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Original contribution

Immunohistochemical pattern of c-MYC protein judged as "+/(weak)+/–" by a new notation correlates with *MYC* gene nontranslocation in large B-cell lymphoma☆,☆☆

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Summary Immunohistochemistry is not only the most important tool for pathologists to establish a final diagnosis, but it can also inform decisions regarding optimal treatment methods. However, there is no universal standard notation for expressing immunohistochemical findings. For a diagnosis of malignant lymphoma, it is important to confirm the presence or absence of *MYC* translocation and communicate these results to a clinical audience. However, the criteria for selecting cases for fluorescence in situ hybridization (FISH) analysis to confirm *MYC* translocation are ill defined. We therefore devised a notation that we termed proportion of immunoreactivity/expression for immunohistochemistry (PRIME notation) based on the cellular proportion showing different antigen-antibody reactivity in immunohistochemistry (CPAR) and used it to examine the relationship between *MYC* translocation and the proportion of c-MYC+ lymphoma cells. We reviewed 82 cases diagnosed as diffuse large B-cell lymphoma or diffuse large B-cell lymphoma coexisting with grade 3A to 3B follicular lymphoma. The most common notation was "+/(weak)+/–" (49/82 cases

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[59.8%]); cases that were CPAR positive, weakly positive, and negative for tumor cells each accounted for about one-third of the total. Unexpectedly, no *MYC* translocation was observed by FISH in this group. Thus, FISH is not needed even if more than half of cells are c-MYC positive by PRIME notation. This is the first report describing a correspondence between immunohistochemical findings and chromosomal abnormality, reflecting findings at the protein and gene levels, respectively.

1. Introduction

Immunohistochemistry, which is indispensable for routine diagnostic work, is one of the most important tools for pathologists to establish a final diagnosis. However, because the manner in which immunohistochemical findings are expressed largely depends on the training background, belief, aesthetic sense, ingenuity, and individuality of each pathologist, there is no universal standard notation. For instance, immunoreactivity (IR) is typically expressed only as positive, weakly positive, or negative, whereas the proportion of immunoreactive cells is ambiguously described as "all," "almost all," "mostly," "partially," "occasionally," "rarely," "very few," and so on. It is therefore difficult if not impossible to compare immunohistochemical findings among many cases, unlike test results that are generally defined by absolute numerical values.

Immunohistochemistry is also important for making decisions regarding treatment approaches. For example, in breast cancer, administration of hormone therapy depends on expression of estrogen receptor [1], and concurrent detection of human HER2, anaplastic lymphoma kinase, and programmed death ligand 1 also plays an important role in treatment selection [1-4]. However, because evaluation methods vary depending on the disease and antibody type, they are inconvenient for routine diagnostic work, and clinical interpretation of actual staining dynamics is challenging.

Allred scores of hormone receptors in the mammary gland [5], IR score [6], and H score [7] are relatively well-established immunohistochemical evaluation methods. However, Allred and IR scores are determined based on staining proportion and strength; the method for calculating the former is ambiguous when positive cells have variable staining intensities. On the other hand, although proportion and strength are reflected in the H score, the method for quantifying positive cells is complicated, and it is difficult to estimate the image of the immunohistochemical specimen from the resultant score (eg, even when the score is 100, it is impossible to estimate whether all cells are weakly positive in terms of staining intensity or whether 10% of cells have strong positivity). Thus, the common problem with existing methods of immunohistochemical evaluation is that the cellular proportion showing different antigen-antibody reactivity in immunohistochemistry (CPAR) cannot be determined objectively.

Apart from companion diagnoses, there have been many recent reports of the relationship between immunohistochemical expression ratio of c-MYC protein (a transcription factor controlling cell proliferation, differentiation, and cell death) and translocation involving the cancer-associated *MYC* gene on chromosome 8q24. *MYC* is involved in the development of various malignancies, but in malignant lymphomas, the translocation partner of *MYC* is usually the immunoglobulin heavy chain (*IGH*) gene located on chromosome 14q32. The chromosomal translocation t(8;14)(q24;q32)/*MYC-IGH* is characteristic of Burkitt lymphoma but is nonspecific because it is also found in diffuse large B-cell lymphoma (DLBCL) and other diseases. Malignant lymphoma with a translocation involving not only *MYC* but also *BCL2* and/or *BCL6* is referred to as double- or triple-hit lymphoma and is reportedly resistant to chemotherapy and associated with poor prognosis [8].

