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Original Article

Distinct Global DNA Methylation Status in B-Cell Lymphomas : Immunohistochemical Study of 5-Methylcytosine and 5-Hydroxymethylcytosine

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Lymphomas are malignant neoplasms composed of lymphoid cells at various developmental stages and lineages. Recent advances in comprehensive genomic analyses in acute myeloid leukemia have revealed prevalent mutations in regulators of epigenetic phenomena including global DNA methylation status. The examples include mutations in isocitrate dehydrogenase 1 (IDH1), IDH2, and ten-eleven translocation 2. These mutations are proposed to inhibit conversion of 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5 hmC), leading to global accumulation of 5 mC. These changes in global DNA methylation status can be visualized immunohistochemically using specific antibodies against 5 mC and 5 hmC. We examined the global DNA methylation status of B-cell lymphomas and that of their normal counterparts by immunohistochemistry for 5 mC and 5 hmC. Non-tumor lymphoid cells inside germinal centers (GC) in reactive lymphoid hyperplasia (RLH) were stained positive for 5 mC, but they were negative for 5 hmC. Similarly, follicular lymphomas, whose postulated normal counterparts are centrocytes in GCs, were 5 mC-positive but 5 hmC-negative by immunohistochemistry. This immunostaining pattern was also observed in Burkitt lymphoma. In contrast, non-tumor lymphoid cells in mantle zones were stained positive for 5 mC as well as for 5 hmC. Likewise, most mantle cell lymphomas, whose postulated normal counterparts are mantle zone B cells in RLH, were stained positive for 5 mC as well as for 5 hmC. This immunostaining pattern was also observed in chronic lymphocytic leukemia/small lymphocytic lymphoma. These results suggest that, in terms of 5 mC/5 hmC immunohistochemistry, B-cell lymphomas with different histological subtypes are associated with distinct global DNA methylation statuses that resemble those of their postulated normal counterparts. 〔*J Clin Exp Hematop 54(1): 67-73, 2014*〕

Keywords: immunohistochemistry, 5-methylcytosine, 5-hydroxymethylcytosine, B-cell lymphoma

INTRODUCTION

Lymphomas are malignant neoplasms composed of lymphoid cells at various developmental and differentiation stages and lineages.[1] They are heterogeneous in terms of their postulated normal counterparts. For example, in the case of B-cell lymphomas, postulated normal counterparts for follicular lymphoma (FL) are centrocytes inside germinal centers (GC) of secondary lymphoid follicles in lymph nodes.[1] On the other hand, postulated normal counterparts for mantle cell lymphoma (MCL) are naïve B cells in mantle zones surrounding GCs in lymph nodes.[1] As other examples, postulated normal counterparts for Burkitt lymphoma are GC or post-GC B cells, while those for chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) are naïve or post-GC memory B cells.[1]

Recent advances in comprehensive genomic analyses of various cancers have revealed recurrent genomic aberrations specific for each. Not only genetic but consequent epigenetic changes that play important roles in the pathogenesis of various cancers have been observed.[2] Epigenetic changes include DNA methylation of CpG islands in regulatory regions of genes and post-translational modification of histones.[3] For example, comprehensive genomic analyses in acute myeloid leukemia have revealed prevalent mutations in regulators of epigenetic phenomena including global DNA methylation status. The examples include mutations in isocitrate dehydrogenase 1 (IDH1), IDH2, and ten-eleven translocation 2 (TET2).[4] These mutations are proposed to inhibit, indirectly or directly, the conversion of 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5 hmC), leading to global accumulation of 5 mC.[5,6] Global accumulation of 5 mC is thought to cause deregulation of the expression of various genes via aberration of their CpG island methylation. These changes in

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global DNA methylation status can be visualized immunohistochemically using specific antibodies against 5 mC and 5 hmC.[5] Interestingly, TET2 mutations were found not only in cases of acute myeloid leukemia but also in some types of T-cell lymphoma, including angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma.[6] These results indicate that both genetic factors and epigenetic factors are involved in tumorigenesis of some subtypes of lymphoma. Furthermore, the prevalence of aberrations in DNA methylation among hematological neoplasms suggests that DNA methylation may be a candidate for a common "bottleneck" among these. However, it was unclear whether the global change in 5 mC and 5 hmC is also observed in lymphomas other than T-cell lymphomas. In addition, it was unclear whether the global change in 5 mC and 5 hmC is a tumor-specific characteristic not observed in non-tumor cells.

