J Clin Exp Hematopathol
Vol. 47, No. 1, Apr 2007

Original Article

Frequent DNA Methylation but not Mutation of the *ID4* Gene in Malignant Lymphoma

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ID4 is a member of the inhibitor of DNA binding (ID) family, and inhibits the binding of basic helix-loop-helix transcription factors to DNA. In some malignant tumors, *ID4* is inactivated by promoter hypermethylation, and is thought to be a candidate tumor suppressor gene (TSG). We have found that the promoter region of the *ID4* gene is frequently methylated in lymphoid cell lines (100%, 9/9), primary diffuse large B-cell lymphoma (95%, 19/20), and follicular lymphoma (100%, 10/10). Somatic mutation of the *ID4* gene was also examined, and no mutations were found. These findings suggest that the *ID4* gene might be inactivated by DNA hypermethylation, and may function as a TSG in malignant lymphoma. 〔*J Clin Exp Hematopathol 47(1) : 15-18, 2007*〕

Keywords: lymphoma, *ID4*, tumor suppressor gene, DNA methylation, combined bisulfite restriction analysis

INTRODUCTION

Lymphomas are malignancies that originate in the lymphoid tissues. Lymphomas are further classified into two major subtypes, Hodgkin lymphoma and non-Hodgkin's lymphoma. Diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) are two major subtypes of non-Hodgkin's lymphoma. FL is characterized by an indolent course with frequent relapses. Ultimately, FL can become resistant to chemotherapy or enter a more aggressive phase of the disease in the form of DLBCL, and patients succumb to their disease. Similar to other types of cancer, the pathogenesis of DLBCL and FL represent a multistep process that involves accumulation of multiple genetic and molecular lesions leading to the selection of a malignant clone[1,2]. In lymphomas, a major step in such processes is the deregulation of oncogenes. Oncogenes sometimes lie close to chromosomal breakpoints and become deregulated or mutated. In malignant lymphomas, several genes, such as *Cyclin D1*[3], *BCL2*[4], and *BCL6*[5], are often affected by specific chromosomal translocations. Important genetic events other than the activation of oncogenes include the alteration of tumor suppressor genes (TSGs). To date, numerous TSG mutations have been reported in lymphoid malignancies, and the clinical importance of such mutations has also been shown by many groups[6,7].

Aberrant CpG methylation in the 5'promoter regions of genes is an epigenetic phenomenon that is the major mechanism for TSG silencing in many cancers[8,9]. Recently, a global assessment of DNA methylation in a mouse model of T/natural killer acute lymphoblastic leukemia was reported[10]. According to this report, *ID4* gene expression was silenced by promoter methylation in human acute and chronic leukemia, and the gene was identified as a putative tumor suppressor[10]. *ID4* is a member of the family of inhibitor of DNA binding (ID) helix-loop-helix proteins; ID proteins function as dominant negative regulators of basic helix-loop-helix transcription factors[11]. Promoter methylation and transcriptional silencing of the *ID4* gene has been reported in gastric cancers[12], colorectal cancers[13], and breast cancers[14]. In addition, the frequency of *ID4* hyermethylation correlates with lymph node metastasis in breast cancer; therefore, *ID4* silencing might prove useful as a genetic marker to predict early metastasis[14]. However, there are few reports describing *ID4* status in malignant lymphoma and information about *ID4* function, expression, and regulation of tumor progression is very limited. The *ID4* gene is located on chromosome 6p23-p22.3[15], where we have observed loss of heterozygosity in malignant lymphoma[16]. These findings have prompted us to investigate whether *ID4* is a TSG for malignant lymphoma.

Received : Aug 5, 2006
Revised : Nov 13, 2006
Accepted : Nov 30, 2006
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MATERIALS AND METHODS

Samples and DNA preparation

DNA was isolated from the biopsied lymph nodes of 20 patients with DLBCL, 10 patients with FL, 3 patients with transformed FL, peripheral blood mononuclear cells (PBMNCs) of 3 healthy volunteers, and 9 lymphoid cell lines (NAMALWA, RAJI, RAMOS, DAUDI, HS-SULTAN, IM-9, THP-6, BALL-1, and JURKAT) by proteinase K digestion followed by phenol-chloroform extraction. Informed consent was obtained from all patients.

