistically significant. The H_O hypothesis is that there is no significant difference in the results of the CD103 counting obtained by visual microscopy evaluation compared to digital image analysis.

Result

Visual microscopy evaluation and digital image analysis of CD103

The visual microscopy evaluation and digital image analysis of CD103 positive lymphocytes counting are shown in Table 1. The Shapiro-Wilk normality test (0.890, p=0.001) was found significant, so data were considered nonparametric. The median of CD103 positive lymphocytes from visual microscopy evaluation was 20, and the median of CD103 positive lymphocytes from digital image analysis was 20. The range of both methods was 0-37.

Table 1. The counting results CD103 positive lymphocytes with visual microscopy evaluation and digital image analysis

Sample No.	Number of CD103 calculated by VME	Number of CD103 calculated by DIA
1	0	2
2	5	5
3	6	7
4	5	5
5	7	7
6	2	4
7	3	4
8	5	5
9	20	20
10	0	2
11	32	30
12	31	30
13	22	22
14	6	6
15	22	20
16	23	22
17	24	24
18	20	20
19	5	6
20	25	24
21	5	5
22	7	7
23	7	7
24	0	0
25	22	22
26	28	29
27	27	27
28	22	22
29	37	37
30	26	26
31	24	24
32	35	35
33	20	20
34	5	6
35	25	25
36	24	24
37	22	22
38	24	23
39	8	8
40	5	5

VME, visual microscopy evaluation; DIA, digital image analysis.

Table 2. The result of the Wilcoxon test

	Median (Minimum-Maximum)	*P*
Number of CD103 by VME	20 (0-37)	0.580
Number of CD103 by DIA	20 (0-37)	

Wilcoxon test, 6 subjects negative ranks, 26 ties, and 8 subjects positive ranks. VME, visual microscopy evaluation; DIA, digital image analysis.

The statistical analysis Wilcoxon test on visual microscopy evaluation and digital image analysis of CD103 count is shown in Table 2. The table shows that the medians of VME group and DIA group were 20. A Wilcoxon Signed-rank test shows no significant difference between Group ($Z = -0.553$, $p > 0.05$). In other words, there is no significant difference between the results of the CD103 count by digital image analysis to visual microscopy evaluation ($p = 0.580$).

Discussion

Digital image analysis in pathology is one of the methodologies in computer-assisted pathology.[17] The approach of assessing digital images of histological slides is gaining momentum in today's laboratory environment. Indeed, most digital image acquisition systems are becoming routine, and associated image analysis solutions are regarded by most as the next significant level in automated histological analysis.[2]

Although pathologist's assessment is the gold standard in diagnosis, there are many warnings with this method. For individuals, fatigue may potentially be a problem in a profession always in demand. These problems are no more a problem with machines, which may be used to increase the output of histological assessment.[4, 17] It is fully recognized, however, that automated image analysis may never progress into a diagnostic circumstance because of the natural differences between individual slides and the importance of an accurate diagnosis. Another major problem relates to IHC staining and its evaluation.[17] IHC staining can vary between slides and between antibodies[2, 17], whereas specimen fixation and processing conditions may cause incompatibility

between samples[14]. This problem suggests an essential condition to standardize staining procedures and sample preparation protocols before using automated image analysis techniques. This problem is specifically vital in clinical situations where IHC testing assists in therapeutic decision-making, such as in breast cancer. There is also often variability between evaluators as another problem of the pathologist's assessment. For example, each pathologist must take up their semi-quantitative scoring system (commonly numerically describing staining as 0, 1+, 2+, or 3+), based on the darkest/lightest staining in the set of slides they are viewing. This method of the semi-quantitative scoring system leads to inconsistencies between pathologists because this approach is simply subjective.[17, 18]

Automated image analysis procedures are beginning to be commonly used for biomarker evaluation in a research context due to the difficulty in the manual scoring of IHC, as discussed previously. This difficulty circulates the IHCtechnique'ss reproducibility as a whole and the human eye's capability to distinguish between 1+ and 2+ scores. Computational evaluations offer the potential to solve this issue for the quantification of the IHC staining.[2, 4, 17]

CD103, also known as integrin αEβ7 (ITGAE), is a transmembrane heterodimer complex that is involved in cell to cell or cell to matrix interaction.[19] CD103 mediates cell adhesion, migration, and lymphocyte homing of the cell through interaction with E-cadherin, which is expressed in epithelial cells.[19] Therefore, CD103 is considered a hallmark of a specific subset of immune cells that resides within mucosal organs" epithelium, including the genital tract, stomach, lung, and skin. The immune cells that express CD103 include effector memory CD8+ T cells. Several studies suggest that CD103 positive TIL in human malignancy is associated with prognosis[11, 13,] and CD103 is a marker of T lymphocyte resident memory that can be detected in acral melanoma tumor-infiltrating lymphocytes. Thus, standardization of the CD103 immunoexpression evaluation is considered necessary because of its impact on prognosis.[13]

The results in this study show that QuPath can be used to calculate the CD103 positive lymphocytes automatically very reliable. Automatic counting by digital image analysis has advantages in determining the time needed to count the CD103+ lymphocytes. Lymphocytes can be hard to identify because the field of view is commonly vast, and the melanin pigment in melanoma slides can obscure the lymphocytes. The time needed to calculate the CD103 positive lymphocytes by visual microscopy evaluation is 15-30 minutes for one slide, depending on tumor size. The time needed by QuPath to calculate CD103 in one whole slide is only 30 seconds. Thus, large amounts of imaging data can be quickly and easily analyzed.[20, 21] Another advantage of automated digital image analysis is minimizing interobserver variability between observers when done visually under a microscope. So that QuPath can be used as an alternative software for biological imaging analysis, especially in the field of histopathology because its use is relatively more manageable with a simple user interface.[17, 21]

Conclusion

Digital imaging analysis techniques play an essential role in the quantitative evaluation of the CD103 in acral melanoma.