In the present investigation, we examined the global DNA methylation status of B-cell lymphomas and that of their normal counterparts by immunohistochemistry for 5 mC and 5 hmC. We found that non-tumor lymphoid cells inside GC of reactive lymphoid hyperplasia (RLH) were stained positive for 5 mC, but they were negative for 5 hmC. Similarly, FLs, whose postulated normal counterparts are centrocytes in GCs, were found to be 5 mC-positive but 5 hmC-negative by immunohistochemistry. In contrast, non-tumor lymphoid cells in mantle zones in RLH were positive for 5 mC as well as for 5 hmC. Likewise, MCLs, whose postulated normal counterparts are mantle zone B cells, were positive for 5 mC as well as for 5 hmC in most cases. The 5 mC/5 hmC immunohistochemistry of Burkitt lymphoma showed a staining pattern similar to that of FL, while examination of CLL/SLL cases revealed a staining pattern similar to that of most MCLs. These results suggest that, in terms of 5 mC/5 hmC immunohistochemistry, B-cell lymphomas with different histological subtypes are associated with distinct global DNA methylation statuses that are similar to those of their postulated normal counterparts.

MATERIALS AND METHODS

Case selection

Seven cases of RLH (chronic tonsillitis), 8 cases of FL, 11 cases of MCL, 8 cases of Burkitt lymphoma, and 11 cases of CLL/SLL were retrieved from the files of the Department of Surgical Pathology, Hyogo College of Medicine Hospital, and those of Kobe City Medical Center General Hospital. Lymphomas were classified according to the current WHO classification criteria[1] with the aid of immunohistochemistry. Briefly, RLH consisted of multiple hyperplastic lymphoid follicles with GCs where many tingible body macrophages were present. FL had follicle-like structures composed of centrocyte-like tumor cells expressing Bcl-2 protein. MCL consisted of diffuse neoplastic expansion of B lymphoid cells expressing CD5 and Cyclin D1 proteins, which resulted in normal follicular structures being obscured. Burkitt lymphoma consisted of diffuse expansion of medium-sized to large neoplastic B lymphoid cells expressing CD10 but not Bcl-2 proteins, whose positivity for Ki-67 was virtually 100%. CLL/SLL consisted of diffuse but vaguely nodular expansion of small neoplastic B lymphoid cells expressing CD23 but not Cyclin D1 proteins. The seven RLH cases included 2 males and 5 females aged from 17 to 61, with a median of 39. The eight FL cases included 5 males and 3 females aged from 53 to 85, with a median of 60.5. Our FL cases consisted of 4 cases of grade 1, 2 cases of grade 2, and 2 cases of grade 3a. The eleven MCL cases included 8 males and 3 females aged from 45 to 85, with a median of 70. The eight Burkitt lymphoma cases included 5 males and 3 females aged from 12 to 70, with a median of 57. The eleven CLL/SLL cases included 7 males and 4 females aged from 56 to 80, with a median of 67. The CLL/SLL cases included 1 case showing partial transformation to large cells (Richter transformation). In this case, immunohistochemical data were obtained from small cell components.

Immunohistochemical analyses

Immunohistochemistry was performed on sections (3 μm thick) of formalin-fixed and paraffin-embedded tissues. NA81 anti-5 mC mouse monoclonal antibody (Merck Millipore catalogue No. 162 33 D3) and anti-5 hmC rabbit polyclonal antibody (Active Motif catalogue No. 39769) were used. For 5 mC immunohistochemistry, the antibody was used at 1:500 dilution and antigen retrieval was carried out by placing the sections in 0.01 M sodium citrate buffer at pH 6.0 and 95°C for 20 min. For 5 hmC immunohistochemistry, the antibody was used at 1:2,000 dilution and antigen retrieval was carried out by placing the sections in 0.01 M sodium citrate buffer at pH 9.0 and 95°C for 20 min. All of the above procedures were performed using Leica BOND-MAX (Leica). Immunoreactive, that is, positive, cells for each antibody were designated as cells whose nuclei were identified in brown. For each case, at least 250 cells were counted and evaluated as either positive or negative. Mean percentages of positive cells ± standard error of the mean (s.e.m.) were calculated for each antibody for each cell type.

