JSHC
Japan Society of
Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 47 (3): 125–131, 2014
doi: 10.1267/ahc.14012

BOB.1-positive Classical Hodgkin's Lymphoma Carries Hypermethylation of Its Promoter as Epigenetic Marker of Gene-silencing Memory

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Received February 18, 2014; accepted May 21, 2014; published online June 26, 2014

Analysis of archival formalin-fixed, paraffin-embedded (FFPE) pathological specimens of three case of Epstein-Barr virus (EBV)-positive diffuse large B-cell lymphoma (DLBCL) and three cases of classical Hodgkin lymphoma (CHL) revealed that hypermethylation of the *BOB.1* gene promoter was exclusively observed in CHL. A discrepancy was observed, however, between the methylation status of the *BOB.1* gene promoter and its expression in the EBV-positive mixed cellular CHL (MCCHL). Since MCCHL lacks the typical B-cell phenotype even in the presence of abundant BOB.1 transcription factors, functional activity of BOB.1 may be lost or reduced by a mechanism other than epigenetic gene silencing. When some tumor-suppressor gene products have lost their biological function, impact or significance of derepression of such genes may be little. Therefore, when interpreting immunohistochemical results for diagnostic or research purposes, it must be borne in mind that apparent positive immunostaining can merely be the result of chromatin remodeling and that such transient expression often has little functional significance. Any apparent positive immunohistochemical result needs to be interpreted carefully with the help of the hypermethylation status as a molecular marker of gene silencing memory.

Key words: Hodgkin's disease, methylation, transcription factor, microdissection

I. Introduction

Malignant lymphoma, a neoplastic disorder of lymphoid tissue, is now grossly classified into Hodgkin (HL) and non-Hodgkin lymphomas (NHL) [8, 19, 20]. HL is characterized by the presence of a small fraction of neoplastic giant cells called Hodgkin/Reed-Sternberg (H/RS) cells surrounded by reactive non-neoplastic inflammatory cells [11, 20]. The normal cellular counterpart of the H/RS cell had long been unidentified due to the variable expression of markers for distinct hematopoietic lineages. Since molecular analyses have revealed the clonal rearrangement of the V, D, and J segments of the IgH chain locus in H/RS cells [8], it is now widely accepted that these cells have descended from B-lymphocytes lacking typical B-lymphocyte-specific transcriptional factors [6, 17, 20]. Among these factors, the B-cell Oct-binding protein 1 (BOB.1) can stimulate B-lymphocyte specific gene transcription by interacting with the octamer-binding transcription factor (OCT-2) [17], and the absence or substantial downregulation of BOB.1 is, indeed, observed in almost all classical HL (CHL) cell lines [17] as well as in primary H/RS cells of all types of CHL [6, 18, 21]. In practical histopathological diagnosis, however, some cases, otherwise typical CHL, show positive BOB.1 immunostaining [4, 7, 22] that is interpreted as merely lack of OCT-2 function or an obstacle to the binding of transcription factors to the target DNA [1].

In this study we examined the association between the methylation status of *BOB.1* gene promoter and BOB.1

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Table 1. *Primer sequences used in polymerase chain reaction (PCR)-based assays, with product size (bp) and annealing temperature*

	Primer sequence	Size	Temp (°C)
First-round PCR			
	(F) 5'-ATTTTTATATTTATATATAGTAGGTT	161	60
	(R) 5'-CAACCAAACCACCTATCACCCCACTA		
Second-round PCR			
Methylated	(F) 5'-GTGTAGGTTGTGGTTGTTCGC	98	60
	(R) 5'-TATACGCGCCCCAACGACCCCG		
Unmethylated	(F) 5'-TAGGTTGTGGTTGTTTGTGTTGT	95	60
	(R) 5'-TATACACACCCCAACAACCCCA		

expression among cases of BOB.1-positive diffuse large B-cell lymphoma (DLBCL), BOB.1-positive CHL and BOB.1-negative CHL.

II. Materials and Methods

Samples

Routinely formalin-fixed (10%) and paraffin-embedded (FFPE) tissue samples of three cases of Epstein-Barr virus (EBV)-positive diffuse large B-cell lymphoma (DLBCL), a case of BOB.1-positive mixed cellularity CHL (MCCHL), a case of BOB.1-negative MCCHL, and a case of BOB.1-negative lymphocyte-rich CHL (LRCHL) were retrieved from the archives of the authors' institutes. All the diagnoses were made by two independent authorized pathologists, according to the criteria of the WHO classification [19]. Written informed consent was obtained from all patients, and the study was reviewed and approved by the local ethics committee at Ehime University.

