DIGITAL IMAGE ANALYSIS OF IMMUNOHISTOCHEMISTRY Ki67

USING QuPath SOFTWARE IN BREAST CANCER

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**Abstracts**

**Background:** Today, pathology services are more developed to the quantitative diagnostic evaluation. This requires detailed accuracy and can be done by using digital image analysis (DIA). Evaluation of Ki67 labelling index (LI) in breast carcinoma needs to be done quantitatively. A visual evaluation of Ki67 LI using light microscopy has high inter-observers variability. To overcome this, evaluation Ki67 could be done digitally with DIA technique by counting the Ki67 LI manually or automatically with the help of bioimage analysis software. QuPath is one of the bioimage analysis software, has characteristic of cross-platform, intended for bioimage analysis and digital pathology. **Objective**: The purpose of this study is to compare the manual and automatic calculation of Ki67 LI digitally. **Methods**: This study was a cross sectional study, total of 240 digital Ki67 images from 30 slides were analyzed by counting manually and automatically using QuPath. **Result**: Statistical analysis using T-test showed no significant difference between the manual and automatic counting of Ki67 LI (p = 0,801, α = 0,05). **Conclusion:** Digital image analysis using QuPath can be used to calculate the Ki67 LI automatically.

Keywords: Ki67, digital image analysis, QuPath, breast cancer

*ANALISIS PENCITRAAN DIGITAL IMUNOHISTOKIMIA KI67 DENGAN PERANGKAT LUNAK QUPATH PADA KANKER PAYUDARA*

***Latar Belakang****: Saat ini pelayanan patologi lebih berkembang kepada dignostik bersifat evaluasi kuantitatif. Untuk itu diperlukan akurasi yang detil dengan menggunakan analisis pencitraan digital. Evaluasi nilai indeks Ki67 pada karsinoma payudara perlu dilakukan secara kuantitatif. Evaluasi indeks Ki67 secara visual menggunakan mikroskop cahaya memiliki nilai variabilitas antar pengamat yang cukup tinggi. Untuk mengatasinya, maka dapat dilakukan teknik analisis pencitraan digital terhadap evaluasi indeks Ki67 baik dengan penghitungan manual atau otomatis dengan bantuan perangkat lunak. QuPath adalah perangkat lunak lintas-platform yang ditujukan untuk analisis pencitraan biologis dan patologi digital.* ***Tujuan****: Tujuan penelitian ini adalah untuk mengetahui perbedaan hasil penghitungan manual dan otomatis nilai indeks Ki67 secara digital.* ***Metode:*** *Penelitian ini menggunakan desain potong lintang. Jumlah total sampel sebanyak 240 pencitraan Ki67 digital dari 30 preparat yang dianalisis menggunakan QuPath secara manual dan otomatis.* ***Hasil:*** *Analisis statistik menggunakan Uji T menunjukkan tidak terdapat perbedaan secara signifikan antara hasil penghitungan nilai indeks Ki67 secara manual dibandingkan dengan otomatis (p = 0,801, α = 0,05).* ***Kesimpulan:*** *Analisis pencitraan digital dengan menggunakan QuPath dapat digunakan dengan baik untuk menghitung nilai indeks Ki67 secara otomatis.*

*Kata kunci: Ki67, analisis pencitraan digital, QuPath, kanker payudara*

**INTRODUCTION**

The development of pathology is very fast. Previous pathology services prioritized on qualitative diagnostic based on holistic pathological judgment and relatively limited clinical informations. At present, pathology services are more developed to dignostics that are semi-quantitative and quantitative evaluations of pathological conditions and biomarker expression. In this case, anatomical pathology becomes more quantitative or analytical. Therefore, more accuracy is needed in the diagnostic process.1 To carry out various types of quantitative pathology diagnostic tasks and researchs, there are many imaging analysis softwares developed for better diagnostic accuracy.2