RESULTS

First, we examined non-tumor secondary lymphoid follicles in RLH (Fig. 1a). As shown in Fig. 1b, we found that lymphoid cells inside GCs were positively stained in nuclei for 5 mC, although the intensity of immunoreactivity was heterogeneous. In comparison, lymphoid cells in mantle zones outside GCs showed strong and homogeneous positive

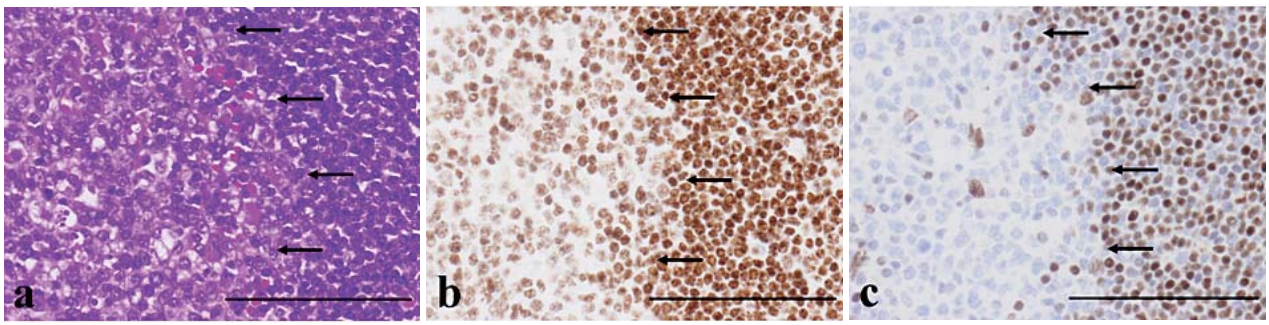

Fig. 1. Representative images of 5−methylcytosine (5 mC)/5−hydroxymethylcytosine (5 hmC) immunohistochemistry of reactive lymphoid hyperplasia. The borderline between germinal centers (GCs) and mantle zones is indicated by *arrows*. The GC is in the left half of each figure, while the mantle zone is in the right half. (***1a***) H&E stain. (***1b***) Immunostaining for 5 mC. (***1c***) Immunostaining for 5 hmC. Brown nuclei are shown to be positive in immunostaining. Both GC B cells and mantle zone B cells were positive for 5 mC (*1b*). On the other hand, GC B cells were not stained but mantle zone B cells were stained for 5 hmC (*1c*). Original magnification: ×400. Scale bar: 100 μm.