Immunohistochemistry

The phenotype of the tumor cells in each lymphoma was reached by the results of immunohistochemical staining with the use of a panel of the antibodies: CD4, CD8, CD15, CD20, CD30, CD57 (DAKO, Japan), BOB.1 (sc955; Santa Cruz, CA, USA), and Oct-2 (clone PT1; Oncogene, CA, USA). Paraffin-embedded sections were immunostained by SuperSensitive™ Link-Label IHC Detection System (BioGenex, CA, USA) according the manufacturers' protocol.

Preparation of Agarose-bead mediated template for methylation-specific polymerase chain reaction (MSP)

Paraffin-embedded samples from hematoxylin and eosin-stained sections were deparaffinized with xylene and subjected to microdissection under a light microscope (Leica Microsystems LMD7000). Typical tumor cells of ten to twenty were selectively separated from surrounding inflammatory cells. The microdissected samples were suspended in 5 μl of 1×TE and mixed with pre-warmed and liquefied low-melting agarose (3.2%) at 1:1 as described [10, 15]. A total of 10 μl agarose beads containing 1×TE and tissue fragments was formed in pre-chilled 250 μl mineral oil and incubated at 50°C overnight in 1 μl of 0.2 μg/ml proteinase K, 10 mM of Tris-HCl (pH 8.0) and 25 mM ethylenediaminetetraacetic acid (EDTA). Aliquots of 100 μl of a 5 M bisulfite solution (3.5 M NaHSO3, Nakarai; 1 mM hydroquinone, Sigma; pH 5.0) were added to each reaction tube containing a single bead. The beads were brought into the aqueous phase by gently inverting the reaction tube. The reaction mixtures were then incubated for 18–24 hr at 50°C in the dark. Treatment was stopped by equilibration against six times wash with 1.5 ml of 1×TE (15 min each) followed by desulfonation twice in 1.5 ml of 0.5 N NaOH (15 min each). The reaction was neutralized with 1/5 volume of 1 N hydrochloric acid. Finally, the beads were washed with 1.5 ml 1×TE (2×5 times mixture). The beads were sliced into small cubes and used directly for MSP. For methylation analysis of the *BOB.1* gene in microdissected H/RS or similar cells, a nested MSP approach was used [20]. First-round PCR for bisulfited DNA was conducted with the use of strand-specific primers for bisulfate-converted single-strand DNA of the promoter, targeting sequences without CpG sites (Table 1). The primers for the first-step PCR amplification, a 161-bp amplicon containing 98-bp CpG sites, were amplified with two primers, (forward) 5'-atttttatatttatatatagtaggtt-3', and (reverse) 5'-caaccaaaccacctatcaccccacta-3', under the following conditions: the hot-start step for 2 min at 98°C, 30 cycles at 98°C for 10 sec, 60°C for 15 sec, 68°C for 30 sec, and the elongation step for 3 min at 72°C. The primers used for the second-step PCR were amplified with two sets of primers for methylated, (forward) 5'-gtgtaggttgtggttgttcgc-3', and (reverse) 5'-tatacgcgcccc aacgaccccg-3', and unmethylated (forward) 5'-taggttgtggttg tttgtgttgt-3', and (reverse) 5'-tatacacaccccaacaacccca-3' under the following conditions: 25 cycles with the same time course and temperature as for the first-step PCR. The PCR products were electrophoresed in a 3% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

III. Results

All three case of the EBV-positive DLBCL contained numerous large tumor cells. HE staining of a typical EBV-positive DLBCL case is illustrated as Fig. 1A (arrows indicate tumor cells). Large tumor cells (Fig. 1B, arrows) as

