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Original Article

Ki-67 expression of immunohistochemistry using computerized image analysis is a useful prognostic marker in follicular lymphomas

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Abstract: Ki-67 is a useful proliferation marker in various tumors including lymphoma. In general, the number of Ki-67 positive cells in immunohistochemistry (IHC) is counted manually for routine pathological diagnosis. However, a manual count is subjective and time consuming. Currently, image analysis is often used for the quantification of positive cells in tissue in IHC. Thus, to determine the pathological prognostic factors for follicular lymphoma (FL), we studied the relationship between Ki-67 expression in IHC and the treatment effect and prognosis using image analysis software. We analyzed 82 newly-diagnosed patients with FL. All patients were treated with rituximab-containing regimens. The median Ki-67 expression was 17.0%. A high expression of Ki-67 tended to be associated with short overall survival ($P = 0.058$). Moreover, Ki-67 expression was significantly lower in patients with FL grade 1-2 than in those with FL grade 3a. This study suggests that image analysis provides an accurate, reproducible, and easy method of measuring Ki-67 expression in IHC in FL, and is possibly a useful marker for treatment selection or prognosis prediction in FL.

Keywords: Follicular lymphoma, Ki-67, image analysis, prognosis

Introduction

Follicular lymphoma (FL) is the most common frequent low-grade B-cell lymphoma in adults [1], and its clinical course is highly variable. Some have indolent forms, yet others progress rapidly or transform into aggressive lymphoma resembling diffuse large B-cell lymphoma (DLBCL) [2, 3]. Several prognostic markers for FL have been determined, including the Follicular Lymphoma International Prognostic Index (FLIPI), the clinical stage, the number of lymph nodes involved, the hemoglobin level, and the lactate dehydrogenase (LDH) level [3]. The histological grade of FL, which is divided into FL grades 1, 2, 3a, and 3b based on the number of centroblasts, is used as a prognostic factor in pathological analysis. Some studies have suggested a correlation between histological grade and prognosis. Overall survival (OS) is similar between grades 1 and 2, but OS for grade 3 is still debated [4, 5]. Although other pathological markers such as Ki-67, Bcl-6, MUM1 protein, p53 protein, and c-Myc have been discussed as prognostic markers in several studies, both high levels of MUM1 protein and Ki-67 are significantly associated with short progression-free survival (PFS) [6]. High levels of Bcl-6 expression show a more favorable OS and time to treatment failure compared with low Bcl-6 expression levels [7]. Furthermore, p53 protein expression > 5% predicts short OS and transformation to DLBCL [8]. Although only case reports have described the use of immunohistochemistry (IHC) to assess c-Myc expression in FL, c-Myc expression might be useful for predicting a more aggressive clinical course [9]. However, there is no consensus for predicting prognostic factors.

In general, the number of positive cells in IHC is counted manually for routine pathological diag-