One of the quantitative evaluation of the biological markers expression is to assess cell proliferation activity. Cell proliferation activity of malignant tumors is an important prognostic factor that has a role in the management of both operative and non-operative therapy. This also applies to breast cancer in order to determine between tumors that have high proliferation or low proliferation.3,4 Dowsett et al5 in their study have recommended the use of Ki67 as a marker of proliferation especially in breast cancer.5,6 Ki67 is a protein expressed in all phases of the cell cycle except the G0 phase.4,5 Proliferating tumor cells show a positive reaction in the cell nucleus against Ki67 antibodies. This characteristic makes Ki67 the best marker to be detected by immunohistochemical techniques in various types of malignant tumors including breast cancer. Various studies provide evidence that a high Ki67 index value indicates an increased risk of recurrence, metastasis, and rapid development of breast cancer.4,7

Ki67 Labelling Index (LI) is the percentage value of the distribution of tumor cells that are positively stained by Ki67 antibodies. Evaluation of Ki67 staining results is generally carried out semi-quantitatively by pathologists using a light microscope at 40X magnification. Evaluation of the Ki67 LI in this way is called visual evaluation by calculating the number of brown (positive) tumor cells from 1000 tumor cells both blue (negative) and brown under a light microscope.8 Because of the important role of Ki67 mentioned above, the reproducibility of the evaluation Ki67 LI is very important. Voros et al9, in his research, showed that the reproducibility of the Ki67 LI that was visually evaluated under a microscope was not optimal because of the high inter-observer variability. Thus, it is necessary to evaluate the Ki67 LI quantitatively which can be done digitally with the help of bioimage analysis software. This technique is called Digital Image Analysis (DIA).

Some softwares for analysis of digital imaging Ki67 are commercially available, but they require considerable costs to obtain the software, and generally can only be used on computers with certain specifications.2,10 In addition to commercial software, researchers have developed software that can be used freely (freeware) for the purposes of digital imaging analysis, one of which is QuPath. QuPath is a cross-platform software aimed at biological imaging analysis and quantitative digital pathology. 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 types of imaging files for pathology and bioscience analysis.6

This study aims to determine the differences in the results of manual and automatic calculation of Ki67 LI performed digitally using QuPath software.

**METHODS**

Ethical clearance was approved by Ethics Committee of the Hasan Sadikin General Hospital, Bandung (LB.02.01/X.6.5/159/2017).

The samples are Ki67 stained preparations from patients diagnosed histopathologically as invasive breast carcinoma in Hasan Sadikin General Hospital Bandung. Total 30 Ki67 stained slide preparations samples were randomly selected from period July 2017 until December 2017.

**Ki67 staining**

The breast cancer tissue in paraffin blocks were randomly selected from the Anatomical Pathology department Hasan Sadikin Hospital 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 in microwave 940C for 20 minutes and followed by cooling at room temperature for 20 minutes and washed with PBS 15 minutes (3x5 minutes), then incubated in Block Peroxidase for 10 minutes, PBS 10 minutes and followed by incubation in Ki67 antibodies (at 40C). After incubation with Ki67, the preparations were then re-incubated with secondary antibodies (Labeled Polymer HRP (Dako Cytomation)) for 60 minutes at room temperature. Lastly, the counter stain 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**

Each Ki67 preparation is divided into 8 compartments, then from each compartment a 400X magnification field is selected that is consistent in one area in each compartment. The chosen field of view is in the right area of the compartment (Figure 1).

Imaging was carried out at 400X magnification using a light microscope Olympus BX5 equipped with a digital camera Olympus XC10 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). Digital imaging of Ki67 preparations was taken using dotSlide software with a resolution of 2560 × 1920 pixels at 8 bit color depth and saved as Joint Photographic Experts Group (JPEG) format. A total of 240 Ki67 images at 400X magnification were obtained and stored on the computer. Ki67 imaging is 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.

**Figure 1**. Compartments on Ki67 slide

**Manual counting of Ki67 digital image analysis**

Imaging taken from each preparation were analysed by QuPath. Manual counting, firstly, is done by activating the show grid to facilitate the cell counting area. Then select the point tool so that the counting panel appears. To start counting cells is done by clicking the cursor on the positive tumor cells. Ki67 positive tumor cells are marked with red points. After finishing clicking positive tumor cells, then add a new annotation to the counting panel to calculate the negative tumor cell Ki67. Click the cursor on the negative tumor cell in the same way as before, the negative tumor cell Ki67 is marked with a blue dot (Figure 2).