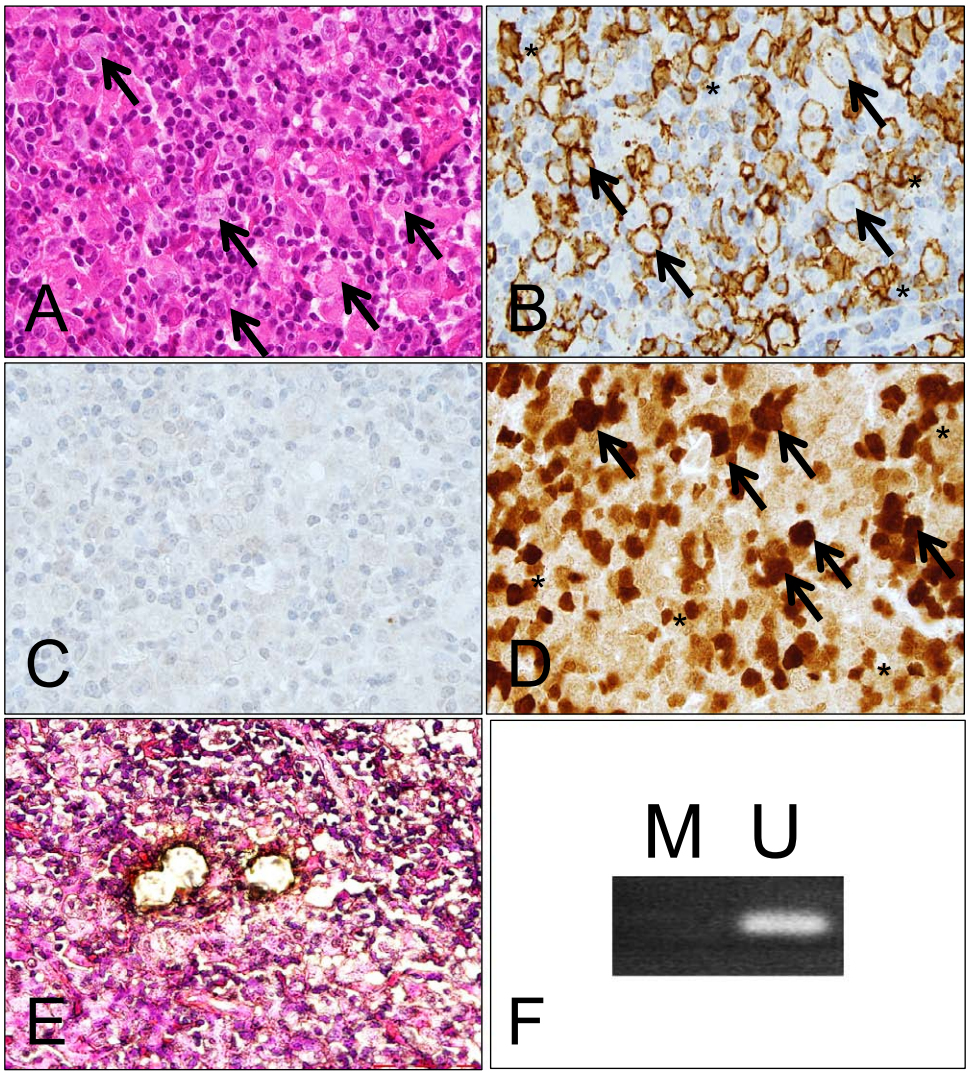

Fig. 1. EBV-positive DLBCL contains numerous large tumor cells with abundant cytoplasm and irregular nuclei (**A**, HE, ×400, arrows). Large tumor cells (**B**, ×400, arrows) as well as infiltrating non-neoplastic small B-cells (**B**, asterisks) are positive for CD20. All kinds of the cells (**C**, ×400) are negative for CD30. Large tumor cells (**D**, ×400, arrows) as well as infiltrating non-neoplastic B-cells (**D**, asterisks) are strongly positive for BOB.1. Large tumor cells selectively collected by microdissection (**E**, ×400) show positive PCR products solely by unmethylated (U) primers (**F**).

well as small infiltrating non-neoplastic B-cells (Fig. 1B, asterisks) stained positive for B-cell marker CD20; all kinds of cells (Fig. 1C) were negative for H/RS marker CD30; and large tumor cells (Fig. 1D, arrows) as well as infiltrating non-neoplastic B-cells (Fig. 1D, asterisks) were strongly positive for BOB.1. In all three cases, samples selectively collected from large tumor cells by microdissection (Fig. 1E, same case as A) showed positive PCR products solely by unmethylated (U) primers (Fig. 1F, same case as A).