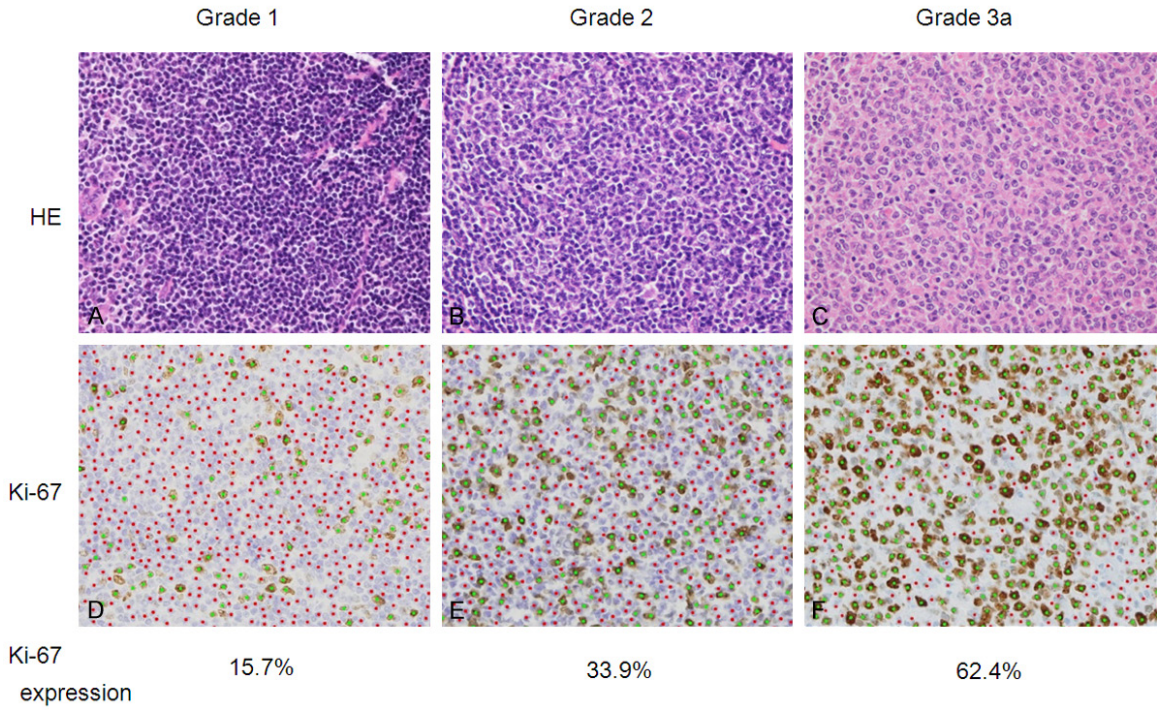

Figure 1. Automated measurement of Ki-67 expression in histological grade 1, grade 2, and grade 3a (A-C); hematoxylin and eosin stain, objective 40× (D-F); Ki-67 expression by immunohistochemistry, objective 40×. Automatic counts of Ki-67 identified 224 positive (green) and 1202 negative (red) cells for expression of 15.7% (D); 467 positive and 908 negative cells for expression of 33.9% (E); and 467 positive and 908 negative cells for expression of 62.4% (F).

nosis. However, a manual count of positive cells in IHC is subjective and time consuming. Currently, image analysis is often used for the quantitation of positive cells in tissue in IHC. In breast cancer, estrogen receptors, progesterone receptors, and HER2 expression are measured using image analysis software, and these methods are established in clinical studies [10, 11]. Moreover, computer-assisted Ki-67 scoring offers a standardized means of tumor cell proliferation assessment that utilizes Ki-67 as a prognostic and predictive marker in breast cancer [12]. Thus, the use of image analysis to measure positive cells in IHC has been established to provide consistent and objective labeling index measurements.

Few studies have investigated the correlation between automated Ki-67 counts and histological grade and disease progression of FL [13, 14]; however, they included histological grade 3b and the treatment effect, and the prognoses were difficult to assess as the various treatments were mixed.

Therefore, to determine the pathological prognostic factors for FL, we studied the relationship between the pathological findings (Ki-67 expression, Bcl-6, MUM1 protein, p53 protein, and c-Myc) in IHC and the treatment effect and prognosis using image analysis software.

Material and methods

Study design and patients

We analyzed 82 newly diagnosed patients with FL from 2003 to 2017 at Showa University Hospital in Tokyo, Japan. Pathological diagnosis was made by two hematopathologists (ES and MT) at our hospital. The study was approved by the research ethics committee of Showa University School of Medicine (approval No.2478) and adhered to the principles of the Declaration of Helsinki. All patients were treated with rituximab-containing regimens as their initial therapy. The following data were obtained: patient sex, age (> 65 years or < 65 years), performance status (0, 1, 2, or 3), FLIPI (low, intermediate, or high risk), laboratory parameters