Confirming the presence or absence of *MYC* translocation in malignant lymphoma and communicating the results are important for pathologists; c-MYC immunohistochemistry has recently been implemented for high-grade B-cell lymphoma. However, immunohistochemical data for c-MYC and the presence/absence of *MYC* translocation have not been consistent [9,10]. Therefore, in malignant lymphoma cases judged as having a high proportion of c-MYC–positive tumor cells by immunohistochemistry and/or an aggressive clinical course, fluorescence in situ hybridization (FISH) analysis of *MYC* should be included to confirm the presence or absence of translocation involving 8q24/*MYC*. However, in many cases, it is unclear whether it is necessary to add FISH analysis of *MYC* to routine work.

To address this question, we devised a notation method based on CPAR and used this to score the proportion of cells positive for c-MYC by immunohistochemistry (c-MYC+ lymphoma cells). We also examined the relationship between *MYC* translocation and the proportion of c-MYC+ lymphoma cells.

2. Materials and methods

2.1. Case selection

We performed c-MYC immunohistochemistry for all B-cell lymphoma cases forwarded to the Registration-Examination-Analysis-Description (READ) system® (Life Science Institute, Tokyo, Japan) between January 2016 and May 2017 from major hospitals in Miyagi Prefecture. Among the cases diagnosed as DLBCL or coexistence of DLBCL and follicular lymphoma

Table 1 Ideal method for evaluating and describing immunohistochemical expression patterns

	Points
1.	CPAR can be visualized in the sample according to the written description.
2.	CPAR can be described under a normal optical microscope without requiring excessive observation time or special equipment.
3.	There is very little difference in notation among diagnosticians.
4.	Cases can be compared across generations, and CPAR can be universally applied.
5.	Multivariate analysis is possible as an extension of point 4 above.

(FL) grade 3A to 3B (G3), 82 cases with "+" in the first position of the c-MYC notation of the IR pattern related to proportion of IR/expression for immunohistochemistry (PRIME) notation (described below) were extracted. As negative subjects, 34 cases of DLBCL or DLBCL + FL/G3 with "(weak)+" or "–" in the first position of the c-MYC notation of the IR pattern were extracted. Specimens were collected from various organs by biopsy or surgical resection. Immunohistochemistry for c-MYC was carried out before obtaining chromosome or FISH analysis results.

2.2. Diagnosis of malignant lymphoma

Malignant lymphoma cases were diagnosed by expert pathologists (hematopathologists) based on morphologic examination (hematoxylin and eosin–stained slides combined with immunohistochemistry) along with flow cytometry, cytogenetic (G-band method with or without fusion FISH), and molecular analyses (detection of IgH/T-cell receptor β genes by Southern blotting with or without polymerase chain reaction) [11]. In this study, split FISH for detection of *MYC* using Carnoy-fixed cells or paraffin sections was performed in cases where the G-band method or fusion FISH failed to detect 8q24/*MYC* gene translocation.

2.3. c-MYC immunohistochemistry

Immunohistochemical staining for c-MYC was performed on the Benchmark Ultra automated immunostainer (Ventana Medical Systems, Tucson, AZ) or the Bond-III automated slide stainer (Leica, Wetzlar, Germany). Formalin-fixed, paraffin-embedded tissue sections were cut at a thickness of