EBV-positive MCCHL contained typical H/RS cells (Fig. 2A, HE, arrows). CD20 was negative in H/RS cells (Fig. 2B, arrows) but positive in infiltrating non-neoplastic B-cells (Fig. 2B, asterisks). CD30 was positive exclusively in H/RS cells (Fig. 2C, arrows) that were positive for EBV (Fig. 2C, insert). BOB.1 was strongly positive in large tumor cells (Fig. 2D, arrows) as well as in infiltrating non-neoplastic B-cells (Fig. 2D, asterisks). H/RS cells selectively collected by microdissection (Fig. 2E) showed positive PCR products by both methylated (M) and unmethylated (U) primers (Fig. 2F).

The typical MCCHL contained a few H/RS cells (Fig. 3A, HE, arrows). While H/RS cells were negative for CD20 (Fig. 3B, arrows), infiltrating non-neoplastic B-cells were positive (Fig. 3B, asterisks). H/RS cells were selectively positive for CD30 (Fig. 3C, arrows), but negative for BOB.1 (Fig. 3D, arrows). BOB.1 was positive in infiltrating non-neoplastic B-cells (Fig. 3D, asterisks). H/RS cells selectively collected by microdissection (Fig. 3E) showed positive PCR products by both methylated (M) and

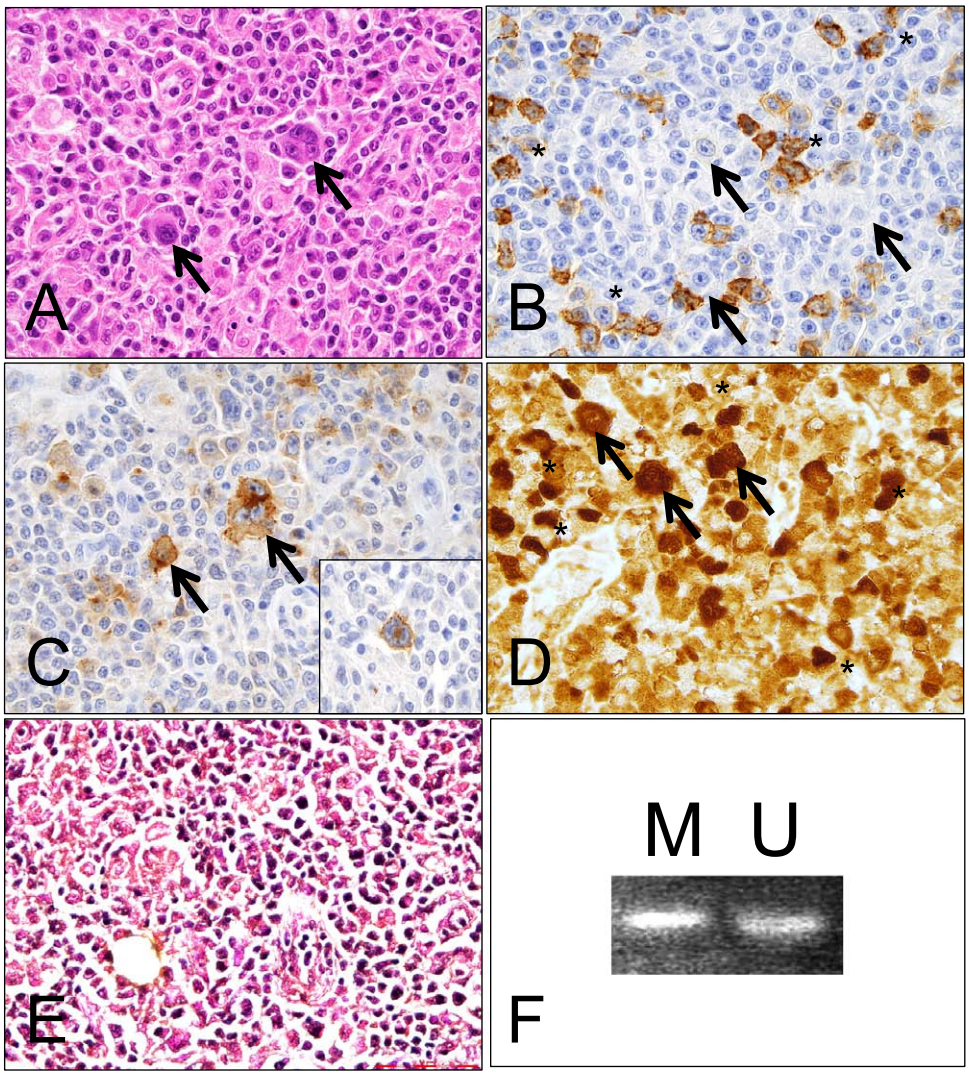

Fig. 2. EBV-positive MCCHL contains typical H/RS cells (**A**, HE, ×400, arrows). CD20 is negative in H/RS cells (**B**, ×400, arrows) but positive in infiltrating non-neoplastic B-cells (**B**, asterisks). CD30 is selectively positive in H/RS cells (**C**, ×400, arrows) that are positive for EBV (×400, insert). Large tumor cells (**D**, ×400, arrows) as well as infiltrating non-neoplastic B-cells (**D**, asterisks) are strongly positive for BOB.1. H/RS cells selectively collected by microdissection (**E**, ×400) show positive PCR products by both methylated (M) and unmethylated (U) primers (**F**).