Table 1. Patient characteristics

Characteristic	No. of Patients (%) N = 82
Age	
Median (range), y	63.5 (31-86)
Sex	
Male	39 (48)
Female	43 (52)
Performance status (ECOG)	
0	28 (34)
1	43 (52)
2	8 (10)
3	3 (4)
4	0 (0)
Ann Arbor stage	
I	2 (2)
II	12 (15)
III	31 (38)
IV	37 (45)
Bone marrow involvement	33 (39)
Histological findings (WHO)	
Grade 1	15 (18)
Grade 2	20 (24)
Grade 1-2	22 (27)
Grade 3a	17 (21)
Unknown	8 (10)
FLIPI	
Low risk	21 (26)
Intermediate risk	20 (24)
High risk	41 (50)
Regimen	
R-CHOP like	71 (87)
Rituximab	9 (11)
R+bendamustine	2 (2)
Rituximab maintenance therapy	
Yes	25 (30)
No	57 (70)

ECOG, Eastern Cooperative Oncology Group; WHO, World Health Organization; FLIPI, Follicular Lymphoma International Prognostic Index; R, Rituximab.

including LDH (elevated or normal) and soluble interleukin-2 receptor (elevated or normal).

Patient material and immunohistochemistry

All lymph node and tissue biopsies were taken prior to the initial treatment. Histological diagnosis was defined according to the 2008 World Health Organization (WHO) classification [14]. We assessed the histopathological findings, including the histological grade (1, 2, or 3a), IHC staining for Ki-67, Bcl-6, MUM1 protein, p53 protein, and c-Myc at the initial diagnosis. The tissues were formalin fixed and paraffin embedded, and serial sections 3-μm thick were cut from paraffin blocks and stained with the following primary antibodies and dilutions: BCL-6 (clone LN22, 1:100; Novocastra, Newcastle, UK), Ki-67 (clone MIB-1, 1:100; Dako, Glostrup, Denmark), c-Myc (clone Y69, 1:200; Abcam PLC, Cambridge, UK), MUM1 protein (clone MUM1p, 1:50; Dako, Glostrup, Denmark), and p53 protein (clone DO-7, 1:50; Dako, Glostrup, Denmark). Following heat-induced epitope retrieval, IHC staining was performed using an automated immunostainer (Histostainer 36 A, Nichirei Biosciences Inc., Tokyo, Japan) according to the manufacturer's protocol.

The IHC expression was calculated using e-count, a digital image analysis software, which automatically measures cell counts in tissue (e-path Institute Inc., Fujisawa, Japan) and is able to calculate the index accurately and efficiently [15]. Positive staining cells were marked with green points, and negative staining cells were marked with red points (**Figure 1**). Cell counts for each tissue section were obtained by averaging the total number of positive centroblasts or centrocytes counted on the immunostained slides in four high-power fields (400× magnification) in four neoplastic follicles. Ki-67, Bcl-6, and MUM1 protein staining were scored as low (< 30% of cells) or high expression (> 30% of cells) [16, 17]. p53 staining was scored as low (< 10% of cells) or high expression (> 10% of cells) [18]. and c-Myc staining was scored as low (< 50% of cells) or high expression (> 50% of cells) [9].

Statistical analysis

Response assessment was classified as complete response (CR), unconfirmed complete response (CRu), partial response (PR), stable disease (SD), or progressive disease (PD) according to the International Working Group Response Criteria [19]. All measurable lesions were assessed by CT and referred for PET/CT. Association with patient characteristics and treatment response was determined by the chi-squared test and the Fisher exact test. PFS was calculated from the day of starting treatment to disease progression, relapse, or death from any cause or remaining alive and disease free.

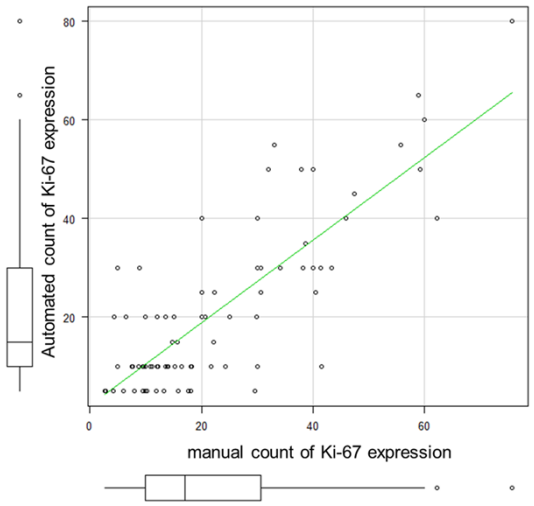

Figure 2. The automated and manual assessment of the identical fields is plotted. Solid line automated Ki-67 counts indicated a positive correlation with the manual count of Ki-67 positive cells (Pearson r = 0.816, $P < 0.001$).

