Promoter Hypermethylation of the Bone Morphogenetic Protein-6 Gene in Malignant Lymphoma

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Abstract

Purpose: Bone morphogenetic proteins (BMPs), belonging to the transforming growth factor-β superfamily, are important regulators of cell growth, differentiation, and apoptosis. The biological effects of BMPs on malignant lymphoma, however, remain unknown. Promoter methylation of the *BMP-6* gene in lymphomas was investigated.

Experimental Design: We investigated *BMP-6* promoter methylation and its gene expression in various histological types of 90 primary lymphomas and 30 lymphoma cell lines. The impact of *BMP-6* promoter hypermethylation on clinical outcome was also evaluated.

Results: *BMP-6* was epigenetically inactivated in subsets of lymphomas. The silencing occurred with high frequency in diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL) in association with aberrant *BMP-6* promoter methylation. The methylation was observed in 60% (21/35) of DLBCL cases and 100% (7/7) of DLBCL cell lines, and in 83% (5/6) of BL cases and 86% (12/14) of BL cell lines. In contrast, other histological types of primary lymphomas studied had little or no detectable methylation (1/49; 2%). The presence of *BMP-6* promoter hypermethylation in DLBCL statistically correlated with a decrease in disease-free survival ($P = 0.014$) and overall survival ($P = 0.038$). Multivariate analysis showed that the methylation profile was an independent prognostic factor in predicting disease-free survival ($P = 0.022$) and overall survival ($P = 0.046$).

Conclusion: *BMP-6* promoter was hypermethylated more often in aggressive types of lymphomas, and the hypermethyaltion is likely to be related to the histological type of lymphomas. *BMP-6* promoter methylation may be a potential new biomarker of risk prediction in DLBCL.

Epigenetic gene silencing represents an important mechanism of inhibition of tumor suppressor gene expression in various cancers (1, 2). In contrast to the DNA mutation that alters gene-encoding sequences, the epigenetic silencing affects primarily gene promoter. Aberrant methylation of the promoter DNA regions rich in CpG island is the key step in the epigenetic gene silencing. In malignant lymphoma, such methylation-dependent gene silencing has been described for a number of genes including the cyclin-dependent kinase inhibitors *CDKN2A*, *CDKN2B*, and *KIP2*, the *TP53* homologue *TP73*, the death-associated protein kinase *DAPK*, the cellular retinol-binding protein 1 *CRBP1*, and other genes with putative tumor suppressor functions, many of which encode proteins with critical functions in cell growth control and apoptosis (3, 4). Finding of additional genes whose expressions are regulated by methylation in malignant lymphomas will identify further genes having important roles in lymphomagenesis as well as prognostic implications of the diseases.

Malignant lymphomas constitute heterogenous groups of lymphoproliferative neoplasms with different bilological and clinical features. Detailed biological mechanisms leading to the development of lymphomas have not been well delineated. There have been several reports suggesting a potential role of transforming growth factor-β (TGF-β) in the pathogenesis of B-cell lymphoproliferative disorders (5-8). The TGF-β signaling regulates tumorigenesis and its pathways are often modified during tumor initiation and progression. There is abundant evidence to show that TGF-β acts as a negative regulator of various types of cancers and induces apoptosis (9-11).

Bone morphogenetic proteins (BMPs), belonging to the members of the TGF-β superfamily, were originally identified as molecules that induce bone and cartilage formations, and are now considered multifunctional cytokines (12). To date, over 20 BMPs have been characterized. Based on amino-acid homology, they can be subdivided into several different classes such as the BMP-2/4 group and the OP-1 group including BMP-5, -6, -7, and -8. In various human

malignacies, as with TGF-β, BMPs modulate cell differentiation and proliferation frequently in an inhibitory manner, and are thought to be involved in tumorigenesis. For example, loss of BMP-2 expression has been reported in cancers of the prostate, colon and stomach (13-15), and inactivation of BMP-3b and BMP-6 has been suggested to promote development of lung cancer (16-18). However, despite the loss of BMPs expression in these cancers, the underlying mechanism has not been well defined.

There has been a lack of unambiguous data in the literature on the specific roles of BMPs in the pathogenesis of malignant lymphoma. As the promoter region of the *BMP-6* gene has rich CpG islands (19), we were interested in investigating the methylation status of the *BMP-6* gene promoter in malignant lymphomas with various histological types. In this study, we found frequent aberrant hypermethylation and the resultant loss of *BMP-6* expression in certain lymphomas, especially in diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL). Prognostic relevance of the *BMP-6* promoter hypermethylation was also evaluated.

Materials and methods

Patients and cell lines. A total of 110 lymph node tissues was obtained by incisional biopsy at presentation from 82 patients with non-Hodgkin lymphoma (NHL), 8 patients with Hodgkin lymphoma (HL), and 20 patients with reactive benign lymphadenopathy. The diagnosis of malignant lymphoma was made by morphology and immunohistochemical analysis according to the World Health Organization (WHO) classification. The histologic type for the NHL samples were DLBCL (n = 35, denoted as case DL1-35), follicular lymphoma (FL; n = 19), mantle cell lymphoma (MCL; n = 7), BL (n = 6), peripheral T-cell lymphoma, unspecified (PTCL-u; n = 8), and angioimmunoblastic T-cell lymphoma (AITL; n = 7). All 19 FL cases were diagnosed as low-grade FL (grade 1 or 2) according to the WHO classification, and MCL cases were all histologically typical MCL, not inclusive of aggressive (blastoid) variants.

Peripheral blood mononuclear cells (PBMCs) from 5 healthy donors were also tested for the presence of *BMP-6* promoter methylation. After obtaining consents for sample drawing and storage, they were stored at –80°C until processing.

The following 30 lymphoma cell lines were also examined in this study: 7 DLBCL lines (Pal-1, OPL-1, OPL-2, OPL-3, OPL-4, OPL-5, and OPL-7); 14 BL lines (Akata, Katata, Raji, Daudi, DG75, Wan, Rael, BL-16, BL-41, Salina, Mutu I, BJAB, Namalwa, and Ramos); a MCL line (SP-53); a HL line (HD-70); 3 primary effusion lymphoma (PEL) lines (KS-1, BCBL-1, and JSC-1); and 3 CD30-positive anaplastic large cell lymphoma (ALCL) lines (DL-40, DL-95, and DL-110) (20). Deglis was established from a polymorphic centroblastic lymphoma (Kiel classification) with dual B-cell and T-cell gene rearrangements (21).