Conflict Of Interest

The authors declare that there is no conflict of interest.

Acknowledgements

The researcher would like to thank the Head and staff of the Anatomic Pathology Department, Medical Faculty Padjadjaran University, Hasan Sadikin General Hospital, who gave permission and a place to conduct this study and assisted with laboratory examinations.

References

[1] Kayser G, Kayser K. Quantitative pathology in virtual microscopy: history, applications, perspectives. Acta histochemica. 2013;115(6):527-32.

[2] Laurinavicius A, Laurinaviciene A, Dasevicius D, Elie N, Plancoulaine B, Bor C, et al. Digital image analysis in pathology: benefits and obligation. Anal Cell Pathol (Amst). 2012;35(2):75-8.

[3] Madabhushi A, Lee G. Image analysis and machine learning in digital pathology: Challenges and opportunities. Med Image Anal. 2016;33:170-5.

[4] Webster JD, Dunstan RW. Whole-slide imaging and automated image analysis: considerations and opportunities in the

practice of pathology. Vet Pathol. 2014;51(1):211-23.

[5] Hendry S, Salgado R, Gevaert T, Russell PA, John T, Thapa B, et al. Assessing Tumor-infiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal for a Standardized Method From the International Immunooncology Biomarkers Working Group: Part 1: Assessing the Host Immune Response, TILs in Invasive Breast Carcinoma and Ductal Carcinoma In Situ, Metastatic Tumor Deposits and Areas for Further Research. Advances in anatomic pathology. 2017;24(5):235-51.

[6] Stanton SE, Disis ML. Clinical significance of tumor-infiltrating lymphocytes in breast cancer. Journal for immunotherapy of cancer. 2016;4:59.

[7] Narayanan S, Kawaguchi T, Peng X, Qi Q, Liu S, Yan L, et al. Tumor Infiltrating Lymphocytes and Macrophages Improve Survival in Microsatellite Unstable Colorectal Cancer. Sci Rep. 2019;9(1):13455.

[8] Lee N, Zakka LR, Mihm MC, Jr., Schatton T. Tumour-infiltrating lymphocytes in melanoma prognosis and cancer immunotherapy. Pathology. 2016;48(2):177-87.

[9] Elder DE, Bastian BC, Cree IA, Massi D, Scolyer RA. Melanocytic tumour classification and the pathway concept of melanoma pathogenesis. In: Elder D, Massi D, Scolyer RA, Willemze R, editors. WHO Classification of Skin Tumours. 4 ed. Lyon: International Agency for Research on Cancer; 2018. p. 66-71.

[10] Desai A, Ugorji R, Khachemoune A. Acral melanoma foot lesions. Part 1: epidemiology, aetiology, and molecular pathology. Clinical and experimental dermatology. 2017;42(8):845-8.

[11] Edwards J, Wilmott JS, Madore J, Gide TN, Quek C, Tasker A, et al. CD103(+) Tumor-Resident CD8(+) T Cells Are Associated with Improved Survival in Immunotherapy-Naive Melanoma Patients and Expand Significantly During Anti-PD-1 Treatment. Clinical cancer research: an official journal of the American Association for Cancer Research. 2018;24(13):3036-45.

[12] Acs B, Ahmed FS, Gupta S, Wong PF, Gartrell RD, Sarin Pradhan J, et al. An open source automated tumor infiltrating lymphocyte algorithm for prognosis in melanoma. Nature communications. 2019;10(1):5440.

[13] Kim Y, Shin Y, Kang GH. Prognostic significance of CD103+ immune cells in solid tumor: a systemic review and meta-analysis. Sci Rep. 2019;9(1):3808.

[14] Bankhead P, Loughrey MB, Fernandez JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. Sci Rep. 2017;7(1):16878.

[15] Abdolhoseini M, Kluge MG, Walker FR, Johnson SJ. Segmentation of Heavily Clustered Nuclei from Histopathological Images. Sci Rep. 2019;9(1):4551.

[16] Ertosun MG, Rubin DL. Automated Grading of Gliomas using Deep Learning in Digital Pathology Images: A modular approach with ensemble of convolutional neural networks. AMIA Annual Symposium proceedings AMIA Symposium. 2015;2015:1899-908.

[17] Madabhushi A, Lee G. Image analysis and machine learning in digital pathology: Challenges and opportunities. Med Image Anal. 2016;33:170-5.

[18] Shaw EC, Hanby AM, Wheeler K, Shaaban AM, Poller D, Barton S, et al. Observer agreement comparing the use of virtual slides with glass slides in the pathology review component of the POSH breast cancer cohort study. J Clin Pathol. 2012;65(5):403-8.

[19] Hardenberg JB, Braun A, Schön MP. A Yin and Yang in Epithelial Immunology: The Roles of the α(E)(CD103)β(7) Integrin in T Cells. The Journal of investigative dermatology. 2018;138(1):23-31.

[20] Kostopoulos S, Cavouras D, Daskalakis A, Bougioukos P, Georgiadis P, Kagadis GC, et al. Colour-texture based image analysis method for assessing the hormone receptors status in breast tissue sections. Conf Proc IEEE Eng Med Biol Soc. 2007;2007:4985-8.

[21] Volynskaya Z, Mete O, Pakbaz S, Al-Ghamdi D, Asa S. Ki67 quantitative interpretation: Insights using image analysis. Journal of Pathology Informatics. 2019;10(1):8.