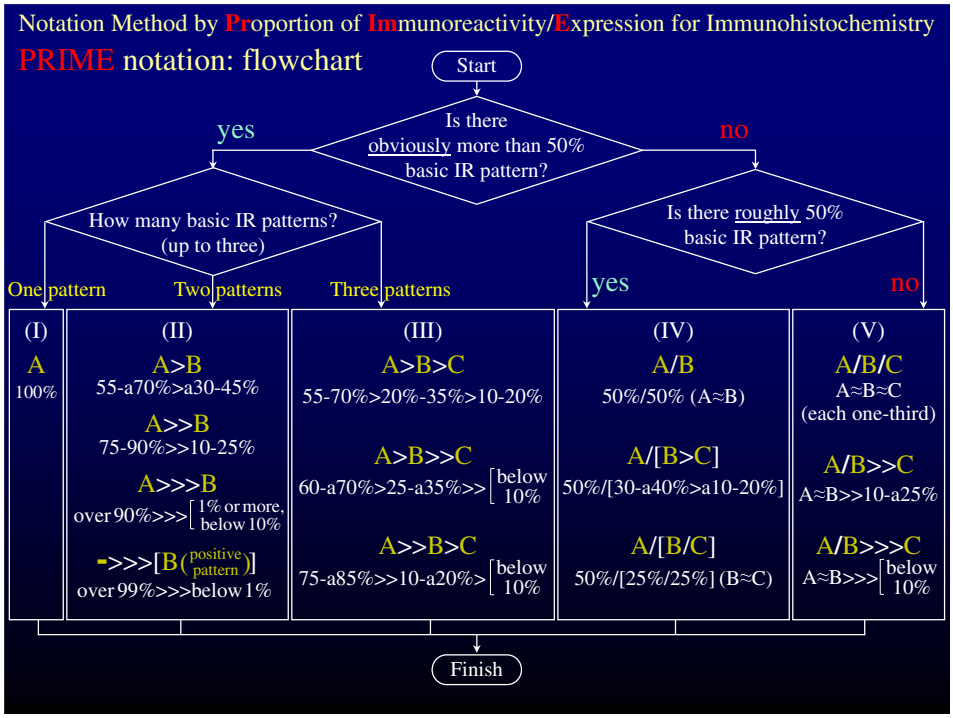

Fig. 1 Flowchart of PRIME notation. In principle, this method is applied only to tumor cells, and visual judgment is made under an optical microscope without using counting instruments. The details of the range of percentages occupied by an IR pattern, various types of IR patterns, inequality signs, and each symbol are described hereinafter: (*a*) Basic IR pattern: "+," "(weak)+," "(very weak)+" and "–"; (*b*) combined IR pattern ("P/Q" expression): "+/(weak)+," "+/(very weak)+," "+/–," "(weak)+/(very weak)+," "(weak)+/–" and "(very weak)+/–" used as one unit same as the basic IR patterns; (*c*) the proportion of tumor cells exhibiting either IR pattern is described using ">" (0-3, with the left IR pattern always being greater than the one on the right) or "/" (0-2). In addition, in the case of 3 inequalities with continuity (X>>>Z) or discontinuity (X>Y>>Z, X>>Y>Z), tumor cells presenting the rightmost IR pattern (Z) are less than 10%; (*d*) for "/" we describe a stronger IR pattern on the left side; (e) "[ ]" are used for less than 1% in (II) or 50% reactivity pattern in (IV); (*f*) *aX%* = around *X%* = [(*X* – 4) to (X + 4)]%.

Table 2 Relationship between *MYC* gene rearrangement and subtype

	MYC gene rearrangement	
	Positive	Negative
DLBCL (n = 55)	14 (25.5%)	41 (74.5%)
DLBCL + FL/G3 (n = 27)	5 (18.5%)	22 (81.5%)
FL/G3A (n = 6)	2	4
FL/G3B (n = 21)	3	18
Total (n = 82)	19 (23.2%)	63 (76.8%)

4 μm and incubated with anti c-MYC primary antibody (rabbit monoclonal primary antibody [Y69]; Roche Diagnostics [Basel, Switzerland] or Abcam [Cambridge, MA]). The immune complex was visualized with hydrogen peroxide substrate and diaminobenzidine tetrahydrochloride chromogen. Appropriate positive and negative controls were included.

2.4. Immunohistochemical evaluation method

We used the PRIME notation to score IR. This method encompasses not only the proportion of tumor cells that are immunohistochemically positive for a specific antigen but also the CPAR of tumor cells for the purpose of identifying the ideal image (Table 1). The distinctive feature of PRIME notation is visual inspection of the whole image of the specimen under an optical microscope without using a counting machine.

