Auto-Analysis for Ki-67 Indices of Breast Cancer Using Specified Computer Software and a Virtual Microscopy

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Abstract: Ki-67 index is one of important markers that is correlated with chemotherapy response and prognosis of breast cancer patients. However, Ki-67 index is not easily provided and are limited by intra-observer error and potentially subjective decision making. We performed this study to develop an objective auto-analysis system to count Ki-67 indices. A total of185 invasive breast cancer cases were used. Immunohistochemical staining was performed using auto-stainer and MIB-1 antibody. The results were stored digitally by virtual microscopy and auto-analyzed by Genie/Aperio software (Vista, CA, USA). As for Ki-67 indices, a good correlation was observed between direct ocular observations and auto-analysis techniques ($r = 0.94$, $p < 0.001$). The index examined by auto-analysis was significantly correlated with nuclear atypia, mitotic counts, and nuclear grade of pT1 breast cancers. Auto-analysis of 5 high power fields was better correlated with nuclear grade than that of whole fields. Further, the Ki-67 index was better correlated with mitotic counts than with nuclear atypia. Auto-analysis can provide results concordant with those obtained by direct ocular observation in a short time. Auto-analysis is more likely to result in an objective observation and provide a means by which to standardize methods for immunohistochemical Ki-67 indices of breast cancer.

Keywords: Breast cancer, Ki-67, auto-analysis, virtual microscopy, immunohistochemistry, prognosis, objective analysis, nuclear grade.

INTRODUCTION

Currently, the therapeutic strategy for breast cancer patients is designed according to their immunohistochemical and molecular findings of several markers, including ER/PgR, HER-2, and Ki-67/Topoisomerase II alpha indices in addition to histopathological findings. These findings are obtained through the collected efforts of many individual pathologists or medical technologists, thus, are limited by intra-observer error and potentially subjective decision making. Standardization of the methods of detection and a means by which to make an objective observation are urgently needed. Recently, computerized auto-image analysis has been used at a few institutions [1]. We developed the auto-analysis system to assess the immunohistochemical findings of breast cancer using the computer software, Genie/Aperio (Vista, CA, USA), and virtual microscopy (Hamamatsu Photonics, Hamamatsu, Japan) [2]. In this study, we evaluated the utility of the auto-analysis of Ki-67 index. Antigen KI-67, also known as Ki-67 or MKI67, is a protein that, in humans, is encoded by the *MKI67* gene (antigen identified by monoclonal antibody Ki-67). Antigen KI-67 is a nuclear protein that is associated with and may be necessary for cellular proliferation. The Ki-67 protein (also known as MKI67) is a cellular marker for proliferation. It is strictly associated with cell proliferation. During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus whereas, in mitosis, most of these proteins are relocated to the surface of the chromosomes. Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis); however, is absent from resting cells (G0) [3]. The Ki-67 index is known as one marker that is correlated with chemotherapy response and prognosis [4, 5]. Therefore, the index is important in the strategy of chemotherapy for breast cancer. At the 12th St. Gallen International Breast Cancer Conference, four intrinsic subtypes of breast cancer were determined by the Ki-67 index with hormonal receptors and HER-2 [6]. However, there is no

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standard for how many cancer cells or fields should be counted for Ki-67 index.

