Development of an Automatic Measurement Method for CD8 and PD-1 Positive T Cells Using Image Analysis Software

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Abstract. *Background/Aim:* With the progress in cancer immunotherapy using immune checkpoint blockade (ICB) therapy, histological observations of tumor-infiltrating lymphocyte (TIL) status are needed to evaluate the antitumor effect of ICB using imaging analysis software. *Materials and Methods:* Formalin-fixed paraffin-embedded sections obtained from colorectal cancer and gastric cancer patients with more than 500 single nucleotide variants were stained with anti-CD8 and anti-PD-1 antibodies. Based on our own algorithm and imaging analysis software, an automatic TIL measurement method was established and compared to the manual counting methods. *Results:* In the CD8⁺ T cell number measurement, there was a good correlation ($r=0.738$ by Pearson test) between the manual and automated counting methods. However, in the PD-1⁺ T cell measurement, there was a large difference in TIL numbers in both groups. After adjustment of

the parameter settings, the correlation between the manual and automated methods in the PD-1⁺ T cell measurements improved ($r=0.668$ by Pearson test). *Conclusion:* An imaging software-based automatic measurement could be a simple and useful tool for evaluating the therapeutic effect of cancer immunotherapies in terms of TIL status.

Since the clinical applications of immune checkpoint blockade (ICB) therapy have had great success in various cancer patients, the number of clinical trials focused on novel therapies, including the combination of ICB with other targeting agents, have been increasing (1, 2). The greater the number of ICB-based clinical trials, the more difficult visual inspections by trained pathologists have become for the evaluation of immune effects by ICB, such as tumor-infiltrating lymphocyte (TIL) measurement and programmed death-ligand 1 (PD-L1) staining.

In fact, many pathologists have intensively studied methods to enumerate TIL numbers accurately and efficiently and demonstrated that a well-functioning immune-scoring system could contribute to determine the TIL status of tumors (3-6). However, manual visual inspections are time-consuming and require manual labor; therefore, an efficient and user-friendly automatic immunohistochemistry (IHC) imaging analysis system is urgently needed. Specifically, since the development of whole slide scanners that can generate ultralarge 2D images in the field of digital pathology, open source bioimage analysis software has been developed, such as ImageJ, Fiji, Icy, and CellProfiler (7-10). However, in terms of high variability and limited reproducibility, it has been difficult for computational researchers to develop state-of-the-art imaging software

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available worldwide. Recently, to solve these problems novel comprehensive opensource software, QuPath (11) has been developed for analyzing a substantial amount of whole slide imaging data mainly in Western countries (12). These developed digital image-analysis software programs are considered to improve the discrepancy in both intra- and interobserver visual analyses in terms of accuracy and reproducibility (13).

In the present study, we developed a novel automatic IHC TIL-measurement algorithm based on WinROOF imaging software (Mitani Corporation, Tokyo, Japan) and different approaches from QuPath software that previously used and compared CD8⁺ and PD-1⁺ T cell numbers in formalin-fixed paraffin-embedded (FFPE) specimens in terms of visual and automatic measurements.

Materials and Methods

Patient characteristics and clinical specimen. Shizuoka Cancer Center launched Project HOPE in 2014 using multiomics analyses including whole exome sequencing (WES) and gene expression profiling (GEP). Ethical approval for the HOPE study was obtained from the Institutional Review Board of Shizuoka Cancer Center (Authorization Number: 25-33). Fifty-two clinical specimens derived from HOPE-registered hypermutator tumors with more than 500 single nucleotide variants (SNVs), which consisted of 26 gastric cancers and 26 colorectal cancers, were obtained and FFPE sections were made and used for IHC analysis.

Immunohistochemistry. For TIL staining, anti-CD8 and anti-programmed death (PD)-1 antibodies (Thermo Fisher Scientific, Waltham, MA, USA) were purchased and used for immunohistochemistry analysis as primary antibodies.

Slides were incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody, and after washing, they were incubated with 3,39-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich Co., St. Louis, MO, USA) until color development. The slides were then counterstained with hematoxylin, and observed under a light microscope (IX71N-23TFL/DIC, Olympus, Tokyo, Japan) equipped with a digital camera (DP70-SET-B, Olympus) and image analysis software (WinROOF, Mitani Corporation). In each section stained with the various antibodies, 10 visual images at high-magnification (200X) were captured and analyzed using WinROOF image-analysis software. The manual counting of stained TILs was performed two times by the reviewer according to the semiquantitative estimation of the density score (14) and the mean value was determined.