The basic IR pattern consists of the following 4 types: 3 positive patterns such as "+," "(weak)+," and "(very weak)+" and 1 negative pattern "−." In addition to the basic pattern, we used "P/Q" expression for the combined IR pattern. Proportionally, almost equal P and Q are selected from the 4 basic patterns and form a new single unit with "/" between them to yield the following 6 patterns: "+/(weak)+," "+/(very weak)+," "+/−," "(weak)+/(very weak)+," "(weak)+/−," and "(very weak)+/−"; the "P/Q" expression is used as one unit as with the 4 basic IR patterns. Another IR pattern such as "(strong)+" would be used only when a certain subtype of malignant lymphoma can be determined, such as strong expression of OCT2 in nodular lymphocyte predominant Hodgkin lymphoma, although we did not use this pattern in this study.

According to the flowchart (Fig. 1), which is a schematized algorithm of our concept, first ("Start"), the result is evaluated based on whether the IR pattern significantly exceeds 50%. An IR pattern is selected from the above-mentioned basic and combined patterns (10 in total). In the case where there is an IR pattern that obviously exceeds 50%, we choose one of (I), (II), or (III), depending on the number of IR patterns. In the case where the IR pattern was approximately 50%, we choose (IV), and in case where the IR pattern was obviously less than 50%, we choose (V). In this way, we apply the findings of IHC to one of (I) to (V), where (I) includes only IR patterns with 100% reactivity and (II) to (V) represent proportions of tumor cells exhibiting varying IR patterns, which are then described using the range of percentages, inequality signs, and symbols

Table 3 Details of PRIME notation pattern

Immunohistologic evaluation	No. of cases	Positive for *MYC* gene rearrangement
+/(weak)+/−	49	0***
+/(weak)+>−	6	4
+/(weak)+>>>−	7	4
+/(weak)+	1	0**
+>(weak)+>−	2	0**
+>(weak)+>>−	9	3**
+>(weak)+	1	1*
+>>(weak)+>−	4	4*
+>>(weak)+	2	2*
+>> >(weak)+	1	1*

***, **, * refer to Result 3.3.

(how to use each of these elements is described hereunder), finally leading to "Finish."

a. The proportion of tumor cells exhibiting either IR pattern is described using ">" (0-3, with the left IR pattern always being greater than the one on the right) or "/" (0-2).
b. For "/" we describe a stronger IR pattern on the left side.
c. "[ ]" are used for less than 1% in (II) or 50% reactivity pattern in (IV).
d. $aX\%$ = around $X\%$ = $[(X - 4)$ to $(X + 4)]\%$.

2.5. Evaluation of validity of the PRIME method

To examine the validity of the PRIME method, 45 cases from the ones examined in this study, including negative subjects, were extracted, presented on a monitor, and evaluated individually by 5 hematopathologists (F. F. and R. I. from Tohoku University, Y. N. from Tohoku Medical and Pharmaceutical University, A. Y.-A. from Iwate Medical University, and Y. O. from the University of Tokyo). In this analysis, the pathologist's assessments were divided into 3 groups: (1) "+/(weak)+/−"; (2) weaker than "+/(weak)+/−," such as "(weak)+/(very weak)+/−" or "(weak)+/−"; and (3) stronger than "+/(weak)+/−," such as "+/(weak)+>>−" or "+/(weak)+>>>−." The interobserver agreements were calculated using Fleiss κ statistic by 2 statisticians (T. N. and N. N.) [12]. Statistical analyses were performed using R version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria).

2.6. Determination of presence/absence of *MYC* abnormalities

The G-band method was used for routine diagnostics, and cases with 8q24/*MYC* translocation were judged as positive for *MYC* translocation. On the other hand, although clonal findings indicating a neoplastic lesion were detected by the G-band method, cases without abnormalities in 8q24/*MYC* were judged as negative for *MYC* translocation. For cases in which the G-band method could not be performed, fusion

FISH of t(8;14)(q24;q32)/*MYC-IGH* or split FISH of *MYC* (Carnoy-fixed cells or paraffin sections) was carried out to confirm 8q24/*MYC* translocation. Positive cases for *MYC* translocation were those in which t(8;14)(q24;q32)/*MYC-IGH* fusions or 8q24/*MYC* split signals were detected in more than 5% of cells.