MATERIALS AND METHODS

185 invasive breast cancer specimens were collected between November 2009 and March 2012. Among those, 43 specimens were examined by auto-analysis, 117 specimens were examined by ocular observation, and 25 specimens were examined by both. 169 of those specimens were surgically resected and 16 were biopsy specimens. Specimen preparation and image analysis were carried out according to the method previously described [2]. Small pieces for immunohistochemistry were cut from each tumor immediately after they were surgically resected. Fixation time in formaldehyde solution was more than 12 hours, but less than 72 hours. Immunohistochemical staining was performed using auto-stainers (Benchmark XT, Roche, Basel, Schweiz) as described previously.[7] The MIB-1 antibody was used (DAKO, 1:50). The results were stored digitally after examination by virtual microscopy (Hamamatsu Photonics, Hamamatsu, Japan). Data analysis was performed with the Genie/Aperio software package on a desktop computer. For the auto-analysis, a combination of the Genie Histology Pattern Recognition tool was used to select tumor cells, and the *Nuclear Quantification v_9.1* cellular analysis tool was used. The average rate of Ki-67 positive cancer cells on 5 high power fields (x40) with a higher rate was examined by ocular observation (approximately 360000μm^2) and auto-analysis (approximately 250000μm^2). The average rate of Ki-67 positive cancer cells on whole fields was examined by auto-analysis. Areas of whole fields varied from specimen to specimen. The average was 18454587μm^2 (range: 2686027-48409141μm^2). Intensity of positivity was not considered. These fields were selected by well-trained medical technologists (MTs) (M.T., J.S., T.T., M.S.) and were checked by authorized pathologists (K.K., K.T.). Ki-67 indices examined by both ocular observation and auto-analysis were also checked by the pathologists. Nuclear atypia was classified into 1, 2, and 3, and mitotic numbers were counted at 10 high power fields (x40). From these results, nuclear grades were assessed 1 to 3 according to the nuclear grading formula of The Japanese Breast Cancer Society [8]. Nuclear grade of pT1 breast cancer of a total of 160 cases were examined by ocular observation and auto-analysis. The use of samples for this study was approved by the Institutional Ethical Board at the Kure Medical Center/Chugoku Cancer Center (NHO-KureH141129).

RESULTS

Correlation Between Direct Ocular Observation and Auto-Analysis Techniques

In 25 cases, we evaluated the correlation between direct ocular observation and auto-analysis techniques. A representative case is shown in Figure **1** in which the

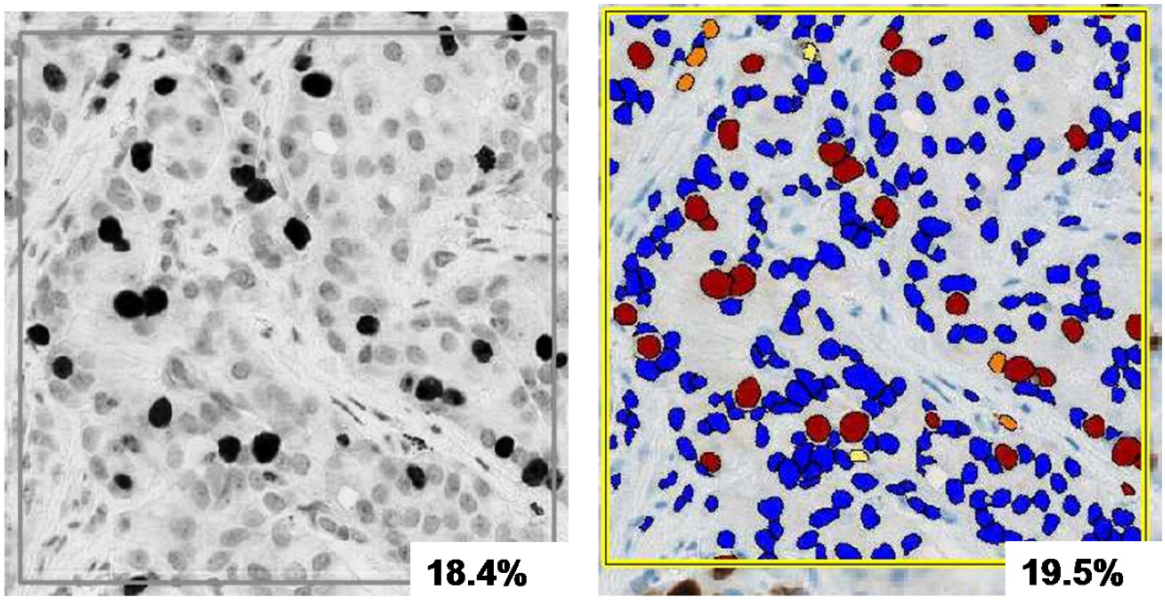

Figure 1: Ki-67 index by ocular observation was 18.4%. An image by the auto-analysis. Only cancer cells are recognized and stromal cells are excluded. Red, orange, and yellow nucleoli are positive, and blue nucleoli are negative. Ki-67 index was 19.5%.