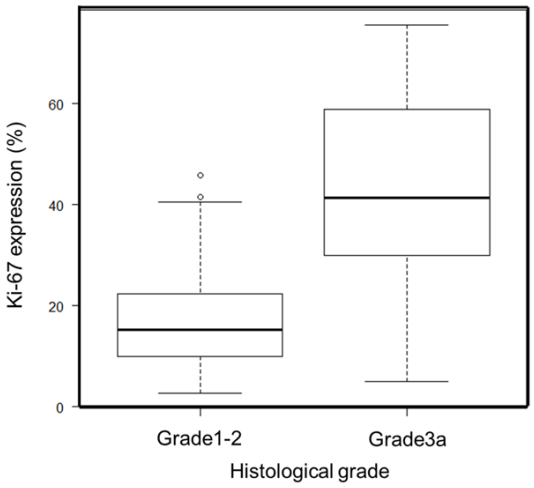

Figure 3. Automated measurement of Ki-67 expression is associated with histological grade. High expression of Ki-67 showed a significant positive correlation with higher histological grade. Grade 1-2 has a median Ki-67 expression of 22.3% and grade 3a expression of 41.4% ($P < 0.001$).

OS was calculated from the day of diagnosis to death from any cause. PFS and OS were estimated by Kaplan-Meier analysis and were compared by the log-rank test. Univariate and multivariate analysis were performed with the Cox proportional hazards model. A *P*-value < 0.05 was considered statistically significant. All statistical analyses were performed using EZR version 1.24 (Kanda Y, Saitama Medical Center, Jichi Medical University, Saitama, Japan).

Result

The patient characteristics are summarized in **Table 1**. The median patient age at diagnosis was 63.5 years (range, 31-86 years), and the median follow-up period was 1723 days (range, 181-5295 days). Histological analysis revealed grade 1-2 in 57 patients (70%) and grade 3a in 17 patients (20%); eight patients (10%) could not be evaluated due to needle biopsy. The median duration from the diagnosis to stating treatment was 38.5 days (range, 1-1400 days). All patients were treated with rituximab-containing regimens: 71 were treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP), 9 with rituximab alone, and 2 with rituximab plus bendamustine. Twenty-five patients (30.5%) received rituximab maintenance therapy after bendamustine treatment.

Figure 1 shows an example of a microscopic field of each histological grade. Automated counting of Ki-67-positive cells in IHC was accurate on visual inspection. Median Ki-67 expression was 17.0% (range, 2.7%-75.6%). Automated Ki-67 counts indicated a positive correlation with the manual count of Ki-67 positive cells (Pearson r = 0.816, $P < 0.001$) (**Figure 2**). Ki-67 expression was significantly lower in patients with FL grade 1-2 than in those with grade 3a; median Ki-67 expression for grade 1-2 was 15.2% and was 41.4% for grade 3a ($P < 0.001$) (**Figure 3**).

Five-year OS was 88.2% (95% CI, 73.8%-94.9%) (**Table 2**). On univariate analysis, high Ki-67 expression tended to be associated with a short OS ($P = 0.058$) (**Figure 4**). In addition, an elevated LDH, PS > 2, and histological grade 3a were significantly associated with short OS ($P = 0.025$, $P = 0.004$, and $P = 0.012$, respectively). Furthermore, FL grade 3a had a shorter OS than FL grade 1-2 ($P = 0.012$) (**Figure 5**). Bcl-6 and p53 expression did not correlate with OS ($P = 0.341$ and $P = 0.593$, respectively).