2.7. Ethics

This study was approved by the ethics committee or the relevant organization of each hospital and institution (Ethics Committee of Tohoku University Graduate School of Medicine, Nos. 2009-28 and 2014-1-573) and complied with the principles outlined in the Declaration of Helsinki.

3. Results

3.1. *MYC* gene rearrangement

Among the 82 extracted cases whose IR pattern in PRIME notation started with "+" there were 55 cases of DLBCL and 27 of DLBCL + FL/G3; 19 (23.2%) were positive for *MYC* translocation, with 3 identified by G band, 6 by fusion FISH, and 12 by split FISH (with some of positive by G band). There was no significant difference between DLBCL and DLBCL + FL/G3 cases in terms of positive rate of *MYC* translocation (25.5% and 18.5%, respectively; Table 2).

3.2. Immunohistochemical staining pattern of c-MYC

The details of PRIME notation are shown in Table 3. Of the 82 cases, "+/(weak)+/–" was the most common (n = 49), followed by "+>(weak)+>>>–" (n = 9), "+/(weak)+>>>–" (n = 7), "+/(weak)+>>–" (n = 6), "+>>(weak)+>–" (n = 4), "+>>(weak)+" (n = 2), "+>(weak)+>–" (n = 2), and "+/(weak)+" (n = 1), "+>(weak)+" (n = 1), and "+>>>(weak)+" (n = 1). Representative c-MYC immunohistochemistry results are shown in Fig. 2.

3.3. Details of PRIME notation pattern

The most common notation by far was "+/(weak)+/–," indicating that CPAR that was positive, weakly positive, and