The number of positive and negative tumor cells will be displayed on the counting panel. The results of the Ki67 LI digital imaging by manual calculation are obtained by the formula:

$$Manual Ki67 LI=\frac{P}{P+N} × 100\%$$

P = The number of Ki67 positive tumor cells

N = The number of Ki67 negative tumor cells

**Figure 2**. Manual counting of Ki67 digital imaging

**Automatic counting of Ki67 digital image analysis**

Imaging of Ki67 from the same preparation, which has been calculated manually, then calculated automatically with the QuPath application by selecting the Analyze → Cell analysis → Positive cell detection button. Then the Cell Detection Parameters dialog box will appear. The parameters used are in accordance with those used for calculating the Ki67 index in a study by Bankhead et al.6 After the positive cell detection parameter is entered, then click Run.

QuPath will display the number of Ki67 positive tumor cells, the number of negative tumor cells Ki67, and the results of digital Ki67 imaging automatic counting in percentage (Figure 3).

**Figure 3**. Automatic counting of Ki67 Digital imaging

**Statistical analysis**

The results of the Ki67 index calculation manually and automatically are statistically analyzed using paired sample T tests. The H0 hypothesis is that there is no significant difference in the results of the Ki67 LI obtained by manually compared to automatically countings. The data obtained was processed using software IBM SPSS Statistics Version 23 for macOS.

**RESULTS**

**Manual and Automatic counting of Ki67 digital image analysis**

The results of the manual and automatic counting of the Ki67 digital image analysis are shown in Table 1. The mean digital image analysis of Ki67 LI with manual counting was 41.83% and with automatic counting was 42.93%. The average difference of the Ki67 LI of manual and automatic counting was 7.02%.

**Table 1.** The results of manual and automatic counting of the Ki67 LI digital image analysis

| **No.** | **Slide codes** | **Manual (%)** | **Automatic (%)** | **Difference (%)** |
| --- | --- | --- | --- | --- |
| 1 | IHC.171076 | 27.50 | 41.50 | 14,0 |
| 2 | IHC.171145 | 82.30 | 60.80 | 21.5 |
| 3 | IHC.171188 | 47.00 | 51.00 | 4,0 |
| 4 | IHC.171217 | 35.70 | 39.80 | 4.1 |
| 5 | IHC.171274 | 13.60 | 27.20 | 13.6 |
| 6 | IHC.171347 | 48.60 | 43.10 | 5.5 |
| 7 | IHC.171519 | 44.40 | 40.90 | 3.5 |
| 8 | IHC.171523 | 40.30 | 43.40 | 3.1 |
| 9 | IHC.172512 | 63.10 | 66.40 | 3.3 |
| 10 | IHC.172576 | 73.20 | 77.10 | 3.9 |
| 11 | IHC.172580 | 29.20 | 40.90 | 11.7 |
| 12 | IHC.172596 | 35.40 | 48.50 | 13.1 |
| 13 | IHC.172606 | 37.30 | 42.30 | 5,0 |
| 14 | IHC.172618 | 27.40 | 32.80 | 5.4 |
| 15 | IHC.172673 | 16.80 | 15.00 | 1.8 |
| 16 | IHC.172676 | 40.90 | 40.00 | 0.9 |
| 17 | IHC.172683 | 48.10 | 45.10 | 3,0 |
| 18 | IHC.172698 | 9.80 | 19.70 | 9.9 |
| 19 | IHC.172701 | 33.00 | 42.40 | 9.4 |
| 20 | IHC.172704 | 64.70 | 63.90 | 0.8 |
| 21 | IHC.172717 | 15.10 | 17.50 | 2.4 |
| 22 | IHC.172718 | 18.20 | 9.60 | 8.6 |
| 23 | IHC.172749 | 70.10 | 66.10 | 4,0 |
| 24 | IHC.172749 | 7.20 | 19.80 | 12.6 |
| 25 | IHC.172913 | 41.00 | 39.30 | 1.7 |
| 26 | IHC.172915 | 44.50 | 40.50 | 4,0 |
| 27 | IHC.172946 | 23.90 | 30.20 | 6.3 |
| 28 | IHC.172949 | 83.90 | 67.10 | 16.8 |
| 29 | IHC.172951 | 52.80 | 44.00 | 8.8 |
| 30 | IHC.172970 | 80.00 | 72.10 | 7.9 |
| Mean: |  | 41,83 | 42,93 | 7,02 |