Five-year PFS was 54.0% (95% CI, 39.8%-66.2%). On univariate analysis, patients with an elevated LDH and not receiving rituximab maintenance therapy were significantly associated with short PFS ($P = 0.022$ and $P = 0.049$, respectively), and histological grade, Ki-67 expression, and the duration from diagnosis to stating treatment did not correlate with PFS ($P = 0.258$,

Table 2. Prognosis factor on progression free survival and overall survival in univariate and multivariate analysis

Factor	No.	PFS	OS		
		Univariate	Univariate	Multivariate	
		P value	*P* value	HR (95% CI)	*P* value
All patients	82				
Age					
< 65	43	0.614	0.158		
≥ 65	39				
PS (WHO)					
< 2	71	0.186	0.004	9.38 (1.13-78.16)	0.040
≥ 2	11				
FLIPI					
Low	21	0.315	0.145		
Int and High	61				
LDH					
Non-elevated	60	0.022	0.025	5.89 (0.77-44.93)	NS
Elevated	22				
SIL-2R					
Non-elevated	28	0.133	0.074		
Elevated	54				
Histological grade					
1-2	56	0.258	0.012	11.35 (1.7-75.67)	0.010
3a	17				
Ki-67 expression					
< 30%	57	0.973	0.058		
≥ 30%	25				
MUM1 expression					
< 30%	82	NA	NA		
≥ 30%	0				
bcl-6 expression					
< 30%	41	0.877	0.341		
> 30%	41				
p53 expression					
< 10%	73	0.387	0.593		
≥ 10%	9				
MYC expression					
> 50%	82	NA	NA		
≤ 50%	0				
Rituximab maintenance					
Yes	25	0.049	0.078		
No	57				

PFS, Progression free survival; OS, Overall survival; PS, Performance status; WHO, World Health Organization; FLIPI, Follicular Lymphoma International Prognostic Index; Int, Intermediate; LDH, Lactate dehydrogenase; sIL-2R, soluble interleukin-2 receptor.

P = 0.973, and *P* = 0.688, respectively). Bcl-6 and p53 expression did not reveal significant data (*P* = 0.877 and *P* = 0.387, respectively). No tissue expressed the MUM1 protein > 30% and c-Myc > 50% (data not shown).

Discussion

Ki-67 is a nuclear nonhistone protein and useful proliferation marker in various tumors, including lymphoma, as it presents in all phas-

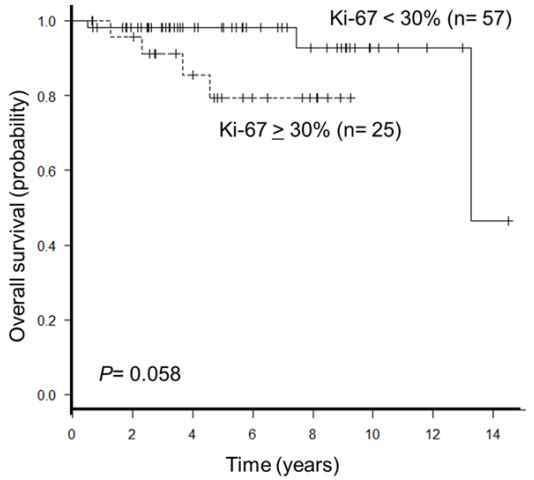

Figure 4. We used a Kaplan-Meier curve for overall survival by comparing low and high Ki-67 expression. Patients with high Ki-67 expression tended to have short OS (P = 0.058).

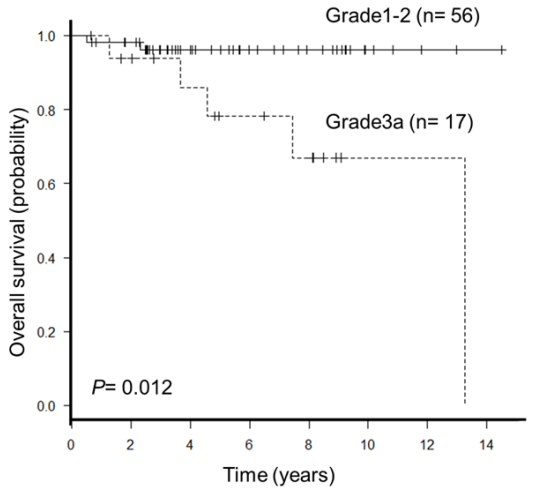

Figure 5. Histological grade 3a of FL is significantly short overall survival than grade 1-2 (P = 0.012).

es (except G0) of the cell cycle [20]. Previous studies have demonstrated a correlation with Ki-67 expression and disease progression, efficacy of therapy, and prognosis in various cancers [16, 21-23]. In FL, Ki-67 expression was significantly higher at grade 2 or 3 compared with grade 1 FL [24]. Some studies have shown that high Ki-67 expression is associated with poor prognosis [25, 26]. Thus, Ki-67 expression is used to assess disease proliferation in the present pathological diagnosis.