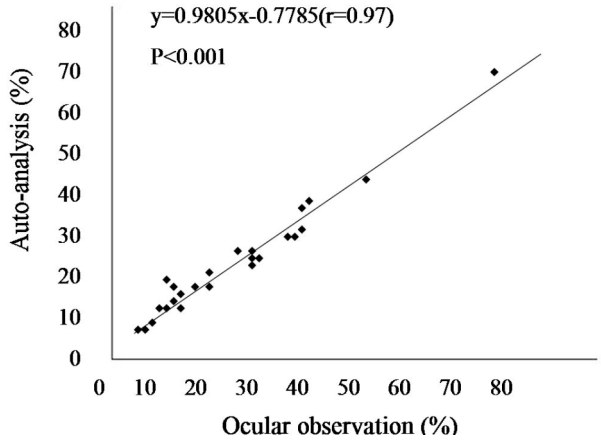

Figure 2: Distribution of Ki-67 index of 25 cases. Correlation coefficient was 0.97 ($p < 0.001$).

Ki-67 index by ocular observation was counted as 18.4%. The figure for the auto-analysis is shown in Figure **1**. The auto-analysis system only recognizes cancer cells; stromal cells are excluded by the system. Red (3+), orange (2+), and yellow (1+) nucleoli are positive, and blue nucleoli are negative for the Ki-67 protein. The Ki-67 index by the auto-analysis was assessed as 19.5%. The distribution of the Ki-67 index of those 25 cases is shown in Figure **2**. The correlation coefficient was 0.97. Good correlation was observed between direct ocular observations and the auto-analysis system ($p < 0.001$), with correlation coefficient of 0.97.

Correlation between Ki-67 Index and Nuclear Atypia, Mitotic Counts, and Nuclear Grade of pT1 Breast Cancer

The Ki-67 index correlated well with nuclear atypia, mitotic counts, and nuclear grade as shown in Table **1**. Ki-67 indices from the auto-analysis of cases with nuclear atypia scores of 1, 2, and 3 were 4.79%, 13.53%, 29.91%, respectively. The Ki-67 indices significantly ($p < 0.0001$) increased with scores of

Table 1: Ki-67 Index of pT1 Cancer by Ocular Observation and Auto-Analysis

	Ocular observation		Auto-analysis (5HPF)		Auto-analysis (WF)	
	n Index (%) ±SD	*P**	n Index (%) ±SD	*P**	n Index (%) ±SD	*P**
NA						
1	15 15.72	13.90 <0.0001	2 4.79	1.22 <0.0001	2 4.54	0.29 0.0002
2	82 19.07	12.37	30 13.53	7.95	30 10.80	6.76
3	18 29.82	19.07	11 22.91	11.05	11 23.24	11.69
MC						
1	58 14.16	7.09 <0.0001	23 11.05	7.92 <0.0001	23 8.88	7.46 0.0002
2	27 23.83	11.27	12 18.80	7.28	12 15.17	6.65
3	19 37.17	20.14	8 33.08	9.75	8 23.32	10.44
NG						
1	64 13.87	6.97 <0.0001	20 8.89	5.54 <0.0001	20 6.97	5.45 <0.0001
2	25 21.58	12.32	12 19.07	6.17	12 17.22	5.66
3	28 34.76	18.04	11 30.69	10.57	11 22.07	11.69
			t-test			
NA						
1 vs. 2		0.3462		0.1365		0.2062
1 vs. 3		0.0236		0.0101		0.0515
2 vs. 3		<0.034		<0.001		0.0001
MC						
1 vs. 2		<0.001		0.0080		0.0197
1 vs. 3		<0.001		<0.001		<0.0001
2 vs. 3		0.0063		0.0014		0.0156
NG						
1 vs. 2		0.0003		<0.0001		<0.0001
1 vs. 3		<0.0001		<0.0001		<0.0001
2 vs. 3		0.0034		0.0038		0.2130

*Analysis of variance for all scores or grades.
NA, nuclear atypia; MC, mitotic counts; NG, nuclear grade; HPF, high power fields; WF, whole fields.

nuclear atypia. Although a similar correlation was found in the ocular observation results, *p*-values for each score from the auto-analysis were lower than were those of the ocular observations. Ki-67 indices by the auto-analysis of cases with mitotic counts score of 1, 2, and 3 were 11.05%, 18.80%, 33.08%, respectively. The Ki-67 indices significantly ($p < 0.0001$) increased with scores of mitotic counts. Almost the same evaluation was found for the correlation for ocular observations. Ki-67 indices of cases with nuclear grades of 1, 2, and 3 were 8.89%, 19.07%, and 30.69%, respectively. Ki-67 indices significantly ($p < 0.0001$) increased with nuclear grade. A similar correlation was found for ocular observations. The Ki-67 indices detected by the auto-analysis tended to be lower than those for ocular observations. In addition, standard deviations for the auto-analysis were smaller than for ocular observations. Significant differences were found between each score except of nuclear atypia 1 versus 2 examined by auto-analysis and ocular observations. This finding could have resulted from a small number of cases examined with a score of 1.