unmethylated (U) primers (Fig. 3F).

Finally, the typical LRCHL contained a few H/RS cells (Fig. 4A, HE, arrow). CD20 was negative in H/RS cells (Fig. 4B, arrow) but positive in infiltrating non-neoplastic B-cells (Fig. 4B, asterisks). Also, H/RS cells were selectively positive for CD30 (Fig. 4C, arrow), but negative for BOB.1 (Fig. 4D, arrows). BOB.1 was positive in infiltrating non-neoplastic B-cells (Fig. 4D, asterisks). H/RS cells selectively collected by microdissection (Fig. 4E) showed positive PCR products by both methylated (M) and unmethylated (U) primers (Fig. 4F). In the three CHLs, microdissected samples from reactive non-neoplastic cells showed no PCR products for the methylated BOB.1 promoter (data not shown). The results are summarized in Table 2.

IV. Discussion

HL, formerly called Hodgkin's disease, is a neoplasm characterized by the presence of clonal malignant H/RS cells surrounded by variable numbers of reactive granulocytes, macrophages, plasma cells, and lymphocytes. While CD30 is the most useful diagnostic marker of H/RS cells [2], the cells fail to exhibit phenotypes typical of any normal cellular counterpart; routine stains for leukocyte common antigen (LCA), T-cell, and B-cell markers are usually negative, albeit B-cell marker CD20 is detected on a minor subset of H/RS cells [3]. Since H/RS cells often constitute less than one percent of the cells in involved tissues, detailed molecular analysis of H/RS cells had been difficult, and their cellular origin had been a longstanding mystery. Recent advances in molecular biology at the