Methylation analysis. Isolated DNA was treated with sodium bisulfite, as described previously (22). Aliquots of the bisulfite-treated DNA were amplified in reaction mixtures containing 67 mM Tis-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1.25 mM each dNTP mixture, 0.5 μM of each primer, and 1U of Ex Taq Hot Start Version polymerase (Takara, Tokyo, Japan). The primers were 5’-GGGGTAAATTTTATGGTGGTT-3’ (sense primer, designated 2F) and 5’-ACCTACRCCCTAACTCCTA-3’ (anti-sense, designated 2R2), which gives a PCR product of 205 base pair (bp), encompassing the *BMP-6* promoter region -521 to -316 bp relative to the transcriptional start site. We also used another anti-sense primer 4R2 (5’-CCTCAATCCTTATCTCTCATA) to amplify a 387 bp-fragment corresponding to the region -521 to -134 bp relative to the transcriptional start site. The PCR condition was 3 min at 94°C followed by 35 cycles of 94°C for 40 sec, 56°C for 30 sec, and 72°C for 30 sec. Combined bisulfite restriction analysis (COBRA) was carried out by overnight digestion of the PCR product at 60°C with a restriction enzyme *Bst*UI (New England BioLabs, Ipswich, MA) which

has the recognition sequence 5'-CGCG-3'. The resultant DNA fragments were electrophoresed on agarose gels and stained with ethidium bromide. The proportion of methylated (M) *versus* unmethylated (U) product (digested *versus* undigested) was quantitated by using a densitometer, determining the density of methylation. The % methylation was calculated as follows: M/(M + U) x 100 (23).

Bisulfite genomic sequencing. The 387 bp-PCR products were purified with the Gel Extraction kit (Bionex, Seoul, Korea), cloned into pGEM-T Easy vector (Promega, Madison, WI), and transformed into *Escherichia coli*. Plasmid DNA from isolated clones containing the insert was purified using the FlexiPrep kit (GE Healthcare Bio-Sciences, Piscataway, NJ). Seven to eight clones for each primary lymphoma sample and cell line were sequenced with the pUC/M13 primer. The DNA sequence was determined by using the BigDye Terminator Cycle Sequencing kit ver.1.1 (Applied Biosystems, Foster City, CA) and an ABI 3130 automated DNA sequencer.

Reverse-transcription (RT)-PCR. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Before RT, a total of 1 μg of RNA was treated with DNase I (Invitrogen) to remove any DNA contaminant. The DNase I-treated RNAs were subjected to RT with ThermoScript reverse transcriptase (Invitrogen), as previously described (24). The cDNAs samples (equivalent to the cDNA amount from 50 ng of initial total RNA) were PCR-amplified, and the products were electrophoresed on agarose gels followed by ethidium bromide staining and visualization under UV light for the presence of DNA bands of appropriate sizes. The sequences of the primers to amplify the *BMP-6* gene by RT-PCR were 5'-CGACAACAGAGTCGTAATCG-3' and 5'-GCATTCTCCATCACAGTAATTG-3', yielding a 195-bp fragment. As a control for RNA

integrity and PCR reactions, the gene encoding β-actin was amplified in parallel with following primers: 5'-ACCTTCAACACCCCAGCCATG-3' and 5'- GGCCATCTCTTGCTCGAAGTC -3', giving a 309-bp fragment.

Western blot analysis. Proteins (corresponding to 2 x 10^4 cells) were subjected to SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane, as previously described (24). The filter was incubated with a 1: 750 dilution of the BMP-6 monoclonal antibody (mAb), and the second-step reaction was performed by incubation of the washed filter with a horseradish peroxidase-conjugated rabbit anti-mouse antibody. Reactive proteins were detected by incubation of the washed filter in the enhanced chemiluminescence system according to the manufacturer's instructions (GE Healthcare Bio-Sciences) followed by exposure to an autoradiographic film. The mAb against β-actin (Sigma, St. Louis, MO) was used in parallel to confirm protein integrity and immunoblot reactions. The BMP-6 mAb recognized a molecular mass at around 64 kDa under reduced conditions, consistent with a size previously reported (25).

5-aza-2'-deoxycytidine (5-aza-dC) treatment. Two lymphoma cell lines, Pal-1 and Rael, were incubated in RPMI 1640 medium supplemented with 10% fetal calf serum in the presence or absence of 3 μM of the demethylating agent 5-aza-dC (Sigma) for 6 days. The cells were split on day 3 with the addition of fresh drug. After the drug treatment, cells were harvested for DNA and RNA extractions.

Statistical analysis. The statistical correlation between variables was analyzed by the two-sided Fisher's extract test. Disease-free and overall survival curves were estimated by the Kaplan-Meier method and were compared with the use of the long-rank test. Multivariate

analysis was performed with Cox's proportional-hazards regression technique to define the prognostic significance of selected covariates, including methylation status. *P*-value of less than 0.05 was considered to be significant.

Results

Methylation analysis of the BMP-6 gene in primary lymphoma samples and cell lines.

Lymph node tissues from pretreated patients with various histological types of malignant lymphomas as well as non-malignant lymph nodes with reactive hyperplasia were tested for the presence of promoter methylation of the *BMP-6* gene by COBRA with PCR products amplified using a primer set of 2F and 2R2. Since aberrant hypermethylation is likely to influence not only the number of methylated CpG but also the density of methylation, we quantitated the methylation density (% methylation) (23). We analyzed the methylation status of 20 non-malignant lymph nodes with reactive lymphadenopathy and PBMCs from 5 healthy donors, none of which showed detectable methylation. Since the densitometric assay demonstrated that % methylatio of these control samples did not exceed 10%, a threshold level of 10% methylation was chosen as evidence of presence or absence of hypermethylation of the *BMP-6* promoter. Figure 1A shows representative results of the *BMP-6* promoter methylation in primary lymphoma samples, and Figure 1B summarizes the distribution of methylation density for each histological group. The promoter methylation of the *BMP-6* gene was found in 21 (60%) of 35 DLBCL, 5 (83%) of 6 BL, and 1 (13%) of 8 PTCL-u. The methylation was not detected in any samples from FL (n = 19), MCL (n = 7), AITL (n = 7), and HL (n = 8). These results indicated a significant higher frequency of *BMP-6* promoter methylation in DLBCL ($P < 0.001$) and BL ($P < 0.001$) compared with other histological types of primary lymphomas studied (1/49; 2%). Unlike cell lines with the fully methylated *BMP-6* gene (as stated below), in these clinical samples, there were always unmethylated bands on COBRA, indicating the

presence of normal tissues in the lymph node samples.

To further investigate the *BMP-6* promoter methylation in malignant lymphomas, we subjected 30 lymphoma cell lines to COBRA. Illustrative examples are shown in Figure 1C, and Table 1 summarizes the results. The *BMP-6* promoter was fully methylated in all DLBCL cell lines analyzed (Pal-1, OPL-1, OPL-2, OPL-3, OPL-4, OPL-5, and OPL-7). The methylation was found in 12 (86%) of 14 BL lines (Akata, Katata, Raji, Daudi, DG75, Wan, Rael, BL-16, BL-41, Salina, Mutu I, and BJAB), 3 (100%) of 3 PEL lines (KS-1, BCBL-1, and JSC-1), and 2 (67%) of 3 CD30-positive ALCL lines (DL-40 and DL-95), whereas neither MCL line SP-53 nor HL line HD-70 had methylated *BMP-6* promoter.

To confirm these results, we repeated COBRA with PCR products amplified with another primer set 2F and 4R2. Representative results are shown in Figure 1D. In good concordance with the results of COBRA using the primer set 2F and 2R2, DLBCL and BL cases showed promoter hypermethylayion of *BMP-6*, whereas other histological types of lymphomas tested had little or no detectable methylation.

