

Association between lymphoma prognosis and aberrant methylation of *ID4* and *ZO-1* in bone marrow and paraffin-embedded lymphoma tissues of treatment-naïve patients

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Abstract. The aim of the present study was to investigate the association between lymphoma prognosis and aberrant methylation of inhibitor of DNA binding factor 4 (*ID4*) and tight junction protein 1 (*ZO-1*) genes in samples isolated from the bone marrow and paraffin-embedded lymphoma tissues of treatment-naïve lymphoma patients. The bone marrow biopsy and paraffin-embedded lymphoma tissue samples from treatment-naïve lymphoma patients were obtained, along with corresponding control samples from subjects without lymphoma and from lymph nodes of chronic cholecystitis and reactive lymphadenitis patients. Methylation-specific PCR (MSP) reactions were performed to analyze the methylation status on the promoter regions of *ID4* and *ZO-1*. *ID4* and *ZO-1* promoter regions in the control group were completely unmethylated, whereas the rates of methylation of *ID4* and *ZO-1* in paraffin-embedded lymphoma tissues of the lymphoma patients were 80.4 and 84.3%, respectively. The methylation positivity rates of both the *ID4* or *ZO-1* genes in lymphoma patients were 92.2%, which was significantly higher compared to the rates in the control group (0%). The methylation positivity rates of the *ID4* and *ZO-1* genes in the bone marrow and paraffin-embedded lymphoma tissues of non-Hodgkin lymphoma patients were significantly higher compared to the rates in the Hodgkin lymphoma patients. The survival rate of lymphoma patients with methylated *ID4* was significantly lower compared to that of patients with unmethylated *ID4*. The methylation of the *ID4* and *ZO-1* genes may be a specific molecular marker for lymphoma diagnosis. The

methylation of the *ID4* gene may be an indicator of poor prognosis in lymphoma patients.

Introduction

Both genetic and epigenetic aberrations play vital roles in tumorigenesis and the development of cancer. DNA methylation is an important epigenetic modification, and aberrant changes in DNA methylation are prominent features in the pathogenesis and development of various diseases, including cancer. Aberrant methylation within the promoter regions of tumor-suppressor genes has been correlated with their silencing and with uncontrolled proliferation of tumor cells (1,2).

There are four types of inhibitor of DNA binding (ID) proteins in mammals, belonging to the helix-ring-helix protein family, and their coding regions are located on chromosome 6p21-22. Animal experiments and *in vitro* cellular assays have shown that the ID4 protein inhibits the proliferation of tumor cells and promotes cell apoptosis (3). In addition, aberrant promoter methylation of the *ID4* gene has been correlated with the pathogenesis and development of hematological malignancies (4). The tight junction protein 1 (*ZO-1*) gene belongs to the membrane guanylate kinase family, and it encodes a tight junction protein involved in many signaling pathways that participate in the regulation of cell proliferation and differentiation (5,6). The aberrant hypermethylation of the promoter region of *ZO-1* was reported in murine leukemia cell lines, and the concomitant suppression of the expression of the *ZO-1* gene was also observed. The *ZO-1* gene may be a novel oncogene in hematological malignancies that was identified using restriction landmark genomic scanning (RLGS) (7). In summary, the alteration of the methylation pattern on the promoter regions of the *ID4* and *ZO-1* genes is a distinctive feature of hematological malignancies when compared to normal cells (8).

Lymphoma is a hematological malignancy which is diagnosed based mainly on pathological examination. However, the diagnosis is not easy, partially because the accuracy of the pathological examination relies on the experience of the clinical practitioner and is dependent on subjective impressions, particularly when the malignant atypical features

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of the tumor cells are not apparent, or when the distinction between benign and malignant hyperplasia is not clear (9,10). Although auxiliary examinations are currently available, such as immunohistochemical tests, gene rearrangement analysis, and T cell receptor (TCR) detection, the diversified pathologic morphologies of lymphoma tissues often result in difficulties with diagnosis. Therefore, there is a need for more sensitive and specific molecular diagnostic methods for the diagnosis, treatment, and prognostic prediction of lymphoma. The highly sensitive methylation-specific PCR (MSP) method has been previously applied to detect methylation of the promoter regions of the *ID4* and *ZO-1* genes in the diagnosis of breast cancer (11). One objective of the current study was to identify the promoter methylation status of the *ID4* and *ZO-1* genes in the lymph node and bone marrow of lymphoma patients.