**Statistical analysis**

Statistical analysis was using paired sample T test on the results of digital image analysis of the Ki67 LI counted manually compared to automatically. Table 2 shows the comparison of the manual and automatic counting of Ki67 LI digital image analysis. It can be concluded that, from the statistical test, there was no significant difference between the results of the Ki67 LI digital image analysis obtained by manual counting compared to automatic counting (p = 0.801).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Mean** | **SD** | **Mean comparison** | **p** | **CI 95%** |
| Manual counting Ki67 LI DIA | 41,83 | 22,68 | 7,02 | 0,801 | 7,86-10,10 |
| Automatic counting Ki67 LI DIA | 42,93 | 18,73 |

**Table 2.** Mean comparison of manual and automatic counting of Ki67 LI digital image analysis

**DISCUSSION**

One sign of malignancies is uncontrolled cell proliferation. In the case of invasive breast carcinoma, the Ki67 LI can be used as proliferation marker and predictive marker for chemotherapy responses and has a tendency towards prognosis.11,12 The Ki67 LI is very important to distinguish between molecular subtypes of Luminal A and Luminal B breast carcinomas. Dowsett et al5, in his study suggested that Luminal B breast carcinoma needs neoadjuvant chemotherapy, whereas Luminal A is not recommended for chemotherapy.5 Thus, standardization of the Ki67 LI analysis is considered important because of its impact on clinical conditions and treatment plans.

Overall, the differences in the results of manual and automatic countings in this study was 7.02%. This difference is better than the results of the study by Kostopoulos et al13, that was 9.8%. The result obtained from automatic counting of Ki67 LI DIA with QuPath tend to be higher (overestimated) than manual counting. Several factors can cause the predictions of the automatic ounting of Ki67 LI with QuPath higher than the manual counting. This can occur because nontumor tissue such as lymphoid tissue and blood vessels in imaging. QuPath cannot distinguish tumor and nontumor tissue areas. As a consequence, the nontumor tissue area will be counted as a tumor cell so that the Ki67 LI obtained is higher than the actual value.

Thus, to improve the accuracy of the results of the automatic counting of KI67 LI, it is better to choose a region that has little nontumor tissue. In this study, the setting of cell detection parameters is shown in Figure 4. This was chosen because with this setting, the Ki67 LI was obtained most accurately and closests to the result of manual counting of Ki67 LI according to the research conducted by Bankhead et al.6

The results show that QuPath can be used to calculate the Ki67 LI automatically very well even though it is not better than the manual counting. The manual counting of Ki67 LI DIA is more accurate because the determination of tumor cells is carried out by pathologists. However, automatic counting has advantages in terms of the time needed to analyze Ki67 digital image. The time needed to analyze Ki67 digital image by manual counting is 15-30 minutes for one image depending on the quality of the image, while the time needed by QuPath to analyze three images of Ki67 at once is 30 seconds. Thus a large amount of imaging data can be quickly and easily analyzed.13 Another advantage of automated digital image analysis is minimizing inter 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 easier with a simple user interface.

In several studies, analysis of digital imaging by Zhong et al8 and Maeda et al14 were done by making virtual whole slide analysis. In this study, imaging was not perform in whole slides but by dividing the preparation slides into 8 compartments. This was because of the tools limitations. From this study, it was found that the method of taking digital images not in whole slides could show good results because there is no significant difference between manual counting compared to automatic counting of digital image. So that even though imaging is not done in whole slides which require relatively expensive tools and software, the method of dividing compartments of preparation slides can still be done for research.

**Figure 4**. The setting parameter of cell detection in QuPath

**CONCLUSSION**

Digital imaging analysis techniques play an important role in quantitative evaluation of the Ki67 LI in invasive breast carcinoma.

Manual counting of Ki67 LI DIA is more accurate than automatic counting. However, automatic counting of Ki67 LI DIA can be done for diagnostic and research purposes because the results of the study show that there are no significant differences between automatic and manual counting of digital images.

**CONFLICT OF INTEREST**

Authors declare that there is no conflict of interest.

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