Direct sequencing of the ID4 gene

We amplified each of the 2 exons, including the exon/intron boundaries, of the*ID4* gene by polymerase chain reaction (PCR) using the following primers: exon1 forward, id4EX1F, 5'-CGACCCTCCCGTCAATTGTT-3'; exon1 reverse, id4EX1R, 5'-TACCCTCCAACCACCGCAC-3'; exon2 forward, id4EX2F, 5'-TGATTTCCGAGGACAATCC A-3'; exon2 reverse, id4EX2R, 5'-GGAGTTTGCTCTCAG AAACG-3' (Fig. 1). Conditions for PCR were as follows: initial denaturing for 5 min at 95℃ followed by 35 or 40 cycles of denaturing for 30 sec at 95℃, annealing for 30 sec at 64℃ (exon1) or 56℃ (exon2), and extension for 45 sec (exon1) or 30 sec (exon2) at 72℃. Each PCR was "hot-started" and the products were separated on 2% agarose gels and visualized by ethidium bromide staining.

The PCR products were collected and purified with SUPREC-EZ (TaKaRa, Kyoto, Japan) and resuspended in 10 μl of H_2O. Cycle sequencing reactions were performed with a BigDye Terminator v1.1 Cycle Sequencing Kit (PE Applied Biosystems, Warrington, UK) using forward or reverse PCR primers, according to the manufacturer's protocol. The sequencing products were precipitated with NaOAc (pH5.2), EDTA, and cold ethanol, washed once, dried and resuspended in 20 μl of Hi-Di-Formamide (PE Applied Biosystems). The sequencing products were analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

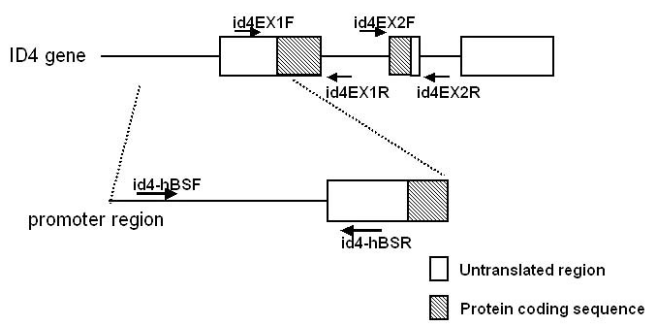

Fig. 1. Genomic structure of the *ID4* gene and locations of primers used in this study. The *ID4* gene has three exons. Open rectangles indicate non-coding regions and shaded rectangles indicate coding regions of *ID4*.

Combined bisulfite restriction analysis (COBRA)

We modified 1 μg genomic DNA from cell lines and clinical samples with sodium bisulfite using a DNA modification kit (BisulFast Methylated DNA Detection Kit; TOYOBO, Osaka, Japan), according to the manufacturer's protocol. Modified DNAs were amplified with the following primers: forward, id4-hBSF, 5'-ATTGGTTGGTTATTTTA GATTTTT-3', and reverse, id4-hBSR, 5'-CTTCCCTCACT AATCCTATAA-3' (Fig. 1). Dr. Christoph Plass (The Ohio State University) kindly informed us regarding use of these sequences for COBRA[9]. The PCR amplification was done in a 20 μl reaction volume with 2 μl of template for 40 cycles of 95℃ for 30 sec, 56℃ for 30 sec and 72℃ for 30 sec. We purified the PCR products with SUPREC-EZ (TaKaRa), and digested them for 4hr at 60℃ with *Bst*UI (New England Biolabs, Beverly, MA, USA), a restriction enzyme that digests CpG sequences retained after the bisulfite treatment due to methylation. Digested products were subjected to electrophoresis in 10% polyacrylamide gels and were visualized by ethidium bromide staining.

RESULTS

Absence of mutations in the ID4 gene in lymphoma samples

We first examined whether lymphomas contain mutations within the*ID4* coding region. Nine lymphoid cell lines and 33 clinical samples (20 DLBCLs, 10 FLs, and 3 transformed FLs) were analyzed. Two exons of the *ID4* gene were amplified by PCR and directly sequenced. We did not find any mutations within the coding region in the tested samples (data not shown).