CD8 and PD-1 gene expression analysis using quantitative PCR. Real-time PCR analysis of CD8 and PD1 genes using a QuantStudio 12K Flex (Applied Biosystems, Foster City, CA, USA) was performed as described previously (15). Total RNA was isolated from the FFPE specimens of 15 colorectal cancers and 15 gastric cancers with hypermutations (more than 500 SNVs). Specific PCR primers for *CD8B*, *PDCD1*, and *GAPDH* were used. The correlation of manual count number or automatic count number of CD8⁺ and PD-1⁺ cells with mRNA gene expression level, shown as delta-delta Ct values in a real time PCR, was investigated using a Pearson correlation test.

Development of an automated measurement process using an established WinROOF-based algorithm. A flow chart demonstrating the manual and automatic T cell measurement processes is shown in Figure 1. Five visual images out of 10 captured images were selected at random. The reviewer performed manual counting of CD8⁺ or PD-1⁺ T cell numbers two times in each section. After counting, the mean positively stained cell number per visual field was determined. Meanwhile, regarding the automatic measurement of positively stained cells, the process is shown in Figure 2. From the automatic setting in CD8⁺ T cell determination, the setting of automatic PD-1⁺ T cell measurement was adjusted to the manual measurement level of PD-1⁺ T cell number.

Color conversion from RGB to HSL (hue, saturation and lightness). In brief, after identifying the cancer areas of the images, DAB-stained reddish color was converted from RGB to HSL. Joblove *et al.* defined another color space or coordinate system other than RGB for color expression or manipulation, and introduced the HSL color space for hue, saturation, and lightness (16). Specifically, the maximum (MAX) value and the minimum value (MIN) of RGB were identified. In the cases of different MAX values, different H values were calculated and the S and L values were determined as follows:

- ① if MAX=R then $H=60 \times (G-B)/(MAX-MIN)$
if MAX=G then $H=120 + 60 \times (B-R)/(MAX-MIN)$
if MAX=B then $H=240 + 60 \times (R-G)/(MAX-MIN)$
- ② $L'=(MAX+MIN)/2$
if $L' \leq 127$ then $S'=(MAX-MIN)/(MAX+MIN)$
if $127 < L'$ then $S'=(MAX-MIN)/(510-MAX-MIN)$
- ③ $S=100 \times S'$, $L=(100/255) \times L'$

Cell radius. The diameter of T cells was approximately 12 μ m, and considering that the cleaved surface of the cell was smaller than that of the large circle, the diffusion of the staining range, 5 to 15 μ m, was set as the diameter range.

Roundness. To separate the deformed dyeing range, assuming that it is elliptical, the circularity was calculated as follows (17):

$$(x/a)^2 + (y/b)^2 = 1 \text{ (Elliptical equation)}$$

Where a is the major axis radius and b is the minor axis radius. When a/b exceeds a certain range, it is judged as multiple.

Statistical analysis. A correlation analysis of the CD8⁺ and PD-1⁺ cell numbers between manual and automatic measurements from IHC specimens of 52 hypermutator tumors with more than 500 SNVs was performed by the Pearson correlation test using EZR software (18) and Microsoft Excel. Values of $p < 0.05$ were considered statistically significant.

Results

Comparison between manual counting and automatic counting in CD8⁺ T cell measurement. The CD8⁺ T cell numbers between manual and automatic counting were compared. Automatic measurement was performed at the first parameter setting adjusted to the manual counting number of CD8⁺ T cells. There were significant correlations

Figure 1. Schematic of the manual and automatic image analysis procedures. Manual measurement was performed two times for each section, and the mean value was determined. Automatic measurement was performed by our own specific algorithm based on WinROOF-based imaging analysis.

Figure 2. Schematic of the automatic measurement procedures based on our own specific algorithm. The process consists of color conversion, segmentation by color thresholding, compactness and elongation, cell size determination, checking roundness etc.

between manual and automatic counting numbers in gastric cancer and colorectal cancer groups (Figure 3A and B).