The MSP (12) technique was utilized in the present study to detect aberrant alterations in the methylation status of the *ID4* and *ZO-1* genes and to evaluate the feasibility of utilizing these changes as epigenetic markers for lymphoma diagnosis, or for detection of minimal residual lymphoma after treatment. A comparison of the methylation statuses of *ID4* and *ZO-1* was performed between lymphoma patients and a control cohort comprised of patients without lymphoma. Meanwhile, the feasibility of using the *ID4* and *ZO-1* methylation status as a molecular indicator for lymphoma diagnosis and prognosis was also evaluated.

Materials and methods

Patients and tissue samples. Paraffin-embedded lymphoma tissue samples were obtained from 92 lymphoma patients treated at the PLA General Hospital (Beijing, China) between May 2006 and October 2009, in whom the diagnosis was confirmed by pathologic morphological analysis and immunohistochemical studies. Paraffin-embedded lymph node samples were also obtained from 10 patients with either chronic cholecystitis or reactive lymphadenitis, as the control set. The bone marrow specimens were obtained before treatment from 90 lymphoma patients admitted to our hospital between February 2008 and February 2010, and their clinical information is summarized in Table I. The therapeutic strategy was determined according to National Comprehensive Cancer Network (NCCN) lymphoma treatment guidelines, or hematopoietic stem cell transplantation was performed if possible. The control samples of bone marrow tissue were from 8 donors who had donated bone marrow for hematopoietic stem cell transplantation. Written informed consent was provided by the patients and their families. The deadline for the last follow-up for all enrolled patients was October 31, 2011.

The HL-60 and K562 cell lines were used as positive and negative controls, respectively, for MSP of the *ID4* and *ZO-1* genes, and the cells were maintained in the Department of Hematology, PLA General Hospital. Deionized water was used as the blank control for MSP.

PCR primers. The methylation-specific primers for the *ID4* gene were *ID4*-MF forward, TCGGAGTTTTCGTTTTCGTT and *ID4*-MF reverse, CGATACTACTCACAACCGCG; *ID4*-UMF forward, TGTTTGAGTTTTGTGTTTTGTT and *ID4*-UMR reverse, CCAATACTACTCACAACCACAC.

The methylation-specific primers for *ZO-1* were *ZO-1*-MF forward, AAAATAAATAGGAAGATTTCGTACGG and *ZO-1*-MR reverse, GAAACTAAACGAAACGAAACGAA; *ZO-1*-UMF forward, GGATAAAAATAAATAGGAAGATT TGTATG and *ZO-1*-UMR reverse, AACAAAACATAACA AAACAAAACAA.

Genomic DNA extraction and base modification. DNA extraction from paraffin-embedded tissues was performed using a commercial kit (Qiagen, USA), and the genomic DNA Purification kit (Promega, USA) was utilized for DNA extraction from the bone marrow. The concentration of extracted DNA was determined using spectrophotometry. Two micrograms of DNA was sheared by passing through 26G syringe needles repeatedly after dilution with water. Then, 5 μ l freshly prepared NaOH solution (3 mol/l) was added to the sheared DNA, and the mixture was incubated at 37°C for 25 min, followed by the addition of 30 μ l freshly prepared hydroquinone solution (10 mmol/l) and 520 μ l sodium bisulfite (3 mol/l) and subsequent incubation at 55°C for 16 h. The DNA was purified from the above mixture using a DNA gel extraction kit (Axygen Biosciences, USA) and stored at -20°C.