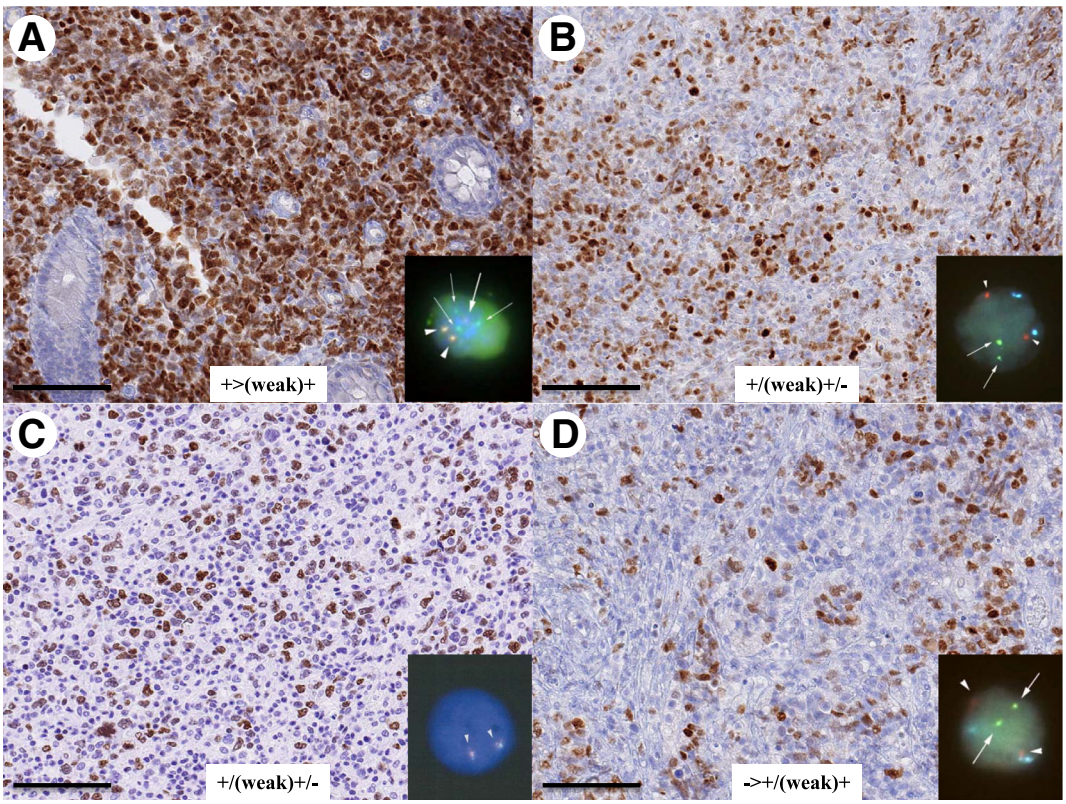

Fig. 2 Photograph of representative c-MYC immunohistochemistry. A, A case of "+>(weak)+" by PRIME notation with positive translocation (there is no "–"; "+" is dominant and includes "(weak)+"). The inset image shows fusion FISH of *IGH/MYC*; yellow (arrowheads) indicates the fusion signal, red (thick arrows) indicates *MYC*, green (thin arrows) indicates *IGH*, and blue corresponds to a CEN8 probe. B, A case of "+/(weak)+/–" by PRIME notation with negative translocation ("+," "(weak)+," and "–" each accounted for about one-third). The inset image shows fusion FISH of *IGH/MYC*; red (arrowheads) indicates *MYC*, green (arrows) indicates *IGH*, and blue corresponds to a CEN8 probe. C, A case of "+/(weak)+/–" by PRIME notation with negative translocation ("+," "(weak)+," and "–" each accounted for about one-third). The inset image shows split FISH of *MYC* and no split signal (no break; arrowheads). D, A case of "–>+/(weak)+" by PRIME notation with negative translocation ("–" is dominant, and the rest are "+" and "(weak)+"). The inset image shows fusion FISH of *IGH/MYC*; red (arrowheads) indicates *MYC*, green (arrows) indicates *IGH*, and blue corresponds to a CEN8 probe. Bar, 100 μm.

negative for tumor cells, and each accounted for about one-third of cases (Table 3/***); surprisingly, none of the 49 cases showed *MYC* translocation. That is, among IR patterns with c-MYC PRIME notation beginning with "+" in DLBCL ± FL/G3 (82 extracted cases), the overall specificity of negativity for *MYC* translocation was 76.8% (63/82 cases); however, among the 49 cases with a notation of "+/(weak)+/–," the specificity of negativity for *MYC* translocation was 100%. On the other hand, all cases with an IR pattern including over 50% "+" and no "–" or over 70% "+" harbored an 8q24/*MYC* translocation (Table 3/*); however, the group with fewer than 30% of cases positive for *MYC* translocation—including those with both "+" and "(weak)+" IR patterns—accounted for about 90% of the total (Table 3/**). There were no instances of *MYC* translocation among the 34 cases whose IR pattern of PRIME notation started with "(weak)+" or "–."

3.4. Validity of PRIME method

Of the 45 cases, 21 cases were judged as "+/(weak)+/–" by F. F. and R. I. (pathologists from the Tohoku University). Of these 21 cases, 16 cases (76.1%; unanimous) and 20 cases (95.2%) were evaluated as "+/(weak)+/–" by all 3 and 2 pathologists from other facilities, respectively. In addition, nobody evaluated as "+/(weak)+/–" cases that showed dyeability was judged to have a possibility of translocation such as "+/(weak)+>>–" or "+>(weak)+>>>–." In the 3 groups described in the Materials and Methods section, κ coefficients among the 5 observers were 0.873 ($P < .001$).

4. Discussion

There are several reports about the relationship between c-MYC immunohistochemistry results and the probability of 8q24/*MYC* translocation. One study evaluated c-MYC immunohistochemistry in increments of 10% and found that cases with *MYC* translocation were positive for c-MYC in more than 50% of tumor cells; another reported 87.8% sensitivity and 36.0% specificity in predicting *MYC* gene translocation in these cases, although CPAR was not considered in either study [9,13]. In the latter report, the specificity of negativity for *MYC* gene translocation was 64.0%, which is not significantly different from the 76.8% of total DLBCL ± FL/G3 cases in our study. However, we found that the specificity of negativity for *MYC* gene translocation was 100% if PRIME notation was limited to cases with "+/(weak)+/–" (Table 3/***). By adopting PRIME notation that considers not only the percentage of positive cells but also CPAR, it was possible to identify a group that was negative for *MYC* gene translocation with extremely high specificity. Thus, even when ~70% of tumor cells are positive or weakly positive for c-MYC, if the expression by PRIME notation is "+/(weak)+/–," it is not necessary to perform FISH analysis of *MYC*. In our study, 49 (59.8%) of 82 extracted cases had the "+/(weak)+/–" notation. The fact that they accounted for more than 50% of cases demonstrates that it is possible to greatly reduce the number of subjects with DLBCL ± FL/G3 with greater than 50% c-MYC–positive cells for whom there is uncertainty as to whether FISH of *MYC* should be performed. In addition, among cases with an IR pattern starting with "(weak)+" or "–," there were no instances of *MYC* translocation; FISH of *MYC* can be omitted for these subjects.