Accordance and discrepancy between manual and automatic counting numbers at the first parameter setting. The CD8⁺ T cell number per visual field obtained from the automatic measurement was similar to that obtained from manual counting, as shown in Figure 4A. However, the automatically counted PD-1⁺ T cell number showed a great difference from the manual counting at the first parameter setting. Therefore, the parameter setting was readjusted to the manual PD-1⁺ T cell number condition, referred to as the second parameter setting (Figure 4B).

Improvement in the correlation between manual counting and automatic counting in PD-1⁺ T cell measurement after readjustment of the parameter setting. The PD-1⁺ T cell numbers between manual and automatic counting were compared. Automatic measurement was performed at the second parameter setting adjusted to the manual counting number of PD-1⁺ T cells. There was a significant improvement in the correlations between manual and automatic counting numbers in the gastric cancer and colorectal cancer groups (Figure 5).

Significant correlation between manual counting and automatic counting in CD8⁺ or PD-1⁺ T cell measurements of hypermutator tumors. The significant correlations between the manual and automatic counts of CD8⁺ or PD-1⁺ T cells in all 52 hypermutator tumors are shown in Figure 6. Each automatic T cell measurement was performed at the first or second parameter setting. The coefficient values were 0.738 (A: CD8⁺ cells) and 0.669 (B: PD-1⁺ cells).

Association of automatic or manual count numbers with mRNA gene expression levels. Regarding gastric cancers, CD8 gene expression was well-associated with both automatic and manually counted numbers; however, PD-1 gene expression had a greater association with manually counted numbers than with automatically counted numbers. Regarding colorectal cancers, neither the automatic count nor the manual count showed a significant association with CD8 and PD-1 gene expression levels (Table I).

Discussion

With advances in cancer immunotherapy using ICB, many novel combination immunotherapies based on ICB and targeting therapeutics have been developed in various stages of

Figure 3. Comparison between manual counting and automatic counting in CD8⁺ T cell measurement. The CD8⁺ T cell numbers between manual and automatic counting were compared. Automatic measurement was performed at the first parameter setting adjusted to the manual counting number of CD8⁺ T cells. (A) Colorectal cancers (red circles), (B) gastric cancers (blue circles). A correlation analysis was performed by the Pearson test. * $p < 0.05$, statistically significant.

Figure 4. Representative colorectal cancer cases were used for the current staining of CD8⁺ or PD-1⁺ T cells. The image shown is a part of the whole image of a single visual field at 200 $\times$, and each counted number of CD8⁺ or PD-1⁺ T cells in the automatic and manual measurements shows the cell number of the whole visual field. The automatic measurement in images A and B is based on the first parameter setting, while the automatic measurement in image C is based on the second parameter setting. SNV# 10543 in A and 1965 in B.

Figure 5. Comparison between the manual counting and automatic counting in PD-1⁺ T cell measurements. Automatic measurement was performed at the second parameter setting adjusted to the manual counting number of PD-1⁺ T cells. (A) Colorectal cancers (red circles), (B) gastric cancers (blue circles). A correlation analysis was performed by the Pearson test. * $p < 0.05$, statistically significant.

Figure 6. The significant correlation between the manual counting and automatic counting in CD8⁺ or PD-1⁺ T cell measurements of hypermutator tumors. Significant correlations between manual and automatic counts of CD8⁺ (A) or PD-1⁺ T cells (B) in 52 hypermutator tumors are shown. Each automatic T cell measurement was performed at the first (A) or second (B) parameter setting. Colorectal cancers are represented by red circles, and gastric cancers are represented by blue circles. A correlation analysis was performed by the Pearson test. * $p < 0.05$, statistically significant.

Table I. Association of auto- or manual count number with mRNA gene expression level.