Comparison of Ki-67 Index on 5 High Power Fields and whole Fields by the Auto-Analysis

The Ki-67 indices on 5 high power fields and whole fields were compared (Figure **3**). A total of 43 pT1 cancers were examined by the auto-analysis. The correlations between these Ki-67 indices and scores of nuclear atypia, mitotic counts, and nuclear grade were

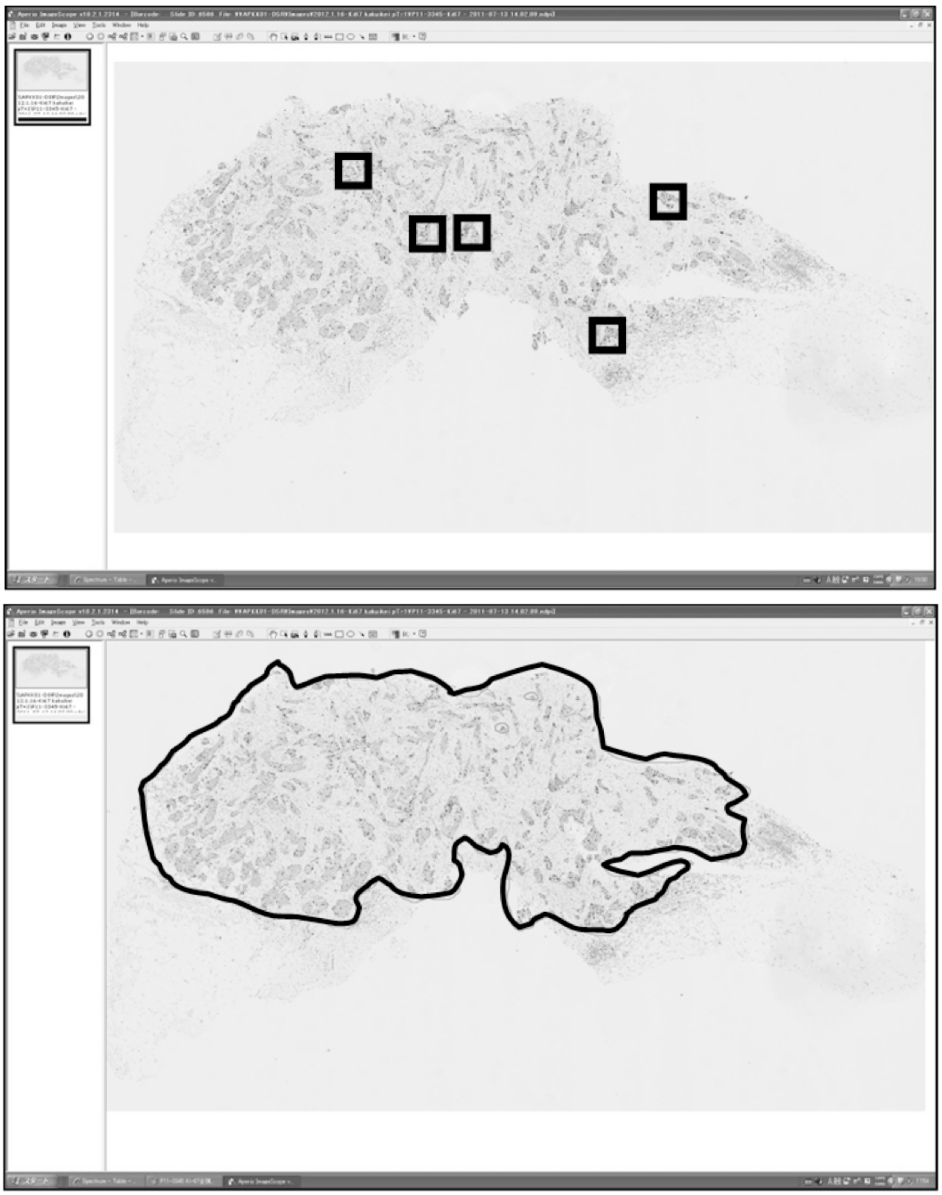

Figure 3: Panoramic views of the auto-analysis on 5 high power fields and auto-analysis on whole fields.