Methylation of the ID4 gene by COBRA

Because *ID4* expression is suppressed in various tumors by DNA methylation, we examined the frequency of *ID4* promoter methylation in our samples by combined bisulfite restriction analysis (COBRA) using PCR primers localized in the promoter regions of the *ID4* gene. The frequency of *ID4* promoter methylation was 100% in lymphoid cell lines (Fig. 2a), 95% in DLBCL samples (Fig. 2b), and 100% in FL and transformed FL samples (Fig. 2c). In addition, we analyzed 3 normal samples as a negative control and found only low amounts of methylation. We had biopsied one case of transformed FL at both the indolent (Fig. 2c, F9) and aggres-

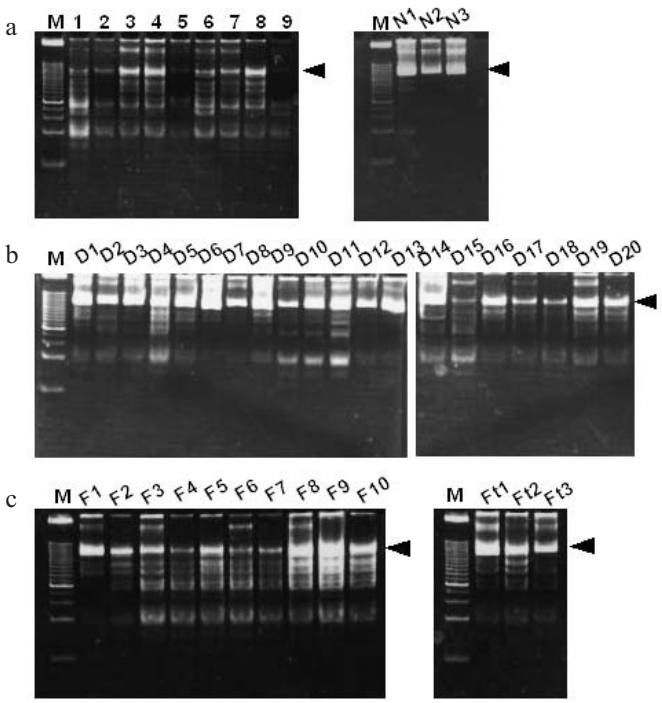

Fig. 2. Methylation analysis by COBRA of the 5' promoter region of the *ID4* gene in lymphoid cell lines and primary lymphomas. PCR products were digested with a restriction enzyme (*Bst*UI) that cleaves CpG sites retained after bisulfite treatment because of methylation. *The arrow* indicates PCR products before enzyme digestion (342bp). Digested fragments correspond to methylated DNA. Methylated fragments were detected in all samples except D12. (a) Lymphoid cell lines and normal PBMNCs. 1: NAMALWA, 2: RAJI, 3: DAUDI, 4: HS-SULTAN, 5: IM-9, 6: THP-6, 7: BALL-1, 8: JURKAT, 9: RAMOS, N1: normal PBMNC 1, N2: normal PBMNC 2, N3: normal PBMNC 3. (b) D1~D20: DLBCL. (c) F1~F10: FL, Ft1~Ft3: transformed FL. M: 25bp DNA ladder.

sive clinical stages (Fig. 2c, Ft2); the COBRA patterns (band size and band intensity) of these two samples were almost identical (Fig. 2c). Our findings indicate that the *ID4* gene was methylated and might be inactivated in almost all malignant lymphomas analyzed here and that the frequency of DNA methylation of the *ID4* gene did not differ between aggressive lymphomas (DLBCL and transformed FL) and indolent lymphomas (FL).

DISCUSSION

In this study, we analyzed the DNA sequence and methylation status of the *ID4* gene, and showed that *ID4* has no mutations but has a high frequency of promoter methylation in lymphoid cell lines and primary lymphoma cells.

Generally, aberrant methylation of CpG islands correlates with loss of gene expression, and such DNA methylation provides an alternative mechanism to gene deletion or mutation for the loss of tumor suppressor function[17-19]. In malignant lymphoma, the $p16^{INK4a}$ and $p15^{INK4b}$ tumor suppressor genes, which encode cyclin dependent kinase (CDK) inhibitors, have been reported to be frequently methylated[19]. In addition, we have observed gene inactivation and DNA hypermethylation of $p57^{KIP2}$, another type of CDK inhibitor, in B cell malignancies at high frequency[9]. Therefore, TSG inactivation by DNA methylation is a major mechanism of lymphoma development and progression. *ID4* has recently been identified as a putative TSG, and its expression is silenced by promoter methylation in human leukemia[10]. Decreased levels of *ID4* mRNA expression in other tumor types have been tightly correlated to promoter methylation[12-14]. In colorectal cancer, the frequency of hypermethylation of the *ID4* gene has been reported to be higher in primary and metastatic cancer than adenoma, and silencing of *ID4* gene expression is thought to be associated with unfavorable prognosis[13]. Here we have investigated *ID4* in lymphoma, and found that there were no somatic mutations in the tested samples, but, as in other hematological malignancies (chronic lymphocytic leukemia and acute myeloid leukemia) very high frequency of hypermethylation was found. These observations suggest that the *ID4* gene may be inactivated by DNA hypermethylation and is a candidate tumor suppressor in malignant lymphoma. The frequency of hypermethylation of the *ID4* gene did not differ among DLBCL, FL, and transformed FL. Samples from one FL case (F9) before and after aggressive transformation showed very similar DNA methylation status in both clinical stages. Thus, the methylation of the *ID4* gene in this patient might not have altered after disease progression.