Groups	Auto-count vs. qPCR	Manual-count vs. qPCR
Gastric cancer (CD8)	$r=0.862$ ($p<0.001^*$)	$r=0.790$ ($p<0.001^*$)
Colorectal cancer (CD8)	$r=0.185$ ($p=0.356$)	$r=0.142$ ($p=0.480$)
All cancers (CD8)	$r=0.271$ ($p=0.052$)	$r=0.226$ ($p=0.056$)
Gastric cancer (PD-1)	$r=0.322$ ($p=0.117$)	$r=0.747$ ($p<0.001^*$)
Colorectal cancer (PD-1)	$r=-0.071$ ($p=0.727$)	$r=-0.229$ ($p=0.251$)
All cancers (PD-1)	$r=0.132$ ($p=0.350$)	$r=0.630$ ($p<0.001^*$)

The correlation of manual count number or automatic count number of CD8⁺ and PD-1⁺ cells with mRNA gene expression levels shown as delta Ct values in a real time PCR, was investigated using a Pearson correlation test. * $p<0.01$.

Table II. Imaging software-based automatic IHC measurement methods.

Authors	Year	Imaging system (software)	Algorithms	Target markers	Descriptions	Ref No.
Varghese <i>et al.</i>	2014	The AxioVisionV4.6 software (Zeiss, Germany)	IHC profiler: quantitative scoring (https://sourceforge.net/projects/ihcprofiler/)	Various many	Comparison study between manual pathological analysis and IHC Profiler resolved in a match of 88.6% in various cancers	[26]
Wanram <i>et al.</i>	2016	The Aperio product, a combination of the Genie [®] Histology Pattern Recognition tool	The Membrane Quantification V9 cellular analysis tool	HLA-class I and tapasin	Significant association between conventional and automated IHC evaluation for MHC class I and Tapasin was shown in 96 cervical cancers	[27]
Mane <i>et al.</i>	2017	Leica application software version 3.5.0 (Germany)	Free open software ImageJ (ACTREC, Navi Mumbai, India)	Tenascin-C	IHC can be quantified using ImageJ analysis software in oral precancerous tissues and OSCC	[28]
Fernandez-Carrobles <i>et al.</i>	2017	The Aperio ScanScope XTScanner	A combination of Macenko's method, Reinhard's method and Non-linear spline mapping method	12 biomarkers	The tests carried out with 8000 bresat cancer TMA images provided an average of 95.94% accuracy	[29]
Tewary <i>et al.</i>	2017	Leica DMi1 microscope (resolution 1,048×768 pixels) and Leica DM750 microscope (resolution 2,048×1,536 pixels)	Machine learning model (random forest), automated Allred score generation	ER and PR	Random forest shows the best correlation with the expert's score (Pearson's correlation coefficient=0.9192) in 50 bresat cancers	[30]
Brown <i>et al.</i>	2019	Developer XD software (Definiens; Munich, Germany)	Segmentation of CTLA-4+ cells was carried out and then applying a color deconvolution to isolate the purple-stained pixels	CTLA-4, FoxP3, CD4, CD8	Image analysis scoring was highly comparable with pathologist scoring for CTLA-4 and CTLA-4/FoxP3 assays	[24]
Chlipala <i>et al.</i>	2020	The Aperio ScanScope XT imaging system (Aperio, Vista, CA) and accompanying Image-Scope software	The annotated areas were analyzed using a modified DAB specific positive pixel count algorithm	10 markers	The image analysis output correlated well with pathology scores	[31]
Yoo <i>et al.</i>	2020	Slides were subsequently scanned by an Aperio AT2 slide scanner (Leica Biosystems)	An open-source software [QuPath (https://qupath.github.io)]-based analytic method established. Using a random forest classifier, TIL numbers were quantified	CD3, CD8	Machine-learning-based image analysis can be useful for extracting quantitative information about the TIME using whole-slide histopathologic images	[25]
Miyata <i>et al.</i>	2021	Winroof with digital camera system (DP70-SET-B, Olympus)	Our own	CD8, PD-1	Automatic measurement of TIL in 56 hypermutator tumors	Ours

clinical trials (1, 2, 19). In addition to the clinical evaluation of responders and adverse effects, methods for making histological evaluations of tumor tissues, namely, the tumor microenvironment, have not been fully developed. In particular, the development of suitable imaging analysis software focusing on TIL enumeration has not yet been successful; thus, manual measurements of TIL numbers after ICB therapy are becoming an increasingly large load to working pathologists. Additionally, high variability among observers and limited reproducibility are inevitable challenges to overcome (20).