Bisulfite sequencing analysis. To confirm the methylation at the restriction enzyme (*Bst*UI)-cleavage sites along with the methylation of other neighboring CpG sites, 5 primary lymphoma samples including 2 DLBCL cases (DL15 and DL32), a BL case (BL1), a FL case (FL3), and a HL case (HL1) as well as 3 lymphoma cell lines (Pal-1, Akata, and SP-53) were subjected to bisulfite sequencing. The 387 bp-PCR products were cloned into a plasmid vector, and seven to eight independent clones were sequenced. As shown in Figure 2, the bisufite sequencing showed good concordance with COBRA. In lymphoma cases found to be methylated by COBRA (DL15, DL32, and BL1), not only the CpG sites at the *Bst*UI cleavage sites but also another CpG sites were partially or fully methylated (Fig. 2A), whereas the unmethylated samples, FL3 and HL1, showed little methylation at CpG sites of the promoter

region. As expected, extensive methylation across the entire CpG islands was seen in the methylated cell lines of DLBCL (Pal-1) and BL (Akata) (Fig. 2B). In contrast, the unmethylaed MCL line, SP-53, showed no evidence of promoter hypermethylation of *BMP-6*.

Association of the BMP-6 promoter methylation with transcriptional gene silencing. To elucidate whether the aberrrant methylation of *BMP-6* is associated with loss of *BMP-6* expression, we analyzed expression of *BMP-6* transcripts in primary lymphoma cells as well as lymphoma cell lines by RT-PCR with cycles that amplified the β-actin cDNA to plateau levels. Representative results are shown in Figure 3A. Primary lymphoma samples lacking the *BMP-6* promoter methylation expressed *BMP-6* mRNA transcripts, whereas samples having the *BMP-6* promoter methylation expressed little or no detectable *BMP-6* mRNAs.

Similarly, the lymphoma cells lines that were methylated at the *BMP-6* promoter (for example, Pal-1, OPL-5, OPL-7, Akata, Daudi, Rael, BJAB, BCBL-1, Deglis, and DL-40) did not show expression of the *BMP-6* RNA transcripts (Fig. 3A and Table 1). In contrast, the cell lines that were unmethylated such as Ramos, Namalwa, SP-53, and HD-70, expressed the *BMP-6* transcripts. Ramos cells, which had been described to express *BMP-6* mRNA, served as a positive control (26). These results indicated that the *BMP-6* promoter hypermethylation correlated with loss of *BMP-6* expression.

We next assessed the association between this epigenetic aberration and transcriptional inactivation of the *BMP-6* gene at the protein levels. Representative results are shown in Figure 3B. Western blot analysis showed that there was a good correlation between the *BMP-6* promoter methylation and BMP-6 protein expression. Methylated primary DLBCL samples (for example, cases DL32 and DL35) did not express the BMP-6 protein, whereas the exact opposite occurred in the unmethylated samples (for example, cases DL13 and DL27). Similarly, the hypermethylated cell lines such as Pal-1, OPL-5, OPL-7, Akata, Rael, BJAB, and

DL-40 lacked expression of the BMP-6 protein, while unmethylated cell lines such as Ramos, Namalwa, DL-110, and SP-53 expressed the protein (Fig. 3B and Table 1).

To confirm that this loss of expression was due to the *BMP-6* promoter hypermethylation, two cell lines Pal-1 and Rael were incubated in the presence or absence of the 5-aza-dC, and methylation status and *BMP-6* mRNA expression were analyzed by COBRA and RT-PCR, respectively. Both Pal-1 and Rael cells had extensive promoter methylation of the *BMP-6* gene, but treatment with 5-aza-dC led to partial demethylation (Fig. 4A). In parallel with the demethylation, there was re-expression of the *BMP-6* transcripts (Fig. 4B), implying that the methylation pattern is associated with transcriptional silencing.

Clinical correlates of the BMP-6 promoter methylation in DLBCL patients. The 35 DLBCL were divided into two groups: those with methylated *BMP-6* promoter (n = 21) and those with unmethylated *BMP-6* promoter (n = 14). The association between the *BMP-6* promoter methylation and clinical characteristics at presentation were analyzed by the Fisher's extract two-tail test. The presence of *BMP-6* promoter methylation was not associated with any difference in the age of onset, gender, clinical stage (Ann Arbor), performance status (Eastern Cooperative Oncology Group), serum lactate dehydrogenase levels, number of sites of extranodal disease, presence of B symptoms, or bone marrow involvement (Table 2). Most importantly, we found that the presence of *BMP-6* promoter methylation was associated with a statistically significant decrease in overall survival (Kaplan-Meier, P = 0.038) and disease-free survival (Kaplan-Meier, P = 0.014) (Fig.5). Multivariate analysis demonstrated that the presence of *BMP-6* promoter methylation was independent prognostic factor for predicting both disease-free survival and overall survival in the series (Table 3). These observations suggested that the *BMP-6* promoter methylation is a likely predictor of poor outcome in patients with DLBCL.

Discussion

BMP-6 is a member of the TGF-β superfamily of signaling molecules that are important negative cell proliferation regulators inducing apoptosis in various types of cells including B lymphocytes (12, 27). BMP-6, like the other BMP members, signals through ligation of type I and type II serine-threonine kinase receptors (BMPRs), which subsequently propagates the signal downstream by phosphorylating receptor activated-Smad proteins (Smad1, Smad5, and Smad8).[12] These Smads then form complexes with the common mediator Smad (Smad4), and are translocated into nucleus where they exert regulation of target genes specific for the BMP pathway. Thus, BMP signaling is similar to the paradigm established by TGF-β signaling. It is logical, therefore, to assume that any functional impairment by genetic alterations or epigenetic inactivation of genes involved in the BMP/TGF-β pathway may predispose to development of malignant diseases (9-11, 28). In fact, several recent findings demonstrated the tumor suppressor function of BMPs. First, BMP-2 inhibited the growth of cancer cells of many origins including cells derived from colorectal, prostatic, and gastric cancers (14, 29, 30). BMP-6 inhibited proliferation of prostate cancer cells by up-regulation of several cyclin-dependent kinase inhibitors (31). Similarly, BMP-7 showed growth inhibitory effect on thyroid carcinoma cells by inducing cell cycle arrest via up-regulation of the cyclin-dependent kinase inhibitors (32). These inhibitory effects of BMPs are consistent with the general characteristics of TGF-β superfamily members. Second, decreased expression of BMPR type II correlated with resistance to the growth-inhibitory effect of BMP-6 in renal cell carcinoma, suggesting that loss of sensitivity to BMP-6 is necessary to achieve the malignant phenotypes (33). Third, loss of *BMP-2* expression has been reported for several cancers, including prostatic, colorectal, and gastric cancers (13-15). Furthermore, epigenetic inactivation of *BMP-3b* and *BMP-6* by gene promoter hypermethylation promoted lung tumor development

(16-18). Thus, molecular alterations involving in the BMP signaling cascade have been demonstrated to be associated with tumorigenesis and/or disease progression in several cancers. In the realm of hematological malignancies, several studies showed that BMPs (BMP-2, -4, -5, -6, and -7) inhibited proliferation and induce apoptosis of myeloma cells (34-37), but little is known about the biological roles of BMPs in other hematological malignancies including malignant lymphoma.