MSP reactions. The total volume of the reaction was 25 μ l, and the mixture consisted of 4 μ g DNA, 2.5 μ l 10X Herman's buffer, 1.25 μ l of 25 mM dNTPs, 0.15 μ l Hot Start *Taq* polymerase, and 3 μ l of each upstream and downstream methylation-specific or unmethylated DNA-specific PCR primers. The PCR reaction settings were as follows: pre-denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 60 sec, 56°C for 40 sec, and 72°C for 50 sec, and a final elongation for 10 min at 72°C. The HL-60 and K-562 cells were used as positive and negative controls, respectively, for each trial, and deionized water was used as the blank control. The products of PCR were separated with 2% agarose DNA electrophoresis, the resolved DNA was stained with ethidium bromide and images were recorded with an ultraviolet transmission analyzer.

The PCR products were recovered and purified from the agarose gel with the DNA gel extraction kit (Qiagen).

Plasmid construction and DNA sequencing. The PCR fragments recovered from the agarose gel were inserted into the pGEM-T Easy vector by T4 DNA ligase-based cloning, and subsequently transformed into competent *E. coli* DH5 α . Positive colonies were obtained by antibiotic resistance screening and blue-white colony screening. One microliter of a bacterial culture grown overnight was used as the template for PCR. The PCR-positive colonies were sequenced by Invitrogen (Beijing, China).

Readout. For the *ID4* gene, complete methylation was indicated by the presence of a 186-bp PCR fragment alone upon amplification with the methylation-specific primers; completely unmethylated DNA was indicated by the presence of a 191-bp PCR fragment alone upon amplification with the unmethylated DNA-specific primers. Partial methylation was indicated by the presence of both the 186- and 191-bp PCR fragments after amplification. Similarly, for the *ZO-1* gene, the presence of a 177-bp or a 179-bp PCR fragment alone indicated complete methylation or a complete lack of methylation, respectively,

Table I. Clinical information of the treatment-naïve lymphoma patients.

Characteristics	Bone marrow	Lymphoma	P-value
Gender			
Male/female	70/20	31/20	0.03
Age in years, mean (range)	44.22±17.56 (13-81)	47.59±17.61 (17-79)	0.28
Height in cm, mean (range)	170.48±6.93 (155-184)	167.35±8.10 (150-186)	0.02
Body weight in kg, mean (range)	68.00±12.25 (45-102)	68.71±11.20 (45.5-89)	0.73
Systolic pressure (mmHg)	120.46±11.53	122.45±12.42	0.34
Diastolic pressure (mmHg)	75.29±8.33	75.94±7.78	0.65
Heart rate (beats/min), mean (range)	81.29±7.84 (60-117)	77.96±6.30 (62-104)	0.02
Medical history			
Healthy (yes/no)	36/54	20/31	0.93
Familial tumor history (yes/no)	11/79	6/45	0.94
Clinical stage of lymphoma			0.14
I	15	12	
II	15	15	
III	16	7	
IV	44	17	
Systemic symptoms B (yes/no)	37/53	23/28	0.65
Lymphoma type			0.32
Non-Hodgkin	78	47	
Hodgkin	12	4	
Pathological type			0.82
B cell	60	37	
T cell	18	10	
Bone marrow involvement (yes/total)	15/90	6/51	0.43

while the presence of both 177- and 179-bp fragments after amplification was indicative of partial methylation. Since the bone marrow of the patients consisted of both lymphoma cells and normal cells, partial methylation and complete methylation were classified as a methylation-positive readout.

Statistical analysis. Quantitative data are expressed as means ± SD, and differences between data sets were analyzed using the Student's t-test. Categorical data were described as absolute frequencies and analyzed by the Pearson's Chi-square test. Cumulative event curves were plotted using the Kaplan-Meier survival method and differences between curves were tested for statistical significance using the log-rank method. P-values <0.05 were considered statistically significant. All data were analyzed using the SPSS version 12.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results

Methylation status of genes amplified from the paraffin-embedded lymphoma and bone marrow tissues. The PCR results showed that 64 out of 92 paraffin-embedded lymphoma tissues were *ID4*-positive and 57 were *ZO-1*-positive, respectively, either in the methylated or unmethylated state,

and that 51 were both *ID4*- and *ZO-1*-positive. Eight out of 10 paraffin-embedded lymphoma tissues from either chronic cholecystitis or reactive lymphadenitis patients were both positive for completely unmethylated *ID4* and *ZO-1* genes. All bone marrow tissues of lymphoma patients were *ID4*- and *ZO-1*-positive, for either methylated or unmethylated DNA.