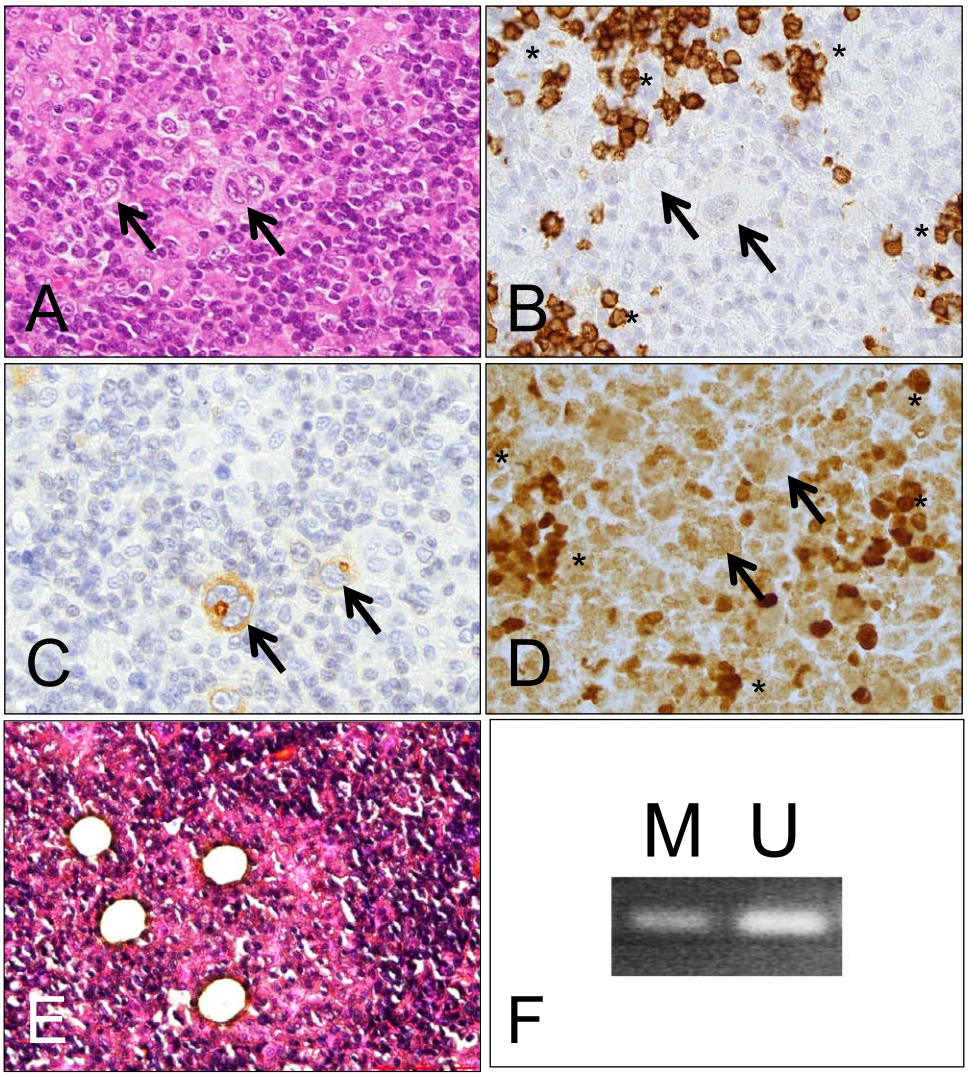

Fig. 3. Typical MCCHL contains a few H/RS cells (**A**, HE, ×400, arrows). While H/RS cells are negative for CD20 (**B**, ×400, arrows), infiltrating non-neoplastic B-cells are positive (**B**, asterisks). H/RS cells are selectively positive for CD30 (**C**, ×400, arrows), but negative for BOB.1 (**D**, ×400, arrows). Infiltrating non-neoplastic B-cells (**D**, asterisks) are positive for BOB.1. H/RS cells selectively collected by microdissection (**E**, ×400) show positive PCR products by both methylated (M) and unmethylated (U) primers (**F**).

single-cell level have disclosed that H/RS cells are transformed B-cells that have lost the expression of most B-cell markers because of decreased B-cell-specific transcription factors, especially those of BOB.1, OCT-2 [6, 18] and PU.1 [9]. Furthermore, the epigenetic gene silencing mechanism by the hypermethylation of the CpG-island located in these gene promoters has been postulated as a primary epigenetic alteration featuring H/RS cells [5, 14]. EBV-infection is, on the other hand, an important etiological factor affecting hypermethylation of tumor suppressor in HL as well as other malignancies [12], and thus we included EVB-positive DLBCL cases as controls for BOB.1 gene methylation.

In the present study, we used the agarose bead-mediated microdissection technique [10, 15] to explore the epigenetic nature of H/RS cells, and confirmed that hypermethylation of the BOB.1 gene promoter was evident in the three CHLs (Fig. 2F, 3F, and 4F) but not in the DLBCL (Fig. 1F). A discrepancy was noted, however, between the methylation status of the *BOB.1* gene promoter and its expression in the EBV-positive MCCHL. Since this MCCHL lacked the typical B-cell phenotype (Fig. 2B) even in the presence of abundant BOB.1 transcription factors (Fig. 2D), functional transcriptional activity of BOB.1 may have been lost or reduced by mechanisms other than epigenetic gene silencing. Indeed, similar phenomena, that genes once silenced by DNA hypermethylation of cancer cells can be significantly but transiently reactivated through chromatin remodeling without any changes in DNA methylation, have been described in several cell lines [16] and in the metaplasia-dysplasia-carcinoma sequence in the Barrett esophagus [13]. Therefore, when interpreting immunohisto-