Of these BMPs, BMP-6 came into the focus of out interest since the *BMP-6* promoter sequence was previously identified as a target for aberrant DNA methylation (19). In this study, we have analyzed methylation status of the *BMP-6* promoter region in primary lymphoma cells and lymphoma cell lines with various histological types. We found intensive promoter methylation with significant high frequency in DLBCL and BL. On the contrary, other histological types of lymphomas tested, including HL, FL (grade 1 or 2), MCL, PTCL-u, and AITL, showed little or no detectable methylation. The *BMP-6* promoter methylation was not observed in any of the lymph node tissues of reactive lymphadenopathy and PBMCs from healthy donors. These findings suggested that the *BMP-6* promoter methylation appears tumor-specific, and that there was a strong trend toward a higher *BMP-6* hypermethylation frequency in subsets of aggressive NHLs such as DLBCL and BL, compared with indolent lymphomas. We also demonstrated methylation-dependent loss of *BMP-6* expression at mRNA and protein levels. Furthermore, the transriptional repression is reversible by treatment with the demethyalting agent 5-aza-dC. Thus, our findings implied a causal relationship between methylation of the *BMP-6* promoter and transcriptional repression. Our data are, to the best of our knowledge, the first demonstration of epigenetic inactivation of a BMP family member in malignant lymphoma.

Importantly, this study reports that the *BMP-6* promoter hypermethylation may provide a novel independent marker for the prognostic assessment of survival in patients with DLBCL.

Because the number of samples analyzed is relatively small, larger randomized prospective studies are required to confirm the prognostic significance of *BMP-6* promoter hypermethylation.

In summary, our study showed for the first time that promoter of the *BMP-6* gene was methylated more often in aggressive types of NHLs such as DLBCL and BL. The *BMP-6* promoter hypermethyaltion is likely to be related to the histological type of malignant lymphomas with more aggressive clinical behavior. The study reported here also showed that *BMP-6* promoter methylation profile seems to be an important marker in predicting the clinical outcome in DLBCL. An understanding of the BMP signaling pathways in lymphoma cells will be valuable in elucidating the molecular mechanisms of the roles of BMPs in lymphomagenesis and for the establishment of novel strategies for their treatment.

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Table 1. *BMP-6* promoter methylation status and *BMP-6* expression in lymphoma cell lines

Cell line	% methylation	Expression	
		RT-PCR	Western blot
DLBCL line			
Pal-1	94	−	−
OPL-1	91	−	NT
OPL-2	96	−	NT
OPL-3	97	−	−
OPL-4	100	−	NT
OPL-5	93	−	−
OPL-7	98	−	−
BL line			
Akata	93	−	−
Katata	30	−	NT
Raji	75	−	NT
Daudi	94	−	NT
DG75	78	−	NT
Wan	38	−	−
Rael	95	−	−
BL-16	95	−	NT
BL-41	29	+	−
Salina	50	−	NT
Mutu I	97	−	NT
BJAB	100	−	−
Namalwa	9	+	+
Ramos	0	+	+
MCL line			
SP-53	0	+	+
HL line			
HD-70	0	+	NT
PEL line			
KS-1	90	−	−
BCBL-1	95	−	NT
JSC-1	96	−	NT
ALCL line			
DL-40	97	−	−
DL-95	97	−	−
DL-110	5	+	+

Abbreviation: NT, not tested.

Table 2. Clinical characteristics in relation to *BMP-6* methylation status in patients with diffuse large B-cell lymphoma

	No. of Patients (%)		
	Methylated ***n*** **= 21 (60%)**	**Unmethylated** ***n*** **= 14 (40%)**	***P*-value***
Age at diagnosis (years)			
≦60	4 (19.0)	5 (35.7)	0.432
＞60	17 (81.0)	9 (64.3)	
Gender			
Male	12 (57.1)	5 (35.7)	0.305
Female	9 (42.9)	9 (64.3)	
Serum LDH			
Normal	8 (38.1)	7 (50.0)	0.511
Elevated	13 (61.9)	7 (50.0)	
Performance status (ECOG)			
0. 1	16 (76.2)	9 (64.3)	0.474
>1	5 (23.8)	5 (35.7)	
Clinical stage (Ann Arbor)			
I, II	14 (66.7)	5 (35.7)	0.094
III, IV	7 (33.3)	9 (64.3)	
Extranodal site			
0, 1	17 (81.0)	12 (85.7)	0.999
>1	4 (19.0)	2 (14.3)	
B symptoms			
Absent	18 (85.7)	12 (85.7)	0.999
Present	3 (14.3)	2 (14.3)	
BM involvement			
Absent	18 (85.7)	11 (78.6)	0.665
Present	3 (14.3)	3 (21.4)	

**P*-values are obtained from the two-tailed Fisher's exact tests.
Abbreviations: LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; BM, bone marrow.

Table 3. Multivariate analysis for overall survival and disease-free survival in DLBCL

Feature	***P*-value**	**Hazard ratio**	**95% confidence interval**
Overall survival			
BMP-6 methylation: present	0.046	0.378	0.145-0.986
Age: >60 years	0.109	2.468	0.817-7.457
Gender: male	0.786	0.888	0.376-2.099
Serum LDH: increased	0.272	1.754	0.643-4.781
Performance status: >1	0.374	1.513	0.607-3.771
Clinical stage: III-IV	0.709	0.852	0.367-1.981
Extranodal site: >1	0.024	3.447	1.180-10.074
B symptom: present	0.788	1.160	0.391-3.440
BM involvement: present	0.800	1.150	0.388-3.407
Disease-free survival			
BMP-6 methylation: present	0.022	0.327	0.125-0.856
Age: >60 years	0.191	2.082	0.693-6.257
Gender: male	0.364	0.674	0.287-1.581
Serum LDH: increased	0.238	1.826	0.671-4.971
Performance status: >1	0.481	1.389	0.557-3.461
Clinical stage: III-IV	0.592	0.792	0.338-1.859
Extranodal site: >1	0.146	2.163	0.764-6.121
B symptom: present	0.984	1.011	0.341-2.998
BM involvement: present	0.940	1.043	0.352-3.092