There were 41 and 43 samples positive for *ID4* and *ZO-1* gene methylation out of the 51 paraffin-embedded lymphoma tissues, and the positivity rates were 80.4 and 84.3%, respectively. Forty-seven samples were positive for either *ID4* or *ZO-1* methylation, and the coverage rate for both genes was 92.2%. The *ID4* and *ZO-1* genes were completely unmethylated in the 8 paraffin-embedded lymph node tissues from either chronic cholecystitis or reactive lymphadenitis patients.

There were 23 and 13 samples positive for *ID4* and *ZO-1* gene methylation, respectively, out of 90 bone marrow samples from lymphoma patients, and the positivity rates were 25.56 and 14.44%, respectively. The *ID4* and *ZO-1* genes of the 8 samples from bone marrow donors were completely unmethylated (Tables II and III, Fig. 1).

Survival curve. The patients with positive MSP results for *ID4* or *ZO-1* isolated from the bone marrow had a lower survival rate compared to the patients with unmethylated *ID4* or *ZO-1*

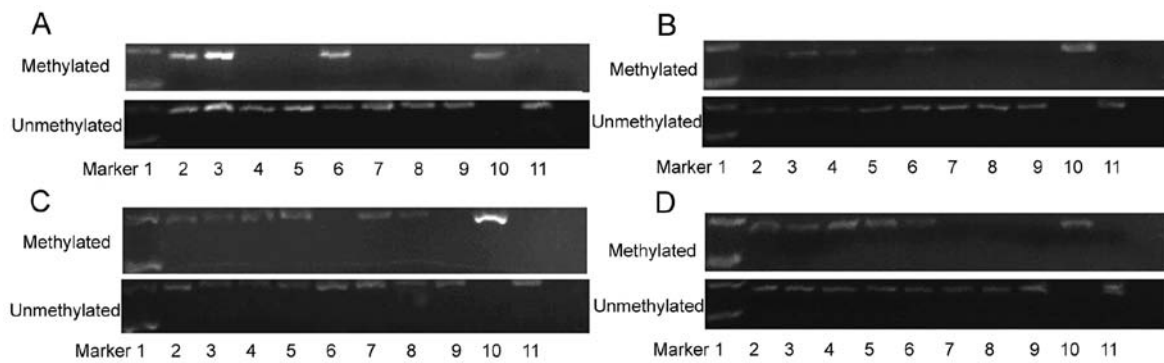


Figure 1. Lanes 1-8 are MSP reactions of samples from lymphoma patients; lanes 9 and 10 are positive and negative controls, respectively, and lane 11 is the blank control. (A) DNA template for *ID4* MSP of samples from bone marrow. (B) DNA template for *ZO-1* MSP from bone marrow of lymphoma patients. (C) DNA template for *ID4* MSP of samples from paraffin-embedded tissues of lymphoma patients. (D) DNA template for *ZO-1* MSP of samples from paraffin-embedded tissues of lymphoma patients.