examined (Table **1**). On whole fields, the Ki-67 indices of cases with nuclear atypia scores of 1, 2, and 3 were 4.54%, 10.80%, 23.24%, respectively. The Ki-67 indices significantly increased with scores of nuclear atypia (p = 0.0002). Ki-67 indices on whole fields of cases with mitotic counts scores of 1, 2, and 3 were 8.88%, 15.17%, 23.32%, respectively. Ki-67 indices significantly increased with the score of mitotic counts (p = 0.0001). Significant differences in the Ki-67 index were found between each score. The Ki-67 indices on whole fields of cases with nuclear grades of 1, 2, and 3 were 6.97%, 17.22%, and 22.07%, respectively. The Ki-67 indices significantly increased with nuclear grade (p = 0.0001). No significant differences were found between nuclear atypia 1 versus 2, 1 versus 3, and nuclear grade 2 versus 3. Generally, p-values for each score on whole fields were higher than those on 5 high power fields.

Time to Examine the Ki-67 Index by Ocular Observation and Auto-Analysis

The auto-analysis shortened the time to examine the Ki-67 index. Although the average time to examine theKi-67 index by ocular observation was 20-30 minutes per 1 specimen, those by auto-analysis were 2-3 minutes per 1 specimen.

DISCUSSION

The Ki-67 index is necessary in the therapeutic strategy of breast cancer. However, conventional examination of the index by ocular observation requires great effort and could be limited by intra-observer error and, potentially, subjective decision making. In this study, we evaluated the utility of the auto-analysis of the Ki-67 index. The present study showed good correlation of the Ki-67 index between direct ocular observation by well-trained MTs and checked by pathologists and auto-analysis techniques. In addition, the auto-analysis shortened the time to evaluate the Ki-67 index. These findings suggest utility of the auto-analysis as a standardized method to detect the Ki-67 index. Nuclear grade is an important factor for breast cancer therapeutic strategy. The grade is determined by scores of nuclear atypia and mitotic counts [8]. In this study, the Ki-67 index examined by auto-analysis was significantly correlated with nuclear atypia, mitotic counts, and nuclear grade of pT1 breast cancer. Mohammed *et al*. also reported accuracy of automated scoring of Ki-67 proliferative activity with another automated system [9]. However, their automated Ki-67 index was inferior in predicting cancer survival in patients with breast cancer. The correlation between the automated Ki-67 index and nuclear grade found in the present study suggests the usefulness of the automated Ki-67 index in predicting cancer survival. At the 12th St. Gallen International Breast Cancer Conference, the Ki-67 index is high when more than 14%, and it is low when less than 14%. The value was reported as significantly associated with cancer survival [5, 6]. In the present study, Ki-67 indices by auto-analysis tended to be lower than those by ocular observation, which suggests the cut-off index may be lower than 14% when examined by auto-analysis (Table **1**). To examine these suggestions, we plan to conduct a study on breast cancer survival and the automated Ki-67 index. The value of the Ki-67 index and quality assurance of immunohistochemical examination by pathologists has recently become more important. However, it has not been decided whether selected fields or whole fields should be counted. Our data on the comparison of 5 high power fields to whole fields suggest 5 high power fields is better than that of whole fields. Concerning the correlation between the Ki-67 index and nuclear grade, significant differences of the index were found between each grade on 5 high power fields; however, no significant differences were found between scores 2 and 3 on whole fields. In the future, the field number selected or the number of cells counted should be standardized. Our present study indicates that well-organized auto-analysis can provide results concordant with those obtained by direct ocular observation in a short time. Tanaka *et al*. demonstrated the usefulness of auto-analysis for ER, PgR, HER-2, and Topoisomerase IIa of breast cancer, not only for Ki-67 [2]. An examination of immunohistochemical specimens in a specified center by auto-analysis that collects many samples from other hospitals is more likely to result in a standardization of the methods for immunohistochemical analysis for breast cancer markers.

DISCLOSURE STATEMENT

The authors have no conflict of interest.

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