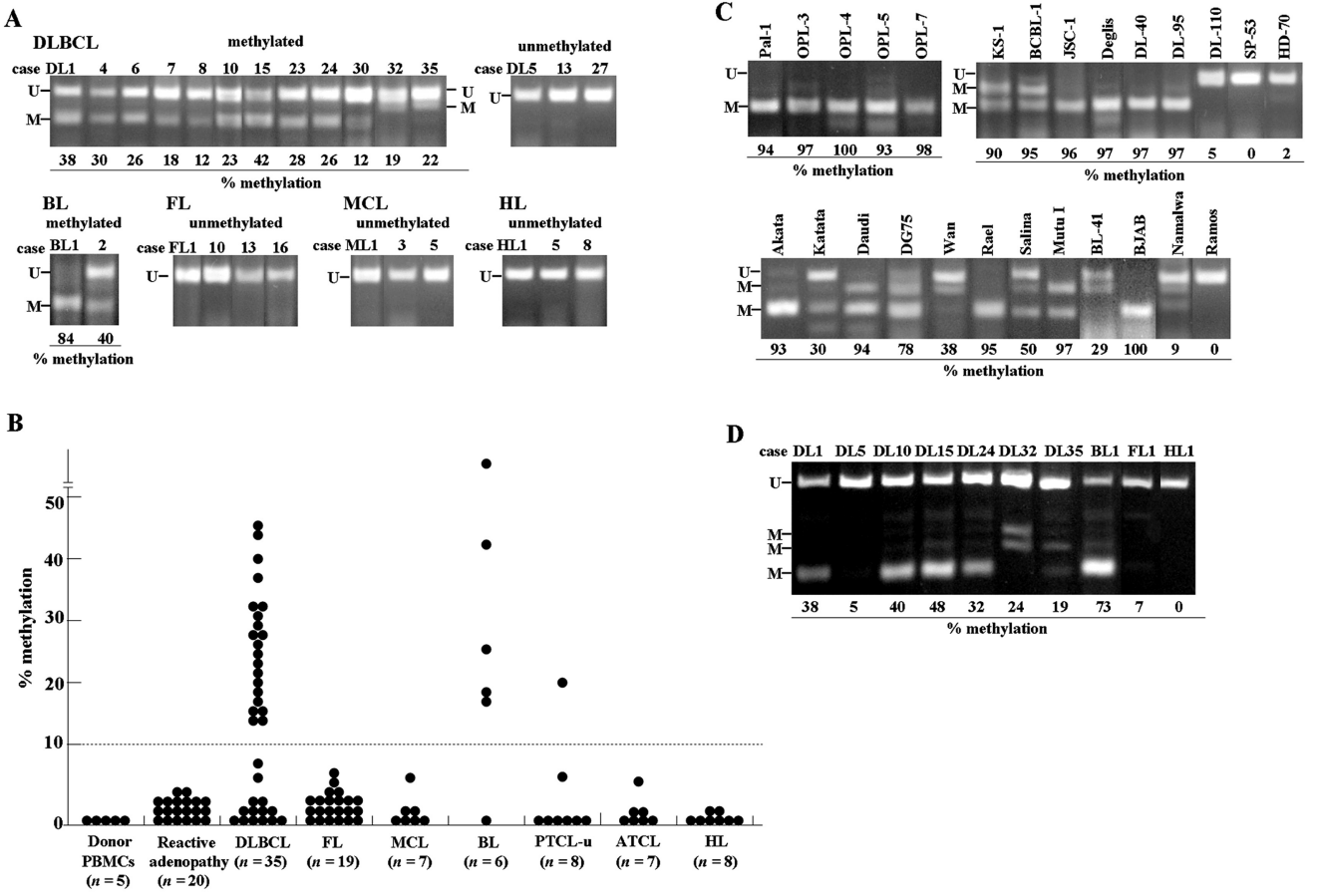
Fig. 1 Representative COBRA results of the *BMP-6* gene promoter. *A*, COBRA results with PCR products amplified with 2F/2R2 primers in primary lymphoma cells, showing frequent aberrant methylation in DLBCL and BL samples, but none of samples from FL, MCL, and HL had methylated *BMP-6* gene. The PCR products (205 bp) from bisulfite-treated DNA were digested with *Bst*U I. DLBCL samples were divided into two groups: methylated and unmethylated samples. Case numbers are shown on top. Unmethylated (U) and methylated (M) bands were quantitated by densitometry. % Methylation (percentage of methylated *versus* unmethylated bands) is shown below each lane. *B*, Intensities of the *BMP-6* promoter methylation in clinical samples. 110 lymph node tissues and PBMCs from 5 healthy donors were analyzed for the *BMP-6* promoter methylation by COBRA. The proportion of methylated *versus* unmethylated bands (as shown in Fig. 1A) was quantitated by a densitometer, and % methylation was calculated. A fraction of >10% (indicated as a dot line) is considered evidence of methylation. *C*, COBRA results (2F/2R2 PCR primers) in lymphoma cell lines. *D*, COBRA results with PCR products (387 bp) amplified with 2F/4R2 primers, showing similar methylation patterns to those shown in Figure 1A.

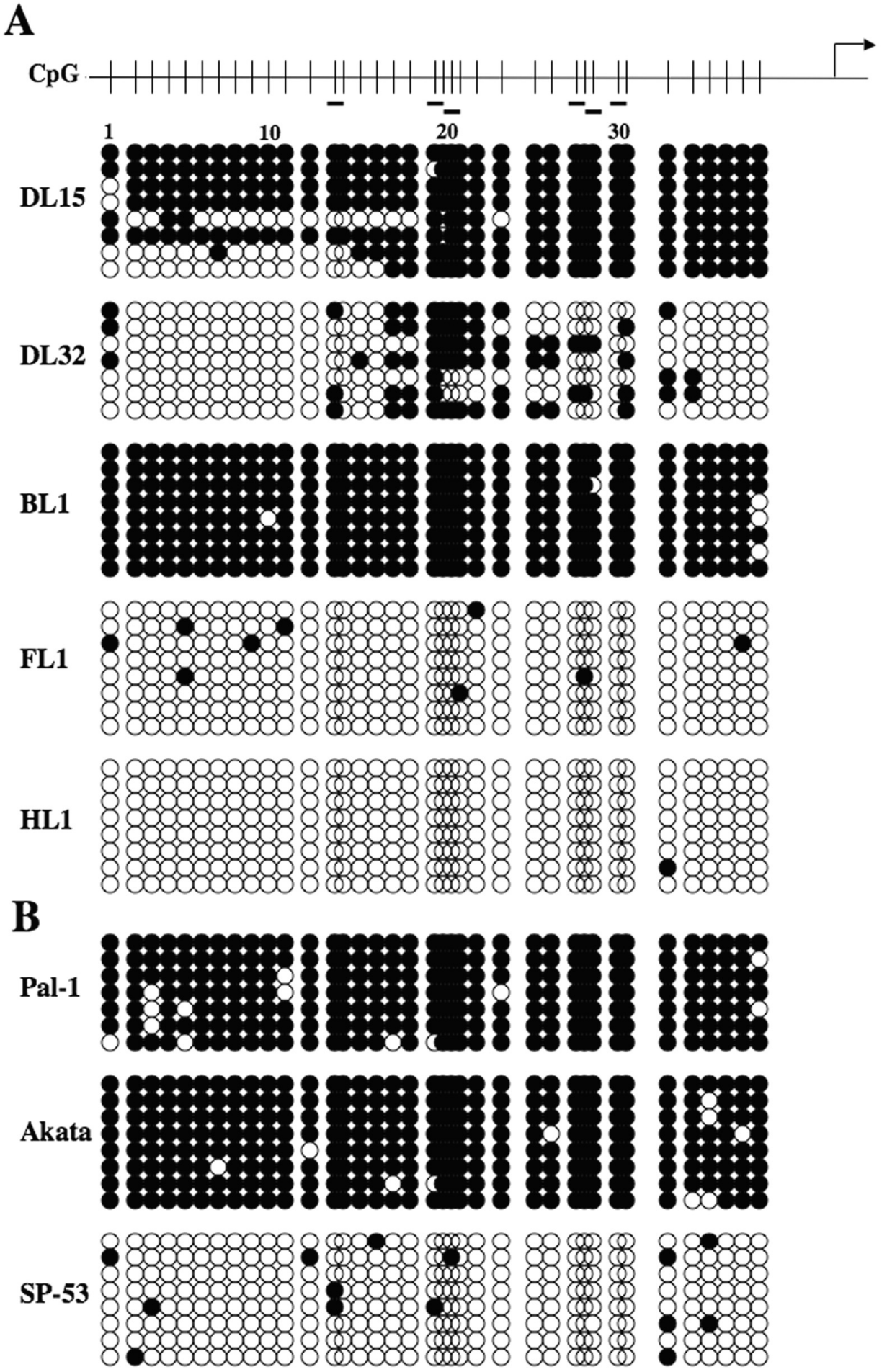
Fig. 2. Methylation patterns of individual bisulfite-sequenced clones of the *BMP-6* promoter in primary lymphoma cells (*A*) and lymphoma cell lines (*B*). Genomic DNA was subjected to sodium bisulfite conversion, PCR amplification with 2F/4R2 primers, cloning, and cycle sequencing. Seven to eight clones from each sample were bisulfite-sequenced to obtain a representative sampling of methylation patterns. Schematic depiction of the CpG island in the *BMP-6* promoter region are shown on top. The 37 CpG sites analyzed are indicated by the vertical bars and numbered from left to right. The translation start site is shown by a horizontal arrow. *Bst*UI sites are underlined. Methylated and unmethylated cytosines are shown by closed and open squares, respectively.