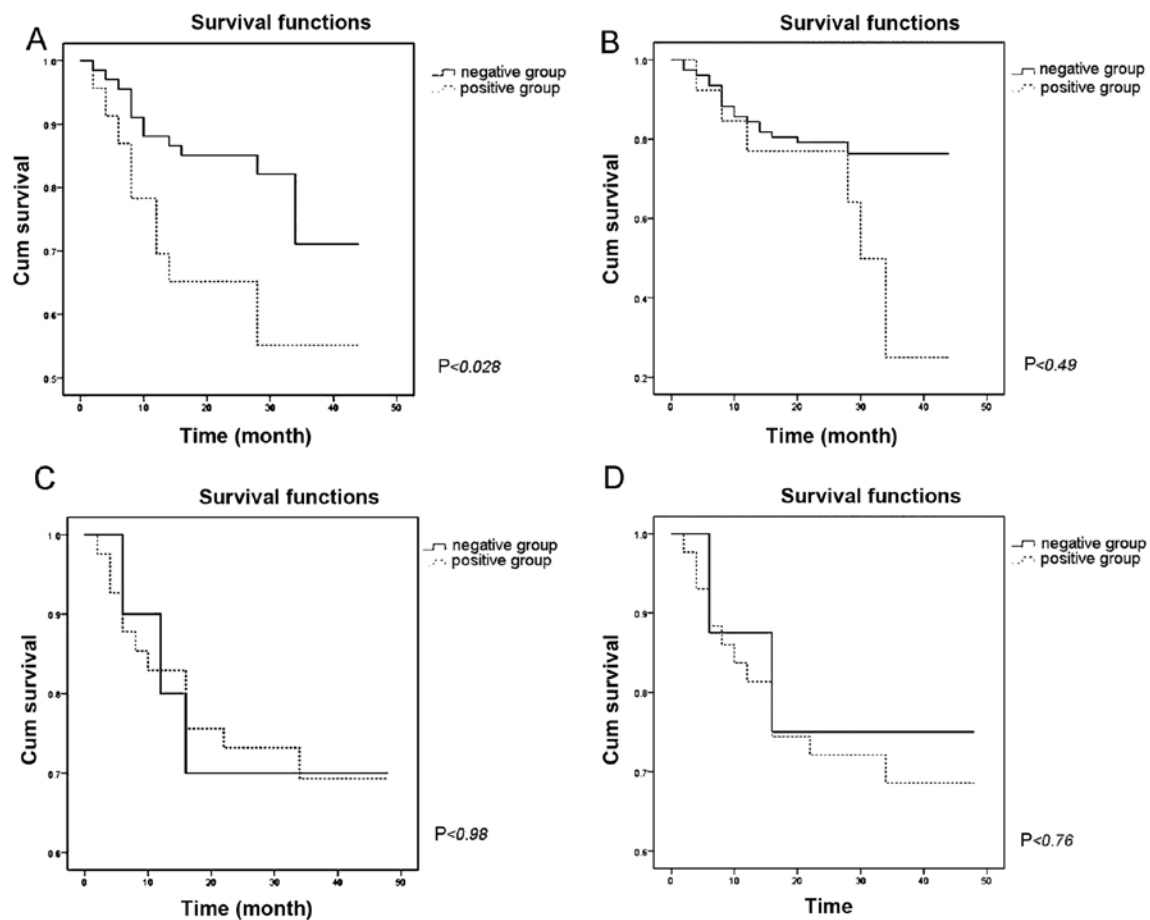


Figure 2. Survival curve comparisons between treatment-naïve lymphoma patients with differential (A) *ID4* and (B) *ZO-1* methylation patterns in bone marrow. Survival curve comparisons between treatment-naïve lymphoma patients with differential (C) *ID4* and (D) *ZO-1* methylation patterns in paraffin-embedded tissues.

genes. A significant difference was observed in the survival rate between the patients with and without *ID4* methylation in the bone marrow ($P<0.05$). On the contrary, the methylation status of *ID4* and *ZO-1* of DNA isolated from the lymphoma tissue did not show any correlation with the survival rate (Fig. 2).