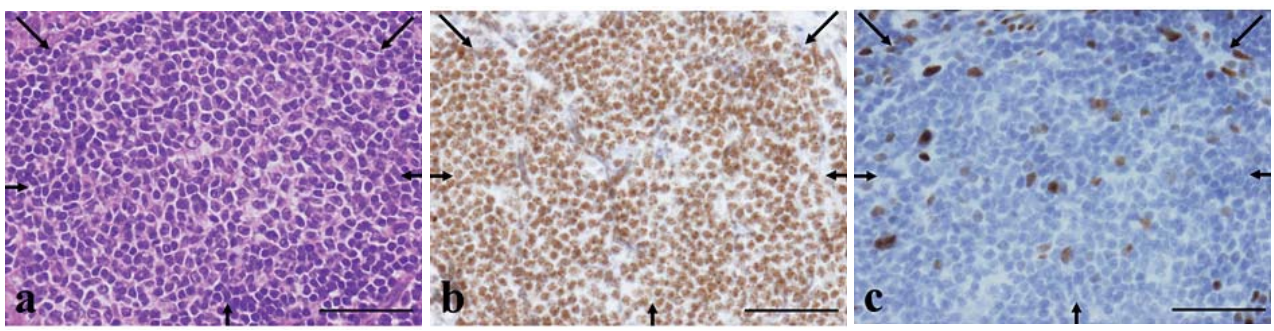

Fig. 2. Representative images of 5−methylcytosine (5 mC)/5−hydroxymethylcytosine (5 hmC) immunohistochemistry of follicular lymphoma. Germinal center-like tumor nodule is indicated by *arrows*. (***2a***) H&E stain. (***2b***) Immunostaining for 5 mC. (***2c***) Immunostaining for 5 hmC. Brown nuclei are shown to be positive in immunostaining. Follicular lymphoma cells were immunohistochemically positive for 5 mC (*2b*), but negative for 5 hmC (*2c*). Original magnification: ×400. Scale bar: 50 μm.

staining for 5 mC (Fig. 1b). In contrast, lymphoid cells inside GCs and those in mantle zones showed a distinct phenotype in immunohistochemistry for 5 hmC. As shown in Fig. 1c, most lymphoid cells inside GCs showed negative staining for 5 hmC. A few 5 hmC-positive cells inside GCs were supposed to include histiocytes, follicular dendritic cells, or endothelial cells, judging from their morphology in the HE image (Fig. 1a). On the other hand, most lymphoid cells in mantle zones showed positive staining for 5 hmC (Fig. 1c). These results suggest that lymphoid cells inside GCs and those in mantle zones have distinct statuses of global DNA methylation.

Next, we examined the global DNA methylation status of FL cells (Fig. 2a), which are postulated tumor counterparts of centrocytes inside GCs. As shown in Fig. 2b, FL cells showed homogeneously positive staining for 5 mC. In contrast, most FL cells were negatively stained for 5 hmC. A few 5 hmC-positive cells inside tumor follicles were supposed to include histiocytes, follicular dendritic cells, or endothelial cells. The 5 mC-positive and 5 hmC-negative immunostaining pattern was quite similar to that observed for non-tumor lymphoid cells inside GCs (Fig. 1b & 1c).

Then, we examined the global DNA methylation status of MCL cells (Fig. 3), which are postulated tumor counterparts of mantle zone B cells. As shown in Fig. 3a, 3c, & 3e, MCL cells showed homogeneously positive staining for 5 mC. In contrast, three staining patterns for 5 hmC immunohistochemistry were observed (Fig. 3b, 3d, & 3f). Of the 11 cases of MCL examined, 1 case showed homogeneous and strong positive staining for 5 hmC (Fig. 3b). Eight cases showed heterogeneous but diffusely positive staining for 5 hmC, although the intensity of the staining was weaker than that of the former pattern (Fig. 3d). In contrast, in the other 2 cases of MCL, most of the tumor cells showed negative staining, although scattered positive cells were observed (Fig. 3f). The last staining pattern was similar to that of FL cases (Fig. 2). However, the frequency of FL-like pattern in MCL cases,