Ki-67 expression in these studies was measured by manual counting, which is subjective, dependent on observer experience, and more time consuming than automated counting for routine pathological diagnostic proposes. Jeroen et al. assessed Ki-67 stained tissue sections of 15 FLs by both manual and automated analysis and observed a good concordance between the two methods [27]. Reproducibility of the automated count was slightly better than for the manual count of positive cells. Additionally, manual assessment required up to 15 minutes for a single microscopic field, but automated assessment of the Ki-67-positive area in IHC was fast. Therefore, their study concluded that automated measurement of the Ki-67-positive surface area in IHC was an alternative to manually counting Ki-67-positive cells. Samols et al. identified 31 FL patients and obtained clinical follow-up data from 25 patients [13]. They assessed software-automated counting of the Ki-67 proliferation index for FL and demonstrated that a high Ki-67 expression was significantly associated with the need to treat. However, their study did not show the association with disease prognosis, as their patient population was small and included histological grade 3b, which was regarded as an aggressive lymphoma. Consequently, our study analyzed a larger population to determine the relationship between Ki-67 expression and prognosis in FL using e-count, the latest version of image analysis software that is able to diagnose the pathological findings of FL with good accuracy, efficiency, and reproducibility. Our group reported that assessment of Ki-67 expression in IHC using e-count is a useful method for diagnosing gastrointestinal neuroendocrine neoplasms [15]. Thus, Ki-67 expression measured using e-count is reliable for diagnosis and prognosis in FL.

In addition, various cutoff levels of Ki-67 expression in IHC have been presented in previous studies. Saito et al. reported that high Ki-67 expression (> 30%) was associated with a worse objective response rate [16]. Koster et al. showed that both PFS and OS were significantly shorter in patients with Ki-67 expression above the median (17.6) [26]. Moreover, Yamamoto et al. concluded that Ki-67 expression > 10% was independently associated with short PFS and OS for FL patients treated with R-CHOP [25]. Those previous studies also included histological grade 3b. Therefore, the present study suggests that patients with Ki-67 expression > 30% had worse OS.

Another finding is that OS was longer in patients with histological grade 1-2 compared to grade 3a. In addition, Ki-67 expression was significantly lower in patients with grade 1-2 versus grade 3a. FL grade 3b is recognized as an aggressive lymphoma such as DLBCL, whereas the behavior of FL grade 3a is controversial. Previous studies have reported that FL grade 1-2 and 3a showed similar OS in patients treated with regimens that did not contain rituximab [28]. In the rituximab era, PFS did not differ between FL grade 1-2 and 3 [29]. Conversely, Koch et al. indicated that FL grade 3a and 3b showed longer PFS compared to grade 1-2 [30]. Our results indicate that FL grade 3a has high Ki-67 expression and poor prognosis and should therefore be considered separately from grade 1-2.

The FLIPI is a useful prognostic index but does not include pathological findings, and it is difficult to determine certain prognostic factors using pathological data. In this study, we found that high Ki-67 expression in IHC using image analysis software tended to have a short OS. Image analysis software can be used for histopathological diagnosis due to objective quantification of Ki-67 expression and it is a useful prognostic indicator in FL. A limitation of our study is that it was retrospective and from a single institution. Therefore, the pathological prognostic factors of a larger number of patients should be analyzed to confirm our results.

Conclusion

In conclusion, this study suggests that image analysis provides an accurate, reproducible, and easy method of measuring Ki-67 expression in IHC in FL and is possibly a useful marker for treatment selection or prognosis prediction in FL.

Disclosure of conflict of interest

None.

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