DNA sequencing results. The CpG methylation sites of individual bacterial clones are shown in Fig. 3. The total methylation

rates of *ID4* and *ZO-1* from 10 randomly picked colonies were 22.08 and 28.4%, respectively (Fig. 3). On the contrary, the total methylation rates of *ID4* and *ZO-1* were 5.0 and 5.1%, respectively, in the paraffin-embedded tissues of subjects without lymphoma, indicating that these genes are overwhelmingly hypomethylated in subjects without lymphoma. The total methylation rates of *ID4* and *ZO-1* of 10 colonies from the paraffin-embedded tissues of lymphoma patients were 69.79

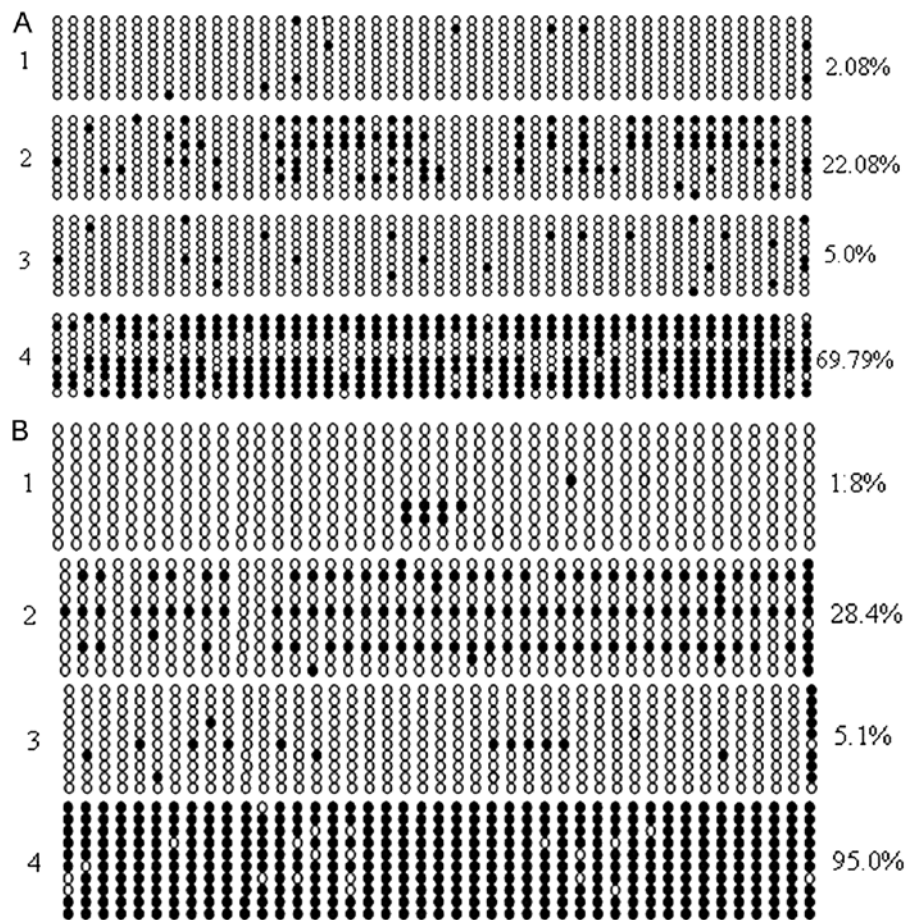


Figure 3. Methylation patterns of promoter regions of the (A) *ID4* and (B) *ZO-1* genes in the treatment-naïve lymphoma patients. Row 1 is the control sample from the bone marrow of subjects without lymphoma; row 2 is the sample from the bone marrow of methylation-positive samples; row 3 is the control sample from the paraffin-embedded samples; and row 4 is the sample from the paraffin-embedded tissues of methylation-positive samples.

and 95.0%, respectively, which were significantly higher than the rates in the control subjects without lymphoma.

Discussion

Genetic and epigenetic aberrations are the two major mechanisms of tumorigenesis. The epigenetic changes are mainly reflected by alterations in the DNA methylation patterns. For instance, the hypermethylation of CpG islands in the promoter region of tumor-suppressor genes and the hypomethylation status of the entire genome have both been found in tumors (13). Usually, one or several tumor-suppressor genes are thought to be responsible for tumorigenesis in certain types of malignancies, and the epigenetic features of the promoter regions of each tumor-suppressor gene are unique (14-17). The pathogenesis and development of lymphoma are closely associated with the methylation status of various tumor-suppressor genes (18-20).