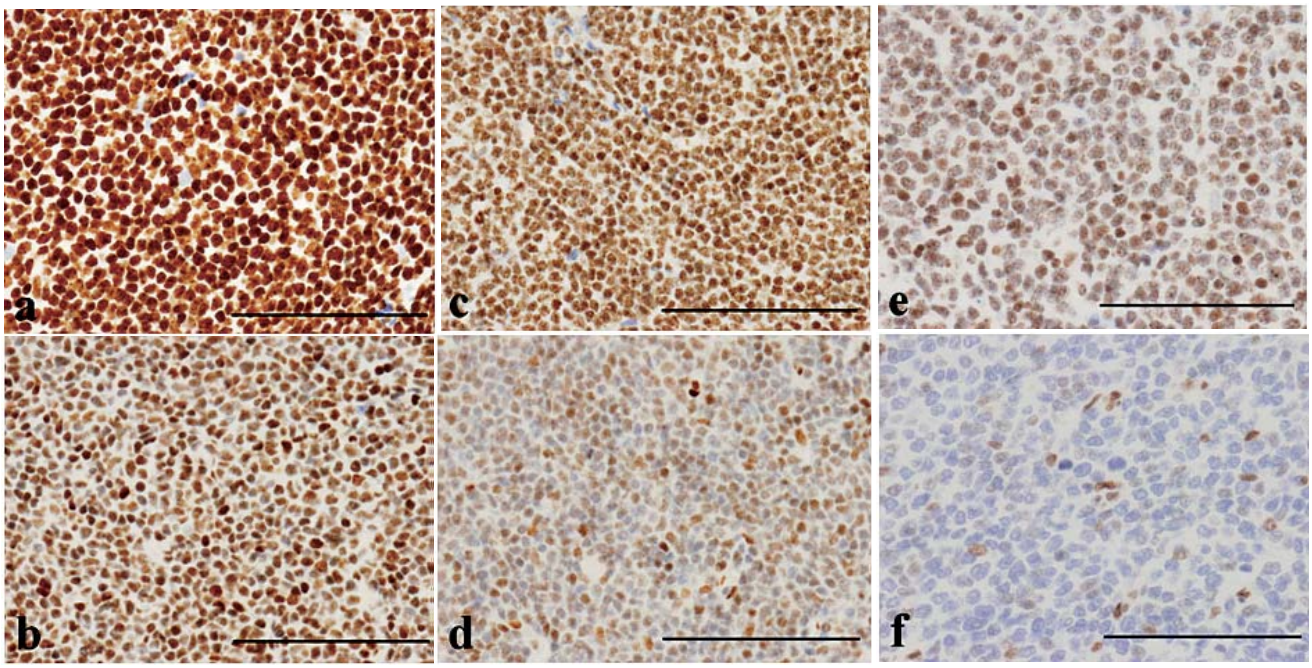

Fig. 3. Three representative cases of 5−methylcytosine (5 mC)/5−hydroxymethylcytosine (5 hmC) immunohistochemistry of mantle cell lymphoma. (***3a***), (***3c***), & (***3e***) Immunostaining for 5 mC. (***3b***), (***3d***), & (***3f***) Immunostaining for 5 hmC. (*3a*) & (*3b*) case 1. (*3c*) & (*3d*) case 2. (*3e*) & (*3f*) case 3. Brown nuclei are shown to be positive. Original magnification: ×400. Scale bar: 100 μm.

which was 2 out of 11, was significantly different from that in FL, which was 8 out of 8 (χ^2 test, $\chi^2 = 12.419$, $p = 0.000425$). Overall, the staining pattern for immunohistochemistry for 5 mC and 5 hmC was similar to that observed for non-tumor mantle zone cells outside GCs (Fig. 1b & 1c).

To confirm these results, we further examined cases of Burkitt lymphoma and CLL/SLL by 5 mC/5 hmC immunohistochemistry. Postulated normal counterparts for Burkitt lymphoma are GC or post-GC B cells, while those for CLL/SLL are naïve or post-GC memory B cells.[1] It was expected that global DNA methylation status of Burkitt lymphoma could be more like that of FL, while that of CLL/SLL could be more like that of MCL. As shown in Fig. 4a & 4b, Burkitt lymphoma cells showed a 5 mC-positive but 5 hmC-negative immunostaining pattern, similar to that of FL cells (Fig. 2b & 2c). On the other hand, CLL/SLL cells showed a 5 mC-positive and 5 hmC-positive immunostaining pattern (Fig. 4c & 4d), similar to that observed in 9 out of 11 cases of MCL examined (Fig. 3a-3d).

Quantitative data of the positive cells for each antibody for each cell type are summarized in Table 1.

DISCUSSION

In this study, we demonstrated that B-cell lymphomas such as FL, MCL, Burkitt lymphoma, and CLL/SLL showed distinct statuses of global DNA methylation in terms of 5 mC/5 hmC immunohistochemistry, which are similar to those of their postulated normal counterparts. In our previous study, we demonstrated that global histone modification profile is well conserved between B-cell lymphomas (FL and MCL) and their postulated normal counterparts.[7] These results suggest that global epigenetic status is relatively similar between B-cell lymphomas and their normal counterparts. In the case of FL, MCL, Burkitt lymphoma, and CLL/SLL, genome-wide comparison of the methylome of lymphoma cells with that of putative normal counterparts revealed some differences of DNA methylation in terms of the genome-wide distribution and/or individual genes.[8-13] However, a distinct global status of 5 hmC in relation to 5 mC in FL, MCL, Burkitt lymphoma, and CLL/SLL has not been reported.