It is very useful from the standpoint of saving time and money that we demonstrate the selection of the cases in which FISH analysis for the translocation of 8q24/*MYC* should be carried out among all cases of DLBCL and/or FL/G3, with c-MYC–positive tumor cells accounting for more than half of all tumor cells. In a small subset of cases where *MYC* translocation was strongly suspected based on immunohistochemical data, translocation was observed (*) but there was also a group (**) with few translocations despite there being many positive cells; in such instances, it is essential to confirm *MYC* translocation by FISH. *MYC* amplification was detected in 3 cases where c-MYC was immunohistochemically confirmed both in terms of a high proportion of cells and high intensity of expression ("+>(weak)+>>–" in PRIME notation), which has been previously reported [8]. However, there were also 3 cases showing *MYC* amplification among those judged as "+/(weak)+/–." The reasons for the increase in protein expression and the significance of c-MYC expression in cases without 8q24/*MYC* translocation in terms of prognosis merit further study.

There have been no attempts to establish a universal notation method based on certain conventions according to the concept of CPAR. In this study, we used PRIME notation with the aim of restricting the type of IR pattern, inequality, and the number of slashes and of unifying the direction of inequality and method of describing immunohistochemistry findings, which has typically been done by individual pathologists using different terminology (Table 1). The flowchart of PRIME notation seems complicated at first sight because it assumes every possibility of CPAR. Because the notation used in practice is limited to some extent, it is easy to apply. Indeed, we have confirmed that there were few difficulties when several pathologists, including those from other institutions, switched from the previous notation to PRIME notation, and there were no significant disparities in notation among pathologists. This saved the time normally required to create appropriate phrases and select vocabulary to adequately describe the findings.

PRIME notation involves visual observation by light microscopy, which is useful for routine medical applications because it does not require a special device. Although quantitative image analyses can be performed using a computer, computer software cannot necessarily recognize or detect only tumor cells, and in diagnostic work where a large number of cases are processed, introducing new equipment can lead to software compatibility issues. It was reported that manual counts and image analysis coincided well with c-MYC immunohistochemistry [13], whereas others have also

demonstrated that counting from images produced accurate results [10]. Thus, examination of immunohistochemical specimens by well-trained pathologists can yield information equivalent to that obtained by image analysis.

The prototype of PRIME notation was invented by one of the authors (R.I.) as an attempt to express CPAR as a known symbol to achieve the ideal image shown in Table 1, on September 29, 2009 and modified on December 7, 2010. A unified method of describing immunohistochemical findings is necessary for epidemiological surveys based on annual and long-term follow-up of malignant lymphoma cases in Miyagi Prefecture (the MIYAGI study) conducted by the Public Interest Foundation Corporation "Ichinohasama Memorial READ Blood Academy," established on October 1, 2008 [11,14]. This system was then systematized and completed by F.F., S.K-F., and R.I. for this submission. In this study, we demonstrated a perfect relationship between PRIME notation and *MYC* translocation, although it was specific to nontranslocation (or lack of translocation) of *MYC*. This is the first report to present a clear correspondence between immunohistochemical findings and chromosomal abnormality (reflecting findings at the protein and gene levels, respectively). PRIME notation can be universally applied to antibodies other than c-MYC; moreover, multivariate analysis of large data sets that include PRIME notation can reveal the associations among gene mutation and/or translocation, degree of malignancy, and patient prognosis.

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