Successful methylation specific PCR was performed in 51 paraffin-embedded lymphoma samples out of the total 92, and 41 and 43 samples were positive for *ID4* and *ZO-1* methylation, respectively, with a positivity rate of 80.4 and 84.3%, respectively. Forty-seven samples were positive for either *ID4* or *ZO-1* gene methylation, and the coverage rate for both genes was 92.2%. Importantly, the *ID4* and *ZO-1* genes of 8 paraffin-embedded lymphoma tissues from either chronic cholecystitis

or reactive lymphadenitis patients were completely unmethylated. Therefore, the methylation of *ID4* and *ZO-1* promoters in lymphoma patients may be an epigenetic feature of lymphoma, and the combined detection of the methylation of *ID4* and *ZO-1* may be a potential highly specific, highly sensitive, auxiliary diagnostic approach for lymphoma.

There are a few factors that may have influenced the detection rate of MSP in the present study. First of all, the DNA isolated from the paraffin-embedded samples may have been degraded or damaged because of prior chemical and physical treatments, such as formalin and temperature changes (21,22). Second, the amount of DNA isolated from the paraffin-embedded samples was relatively low, and the final concentration was only 50 $\mu\text{g}/\mu\text{l}$. The quality of DNA was also problematic, since the ratio of absorption at 260 and 280 nm was usually above 1.9, indicating contamination with RNA or protein. Moreover, the severe degradation of DNA along with the prolonged storage of paraffin-embedded samples may be another reason for the low detection rate of DNA methylation.

Of the 90 bone marrow samples from the lymphoma patients, 23 and 13 samples were positive for *ID4* and *ZO-1* gene methylation, respectively, and the positivity rate was 25.56 and 14.44%, respectively. Interestingly, the *ID4* and *ZO-1* genes of the 8 bone marrow donors were completely unmethylated. The patients with positive *ID4* or *ZO-1* gene methylation in bone marrow had lower survival rates compared

Table II. Correlation between the *ID4* and *ZO-1* gene methylation status in paraffin-embedded tissues and the lymphoma pathological type, stage and prognosis.

	Tissue samples Total n	<i>ID4</i> methylation n (%)	<i>ZO-1</i> methylation n (%)	P-value (<i>ID4</i>)	P-value (<i>ZO-1</i>)
Lymphoma	51	41 (80.4)	43 (84.3)		
NHL	47	40 (85.1)	41 (87.2)	0.004	0.05
HD	4	1 (25)	2 (50)		
Pathological type					
B cell	37	31 (83.8)	31 (83.8)	0.62	0.17
T cell	10	9 (90)	10 (100)		
Clinical stage					
I/II	26	22 (84.62)	20 (76.9)	0.44	0.14
III/IV	25	19 (76)	23 (92.0)		
IPI					
0-2	39	33 (84.61)	34 (87.18)	0.83	0.98
3-5	8	7 (87.5)	7 (87.5)		

ID4, DNA binding factor 4; *ZO-1*, tight junction protein 1; NHL, non-Hodgkin lymphoma; HD, Hodgkin lymphoma; IPI, International Prognostic Index.

Table III. Correlation between the *ID4* and *ZO-1* gene methylation status in bone marrow samples and the lymphoma pathological type, stage and prognosis.

	Bone marrow samples Total n	<i>ID4</i> methylation n (%)	<i>ZO-1</i> methylation n (%)	P-value (<i>ID4</i>)	P-value (<i>ZO-1</i>)
Lymphoma	90	23 (25.56)	13 (14.44)		
NHL	78	21 (26.92)	12 (15.38)	0.4483	0.5177
HD	12	2 (16.67)	1 (8.33)		
Pathological type					
B cell	60	16 (26.67)	7 (11.67)	0.9257	0.09659
T cell	18	5 (27.78)	5 (27.78)		
Clinical stage					
I/II	30	10 (33.33)	4 (13.33)	0.2316	0.8321
III/IV	60	13 (21.67)	9 (15)		
IPI					
0-2	67	16 (23.88)	7 (10.45)	0.5340	0.06564
3-5	23	7 (30.43)	6 (26.09)		