The results in this paper suggest the possibility that some kind of "epigenetic memory" of cells of origin may persist in some types of B-cell lymphoma. This is reminiscent of "epigenetic memory" of cells of origin in induced pluripotent stem cells (iPSCs).[14,15] In fact, the differentiation spectrum of iPSCs was affected by which cells of origin the iPSCs were derived from.[14,15]

We also demonstrated that the transition of immunohistochemical status of 5 mC/5 hmC occurs between mantle zone B cells and lymphoid cells inside GCs. It was reported that mutations in IDH1, IDH2, or TET2 were associated with a 5 mC-positive and 5 hmC-negative immunophenotype. However, these mutations were not previously reported in FL,

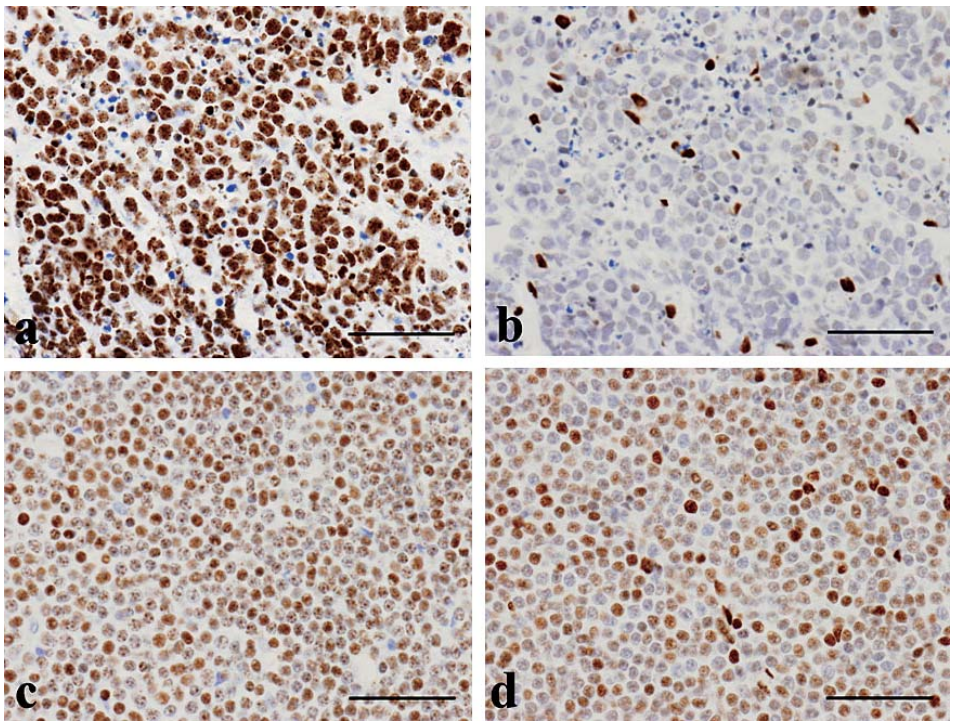

Fig. 4. Representative images of 5−methylcytosine (5 mC)/5−hydroxymethylcytosine (5 hmC) immunohistochemistry of Burkitt lymphoma and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL). (***4a***) & (***4c***) Immunostaining for 5 mC. (***4b***) & (***4d***) Immunostaining for 5 hmC. (*4a*) & (*4b*) Burkitt lymphoma. (*4c*) & (*4d*) CLL/SLL. Brown nuclei are shown to be positive. Original magnification: ×400. Scale bar: 50 μm.