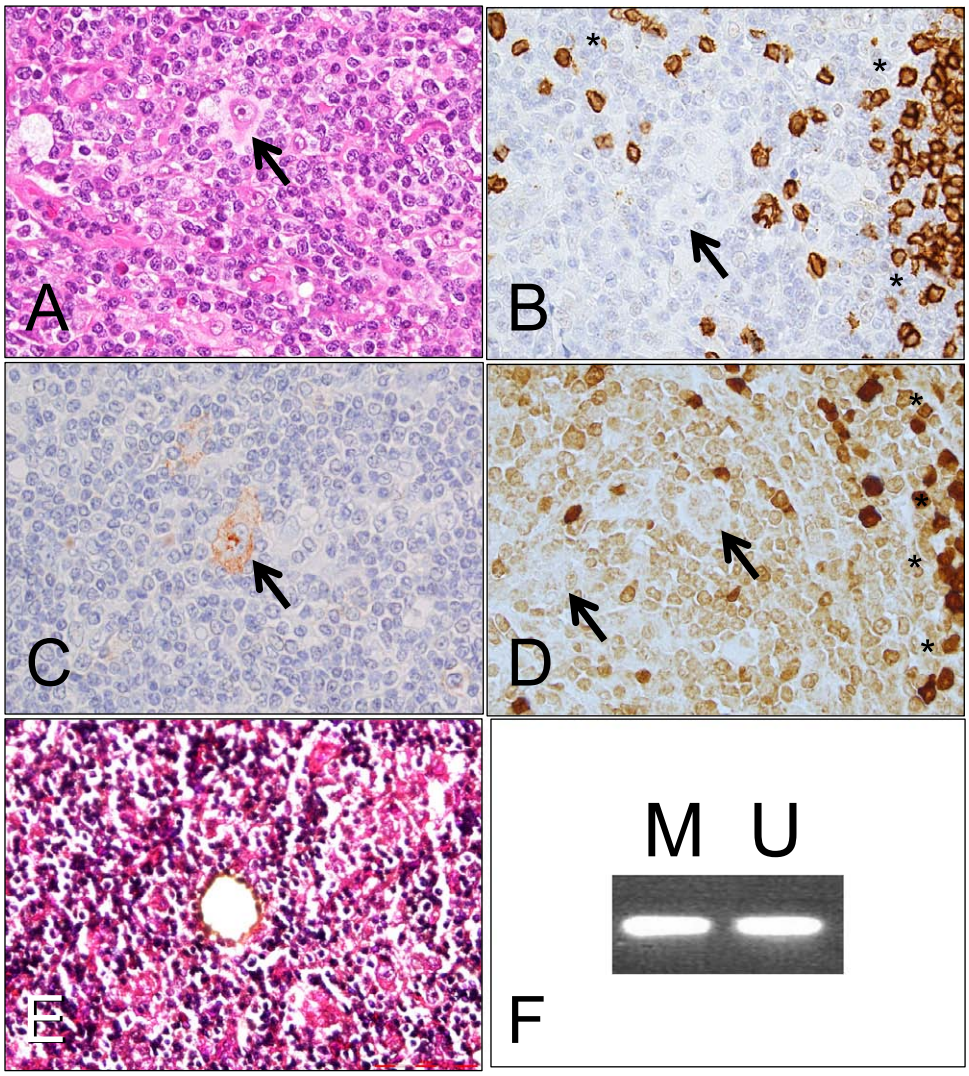

Fig. 4. Typical LRCHL contains a few H/RS cells (**A**, HE, ×400, arrow): CD20 is negative in H/RS cells (**B**, ×400, arrow) but positive in infiltrating non-neoplastic B-cells (**B**, asterisks). Also, H/RS cells are selectively positive for CD30 (**C**, ×400, arrow), but negative for BOB.1 (**D**, ×400, arrows). Infiltrating non-neoplastic B-cells (**D**, asterisks) are positive for BOB.1. H/RS cells selectively collected by microdissection (**E**) show positive PCR products by both methylated (M) and unmethylated (U) primers (**F**).

Table 2. *Summary of immunohistochemistry findings and MSP*

	CD20	CD30	BOB.1	Methylation
DLBCL (EBV)	+	–	+	–
MCCHL (EBV)	–	+	+	+
MCCHL	–	+	–	+
LRCHL	–	+	–	+

chemical results for diagnostic or research purposes, we must bear in mind that apparent positive immunostaining could merely be the result of chromatin remodeling [16] and that such transient expression often has little functional significance.

In conclusion, the agarose-bead mediated technique is an effective tool for retrospective morphology-oriented epigenetic analyses. When characterizing tumor cells by immunophenotyping, any apparent positive immunohistochemical result needs to be interpreted carefully with the help of the hypermethylation status as a molecular marker of gene silencing memory.

V. Acknowledgments

We thank Ms Yuki Takaoka for technical assistance.

VI. References

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