ID4, DNA binding factor 4; *ZO-1*, tight junction protein 1; NHL, non-Hodgkin lymphoma; HD, Hodgkin lymphoma; IPI, International Prognostic Index.

to the patients with unmethylated *ID4* or *ZO-1* genes. In addition, a significant difference was observed in the survival rate between the patients with and without *ID4* gene methylation in the bone marrow, indicating that the methylation status of the *ID4* gene in the bone marrow may be associated with a more favorable prognosis in lymphoma patients. Further studies in a larger cohort of patients are needed to evaluate the generalizability and clinical utility of this observation.

In the present study, we found that the MSP-derived methylation detection rate for the *ID4* and *ZO-1* genes was higher

in samples from paraffin-embedded lymphoma tissues than in those from fresh bone marrow. This may be due to the large amount of lymphoma cells in the paraffin-embedded tissues, and may be also due to the fact that the bone marrow is not involved during the early stages of lymphoma. We also found in the present study that the methylation status of *ID4* and *ZO-1* in non-Hodgkin lymphoma patients was higher than that in Hodgkin lymphoma patients, in DNA samples derived either from the paraffin-embedded tissue or from fresh bone marrow (Tables II and III). Meanwhile, the stages, the cellular type of

lymphoma, and the International Prognostic Index were not correlated with the methylation status of the *ID4* and *ZO-1* genes (Tables II and III). Moreover, the survival rate of the patients with methylated *ID4* or *ZO-1* in samples derived from the paraffin-embedded tissues was not significantly different from that of patients with unmethylated *ID4* and *ZO-1* genes, indicating that *ID4* and *ZO-1* methylation in the bone marrow is of particular significance in lymphoma.

In order to analyze the CpG island methylation status in the promoter regions of the *ID4* and *ZO-1* genes, bisulfite sequencing was performed for individual clones of *ID4* and *ZO-1* amplified from the bone marrow of healthy individuals and the lymphoma patients without prior treatment. Similar to the MSP results, the bisulfite sequencing results showed that the total methylation rates of *ID4* and *ZO-1* were 22.08 and 28.4%, respectively. On the contrary, the total methylation rates of *ID4* and *ZO-1* were 5.0 and 5.1%, respectively, in paraffin-embedded tissues of subjects without lymphoma, indicating that these genes are predominantly in a hypomethylated state in healthy subjects. The total methylation rates of *ID4* and *ZO-1* genes of 10 colonies derived from the paraffin-embedded tissue samples of lymphoma patients were 69.79 and 95.0%, respectively, which were significantly higher than the rates in the control subjects without lymphoma. The CpG island methylation status in the promoter regions of the *ID4* and *ZO-1* genes was also low in the DNA isolated from bone marrow of the subjects without lymphoma. Overall, the methylation patterns of the *ZO-1* and *ID4* genes were altered in the lymphoma patients, indicating that the aberration of methylation patterns of the *ZO-1* and *ID4* genes may be related to the pathogenesis and development of lymphoma.

In the present study, we also showed that the methylation rates of the *ID4* and *ZO-1* genes were 80.4 and 84.3%, respectively, in samples derived from 51 paraffin-embedded lymphoma tissues. Control samples only contained completely unmethylated *ID4* and *ZO-1* DNA, indicating that the methylation of *ID4* and *ZO-1* is highly specific to patients with lymphoma diagnosis. Therefore, the methylation pattern of the *ID4* and *ZO-1* genes may be a potential biomarker of lymphoma (5,22), which could be used in the early diagnosis of lymphoma. Moreover, patients with a positive methylation status of *ID4* in the bone marrow had significantly lower survival rates than patients whose specimens lacked *ID4* methylation. These data suggest that the aberrant methylation status of the *ID4* gene isolated from the bone marrow may be one indicator of poor prognosis in lymphoma. Further studies will help establish the clinical value of the methylation status of the *ID4* and *ZO-1* genes as diagnostic and prognostic markers in lymphoma.

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