Table 1. Percentage of positive cells of immunohistochemistry for 5 mC and 5 hmC in the cases of reactive lymphoid hyperplasia, follicular lymphoma, mantle cell lymphoma, Burkitt lymphoma, and chronic lymphocytic leukemia/small lymphocytic lymphoma

Lymphoid tissues/lymphoma	5 mC	5 hmC
Germinal center of reactive lymphoid hyperplasia	98.7 ± 0.4	15.8 ± 3.0
Mantle zone of reactive lymphoid hyperplasia	99.3 ± 0.3	96.7 ± 0.7
Follicular lymphoma cells	98.8 ± 1.3	9.2 ± 1.3
Mantle cell lymphoma cells	98.6 ± 0.2	82.6 ± 9.8
Burkitt lymphoma cells	96.4 ± 2.6	10.7 ± 4.1
Chronic lymphocytic leukemia/small lymphocytic lymphoma cells	95.8 ± 4.7	87.9 ± 9.5

5 mC, 5-methylcytosine; 5 hmC, 5-hydroxymethylcytosine. The values show mean percentage ± standard error of the mean.

MCL, Burkitt lymphoma, and CLL/SLL. The following question thus arises: What is a candidate factor involved in changes of global DNA methylation during the developmental transition from naïve mantle zone B cells to GC B cells? One of the possible factors involved in this transition is an epigenetic regulator, Enhancer of zeste homolog 2 (EZH2). EZH2, a subunit of a multi-enzyme complex known as polycomb repressive complex 2, is a SET domain-containing methyltransferase responsible for the trimethylation of lysine residue 27 of histone H3 (H3K27me3).[16] H3K27me3 is a transcriptionally repressive epigenetic mark. It was demonstrated that an increase in H3K27me3 by EZH2 leads to an increase in 5 mC in EZH2 target genes.[17] Comprehensive genomic analysis of diffuse large B-cell lymphomas revealed gene mutations in factors regulating epigenetics, including histone modification enzymes and chromatin remodeling factors.[18] Mutations in the SET domain of EZH2 were identified in 7.2% of FLs and 21.7% of diffuse large B-cell lymphomas with GC phenotype.[19] Mice conditionally expressing an EZH2 mutation observed in human lymphomas showed GC

hyperplasia.[20] EZH2 deletion or pharmacologic inhibition suppressed GC formation and functions in mice.[20] These results suggest that inhibitors of EZH2 may be effective for some types of B-cell lymphoma, particularly those showing GC-like phenotypes, including FLs.

Factors more directly involved in the transition of global DNA methylation status may be DNA methyltransferase (DNMT) 1 and activation-induced cytosine deaminase (AID). The expression of both is upregulated in GC B cells.[21,22] Interestingly, it was recently reported that AID may be involved in global DNA demethylation in primordial germ cells.[23] It is tempting to speculate that the balance between methylation by DNMT1 and demethylation by AID may be important for the transition of global DNA methylation status from mantle zone B cells to GC B cells.

Epigenetic therapies have been shown to be effective for some hematological malignancies.[24] Examples include 5-azacytidine for myelodysplastic syndrome and histone deacetylase inhibitors for cutaneous T-cell lymphomas.[24] We demonstrated that FLs showed a 5 mC-positive and 5 hmC-negative immunohistochemical phenotype. Recently, it was reported that a large number of other malignant tumors show a 5 mC-positive and 5 hmC-negative phenotype, just like the FLs in this study, including malignant melanoma.[25,26] In the case of malignant melanoma, some cases harbor mutations in IDH1 or IDH2, while others show loss of TET2 protein expression.[26] It was reported that compensation for the loss of TET2 protein expression by its exogenous overexpression suppressed the aggressive behavior of human malignant melanoma cells in xenograft mouse models.[26] This indicates that compensation for the TET2 protein can be a novel therapeutic approach for malignant tumors with a 5 mC-positive and 5 hmC-negative phenotype, including FLs as demonstrated in this study. In the same way, differences in global DNA methylation status between MCLs and FLs can be therapeutically exploited. It is an intriguing possibility that the aggressive clinical behavior of MCLs can be reduced (transformed) to a less aggressive form of FLs by exploitation and manipulation of global DNA methylation status.

At the same time, it should be kept in mind that relative conservation of global DNA methylation status between non-tumors and tumors, that is, the presumed presence of "epigenetic memory", may lead to side effects on non-tumorous tissue, when the methylation status is therapeutically targeted.

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