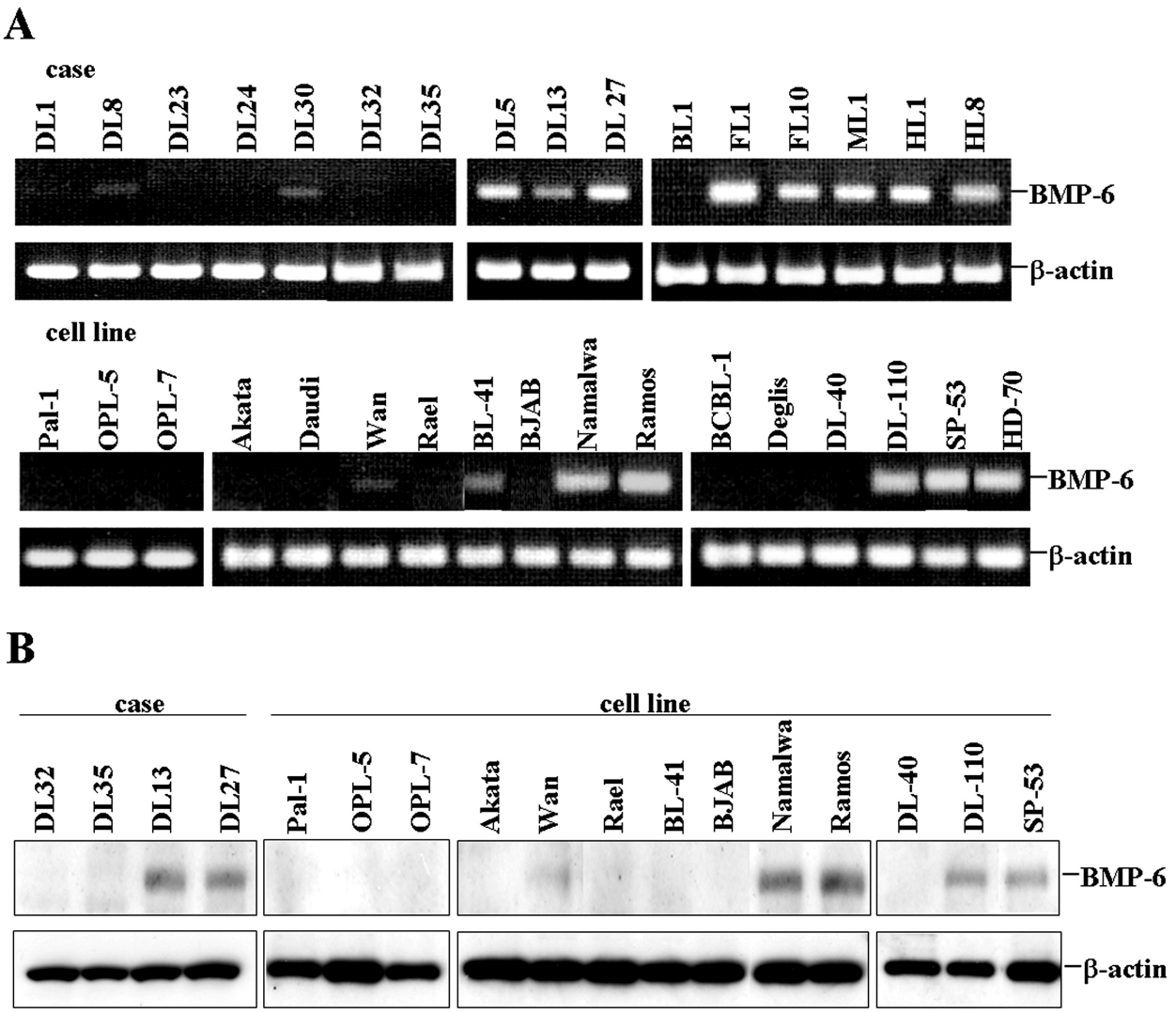
Fig. 3. Analysis of *BMP-6* expression. *A*, RT-PCR analysis for expression of *BMP-6* mRNA in primary lymphoma cells (upper panel) and lymphoma cell lines (lower panel). Lymphoma samples lacking *BMP-6* methylation expressed *BMP-6* mRNAs, whereas samples with *BMP-6* methylation expressed little or no detectable *BMP-6* mRNA transcripts. β-*Actin* was used as a control for cDNA integrity and quality. *B*, Western blot analysis for expression of BMP-6 protein in primary lymphoma cells and lymphoma cell lines. Proteins were assessed by SDS-PAGE followed by blotting onto membranes and detection with BMP-6 mAb. Lymphoma cells carrying *BMP-6* promoter methylation did not express BMP-6 protein, whereas unmethylated cells expressed the protein. β-Actin is shown as a loading control.