Recently, novel imaging analysis systems contributing to automatic IHC evaluation without manual labor by pathologists, such as ImageJ, Fiji, Icy, CellProfiler, QuPath, and deep learning methods, have been studied at a clinical research level in the field of digital pathology (21). In particular, QuPath is a new bioimage analysis software designed to meet the growing need for a user-friendly, extensible, open-source solution for digital pathology and whole slide image analysis. These analysis systems seem to be somewhat expensive to ordinary pathological laboratories in terms of the setup of imaging scanner equipment (22).

In the present study, we established a simple automatic TIL number measurement method based on parameter settings adjusted to manually measured TIL numbers using IHC sections of hypermutator tumors.

First, we focused on the enumeration of CD8⁺ and PD-1⁺ T cells inside tumor tissues. The reason is that the CD8⁺PD-1⁺ T cell population is well known as exhausted effector T cells, and a high accumulation of CD8⁺PD-1⁺ T cells inside the tumor is a hallmark of good responders to ICI therapy and good prognosis (23).

Second, we used specific parameter settings suitable for each target protein and tried to adjust the parameter many times to manually measured numbers. We observed that the parameter setting may depend on the different staining patterns of each protein, even for markers expressed on the same T cell, such as CD8 and PD-1. Specifically, the first parameter setting suitable for CD8⁺ T cell counting did not perform well in PD-1⁺ T cell counting, therefore we made an adjustment to the second parameter setting.

Third, we aimed to develop automatic measurements specific to TIL counting. In recent years, the development of imaging analysis algorithms has mainly focused on positivity and intensity determination for cancer-associated biomarkers in IHC specimens. The few studies that were involved in the development of automatic TIL number measurements are shown in Table II (24-31).

Brown *et al.* reported that image analysis scoring based on a color deconvolution algorithm was highly comparable with pathologist scoring for CTLA-4 and CTLA-4/FoxP3 staining of regulatory T cells (24). Meanwhile, Yoo *et al.* demonstrated that machine-learning-based image analysis such as QuPath can be useful for extracting quantitative information about the

TMIT (tumor microenvironment immune types) using whole-slide histopathologic images (25).

Regarding the correlation analysis between manual and automatic measurements of TIL numbers, our method is comparable to other methods reported in Table II. Regarding the correlation between automatic and manual counting in CD8⁺ TILs, our efficiency by Pearson's test was 0.738, while the efficiency in the study by Yoo *et al.* (25) was 0.657–0.707. These results suggest that a commercially available imaging analysis software-based simple automatic measurement system for TIL numbers can be worthwhile to ameliorate hard manual labor, such as manual TIL counting by pathologists. Additionally, we investigated the association of the automatic or manual count number of CD8⁺ and PD-1⁺ T cells with the mRNA gene expression levels measured by real-time PCR (Table I). Regarding the TIL numbers in gastric cancers, CD8 gene expression was well-associated with both automatically and manually counted numbers; however, PD-1 gene expression had a higher association with manually counted numbers than with automatically counted numbers. These results suggest that the parameter condition in the automatic measurement of PD-1⁺ cells still needs to be improved.

Moreover, beyond IHC imaging analysis, genetic TIL number semiquantitative enumeration based on highly advanced single-T cell RNA sequencing and cy-time-of-flight (TOF) mass spectrometry technologies has been developed efficiently (32, 33) and can be applicable to TIL status evaluation before and after ICI therapy; however, because of the expensiveness of this professional analysis system, it will take a long time until the RNA-seq system is used at the clinical level.

Finally, based on the successful result of our own automatic TIL measurement, we will apply the automatic method to immunofluorescent CD8 and PD-1-stained specimens beyond IHC specimen analysis. Therefore, we will be able to improve the accuracy and utility by developing a multicolor analysis system on the same specimen.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

HM and YA participated in the design of the study and drafting of the manuscript and were responsible for competing the study. AI, CM, AK, KW and TA performed immunological experiments including immunohistochemistry. TN, RK, AN and Keita Mori were responsible for the statistical analysis and reviewed the manuscript. AS and MT participated in collecting clinical specimens and KU and KO were responsible for genetic analysis of clinical samples. TK, Koji Muramatsu and TS were responsible for preparing pathological specimens for TIL analysis. KY reviewed the manuscript. All Authors read and approved the final draft.

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