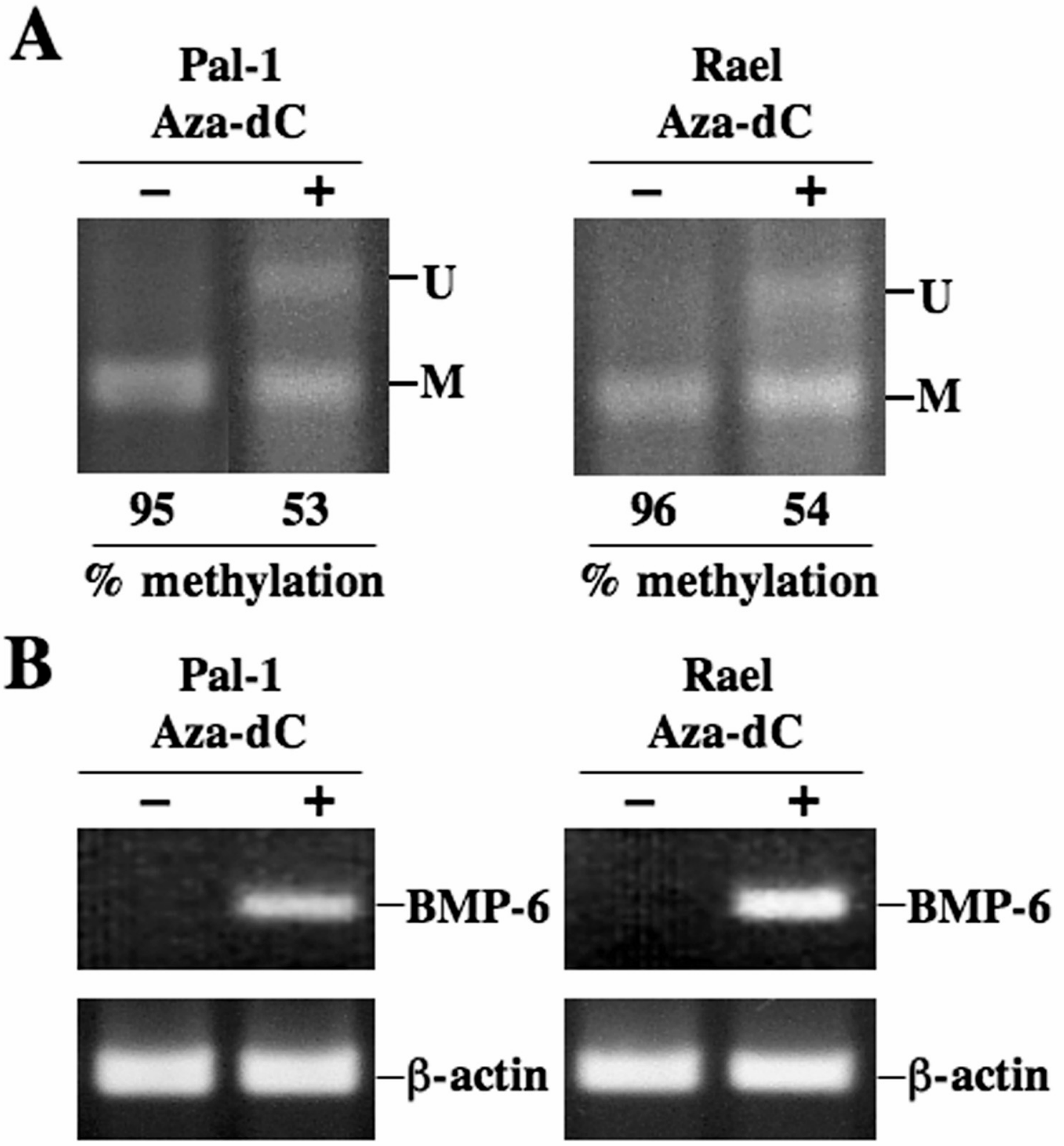
Fig. 4. Demethylation of the *BMP-6* promoter reverses transcriptional silencing. *A*, COBRA for the *BMP-6* promoter methylation in Pal-1 and Rael cells that were grown in the presence (+) or absence (-) of 5-aza-dC, showing partial demethylation in 5-aza-dC-treated cells. % Methylation is shown below each lane. U: unmethylated fragment; M: methylated fragment. *B*, RT-PCR analysis showed methylation-dependent restoration of *BMP-6* mRNA transcripts. β-*Actin* was used as a control for cDNA integrity and quality.

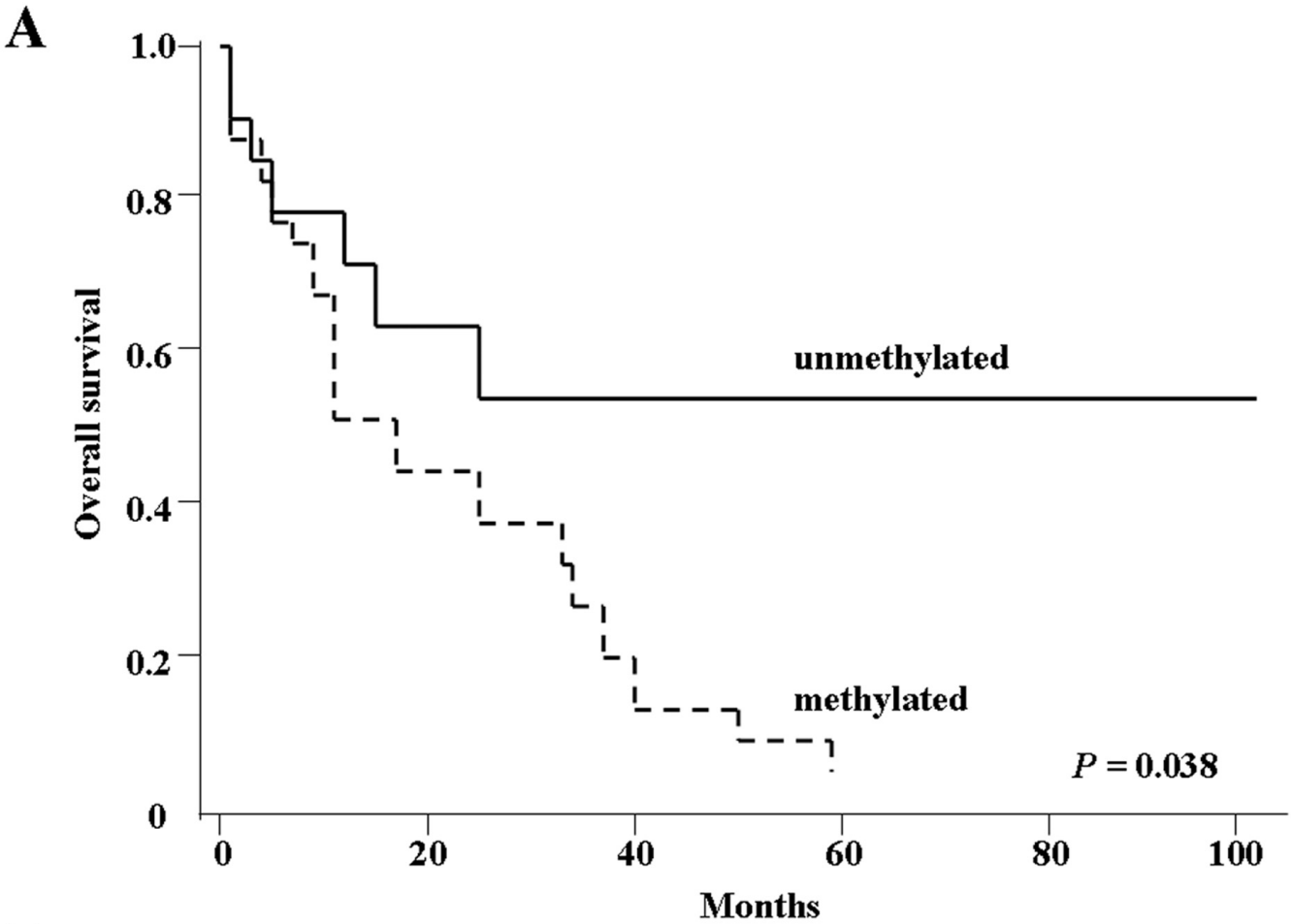
Fig. 5. Overall survival and disease-free survival of patients with DLBCL according to *BMP-6* promoter methylation status during the follow-up period. Overall survival (*A*) and disease-free survival (*B*) in patients with *BMP-6* promoter methylation were significantly lower than in those without *BMP-6* promoter methylation ($P = 0.038$ and $P = 0.014$, respectively, log-rank test).

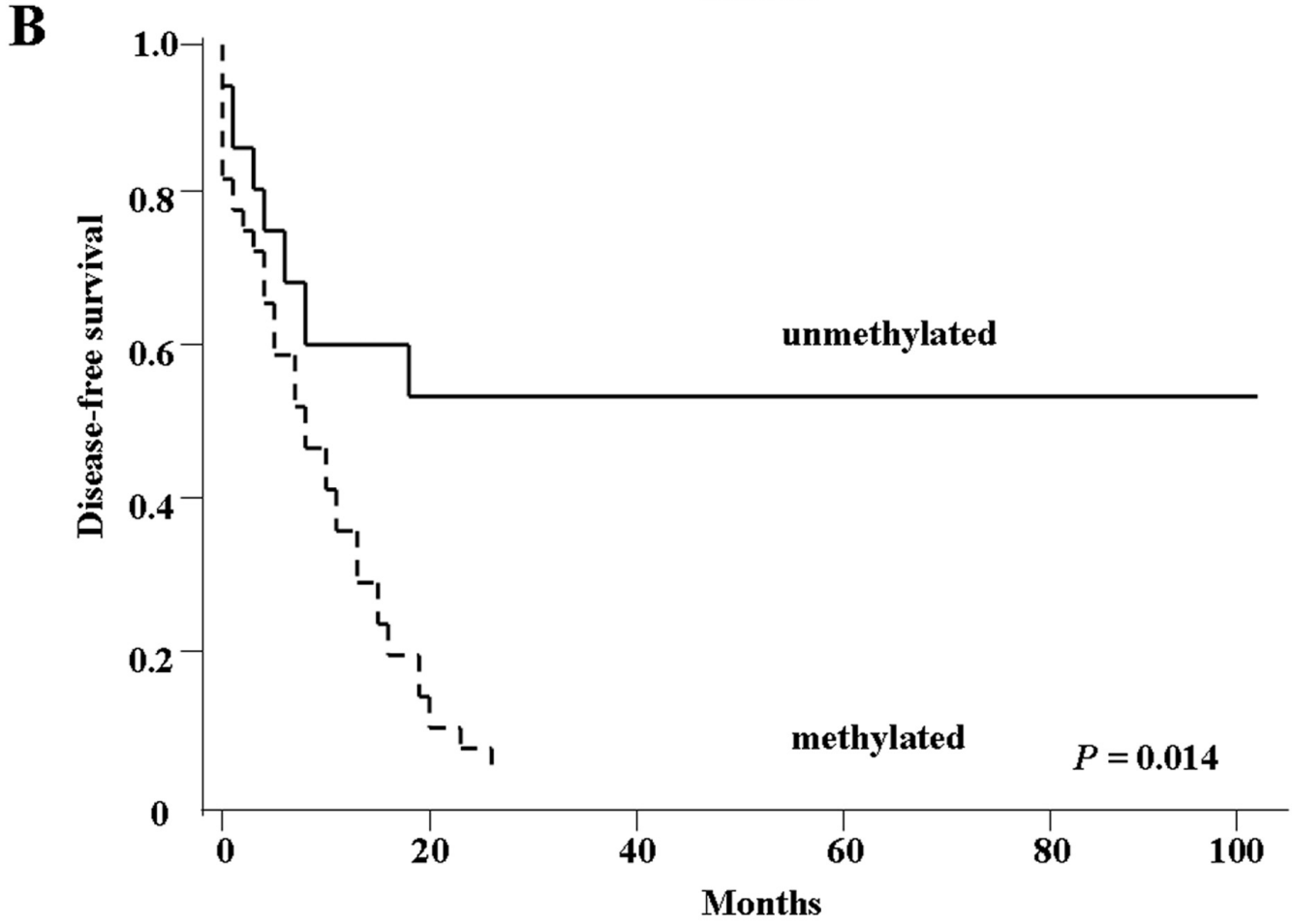

A
DLBCL
methylated
case DL1 4 6 7 8 10 15 23 24 30 32 35
U
M
38 30 26 18 12 23 42 28 26 12 19 22
% methylation
unmethylated
case DL5 13 27
U
BL
methylated
case BL1 2
84 40
% methylation
FL
unmethylated
case FL1 10 13 16
MCL
unmethylated
case ML1 3 5
HL
unmethylated
case HL1 5 8
B
% methylation
0
10
20
30
40
50
Donor PBMCs (n = 5)
Reactive adenopathy (n = 20)
DLBCL (n = 35)
FL (n = 19)
MCL (n = 7)
BL (n = 6)
PTCL-u (n = 8)
ATCL (n = 7)
HL (n = 8)
C
Pal-1 OPL-3 OPL-4 OPL-5 OPL-7
94 97 100 93 98
% methylation
KS-1 BCBL-1 JSC-1 Deglis DL-40 DL-95 DL-110 SP-53 HD-70
90 95 96 97 97 97 5 0 2
% methylation
Akata Katata Daudi DG75 Wan Rael Salina Mutu I BL-41 BJAB Namalwa Ramos
93 30 94 78 38 95 50 97 29 100 9 0
% methylation
D
case DL1 DL5 DL10 DL15 DL24 DL32 DL35 BL1 FL1 HL1
U
M
38 5 40 48 32 24 19 73 7 0
% methylation


A
CpG
1
10
20
30
DL15
DL32
BL1
FL1
HL1
B
Pal-1
Akata
SP-53


A
case
DL1
DL8
DL23
DL24
DL30
DL32
DL35
DL5
DL13
DL 27
BL1
FL1
FL10
ML1
HL1
HL8
BMP-6
β-actin
cell line
Pal-1
OPL-5
OPL-7
Akata
Daudi
Wan
Rael
BL-41
BJAB
Namalwa
Ramos
BCBL-1
Deglis
DL-40
DL-110
SP-53
HD-70
BMP-6
β-actin
B
case
cell line
DL32
DL35
DL13
DL27
Pal-1
OPL-5
OPL-7
Akata
Wan
Rael
BL-41
BJAB
Namalwa
Ramos
DL-40
DL-110
SP-53
BMP-6
β-actin


A
Pal-1
Aza-dC
–
+
U
M
95
53
% methylation
Rael
Aza-dC
–
+
U
M
96
54
% methylation
B
Pal-1
Aza-dC
–
+
BMP-6
β-actin
Rael
Aza-dC
–
+
BMP-6
β-actin


A
1.0
0.8
0.6
0.4
0.2
0
Overall survival
unmethylated
methylated
P = 0.038
0
20
40
60
80
100
Months


B
1.0
0.8
0.6
0.4
0.2
0
Disease-free survival
unmethylated
methylated
P = 0.014
0
20
40
60
80
100
Months