We observed methylation of the *ID4* gene in IM-9 cells, which are EBV transformed B cells. This finding is interesting in the context of immortalization by EBV infection and DNA methylation of cellular genes. The hypermethylation of the *ID4* gene was thought to be a common molecular marker of malignant lymphoma, but not to be an indicator of disease progression. Indeed, any correlation between lymphomagenesis and *ID4* gene methylation remained to be examined.

In conclusion, hypermethylation of the *ID4* promoter is frequently found in B cell lymphoma, and the *ID4* gene is a candidate tumor suppressor for FL and DLBCL.

ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid for Cancer Research (15-11) from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

1 Lossos IS: Molecular pathogenesis of diffuse large B-cell lymphoma. J Clin Oncol 23: 6351-6357, 2005

2 de Jong D: Molecular pathogenesis of follicular lymphoma: A

cross talk of genetic and immunologic factors. J Clin Oncol 23 : 6358- 6363, 2005
3 Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM, Arnold A : A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature 350 : 512- 515, 1991
4 Tsujimoto Y, Cossman J, Jaffe E, Croce CM : Involvement of the bcl-2 gene in human follicular lymphoma. Science 228 : 1440- 1443, 1985
5 Baron BW, Nucifora G, McCabe N, Espinosa R 3rd, Le Beau MM, McKeithan TW : Identification of the gene associated with the recurring chromosomal translocations t (3 ; 14) (q27 ; q32) and t (3 ; 22) (q27 ; q11) in B-cell lymphomas. Proc Natl Acad Sci USA 90 : 5262- 5266, 1993
6 Ichikawa A, Kinoshita T, Watanabe T, Kato H, Nagai H, Tsushita K, Saito H, Hotta T : Mutations of the p53 gene as a prognostic factor in aggressive B-cell lymphoma. N Eng J Med 337 : 529- 534, 1997
7 Nagai H, Kinoshita T, Ichikawa A, Murate T : Malignant lymphoma and tumor suppressor genes. J Clin Exp Hematopathol 42 : 11- 24, 2002
8 Jones PA, Baylin SB : The fundamental role of epigenetic events in cancer. Nat Rev Genet 3 : 415- 428, 2002
9 Li Y, Nagai H, Ohno T, Yuge M, Hatano S, Ito E, Mori N, Saito H, Kinoshita T : Aberrant DNA methylation of $p57^{KIP2}$ gene in the promoter region in lymphoid malignancies of B-cell phenotype. Blood 100 : 2572- 2577, 2002
10 Yu L, Liu C, Vandeusen J, Becknell B, Dai Z, Wu YZ, Raval A, Liu TH, Ding W, Mao C, Liu S, Smith LT, Lee S, Rassenti L, Marcucci G, Byrd J, Caligiuri MA, Plass C : Global assessment of promoter methylation in a mouse model of cancer identifies *ID4* as a putative tumor-suppressor gene in human leukemia. Nat Genet 37 : 265- 274, 2005
11 Massari ME, Murre C : Helix-loop-helix proteins : regulators of transcription in eukaryotic organisms. Mol Cell Biol 20 : 429- 440, 2000
12 Chan AS, Tsui WY, Chen X, Chu KM, Chan TL, Chan AS, Li R, So S, Yuen ST, Leung SY : Downregulation of ID4 by promoter hypermethylation in gastric adenocarcinoma. Oncogene 22 : 6946- 6953, 2003
13 Umetani N, Takeuchi H, Fujimoto A, Shinozaki M, Bilchik AJ, Hoon DS : Epigenetic inactivation of ID4 in colorectal carcinomas correlates with poor differentiation and unfavorable prognosis. Clin Cancer Res 10 : 7475- 7483, 2004
14 Umetani N, Mori T, Koyanagi K, Shinozaki M, Kim J, Giuliano AE, Hoon DS : Aberrant hypermethylation of ID4 gene promoter region increases risk of lymph node metastasis in T1 breast cancer. Oncogene 24 : 4721- 4727, 2005
15 Rigolet M, Rich T, Gross-Morand MS, Molina-Gomes D, Viegas-Pequignot E, Junien C : cDNA cloning, tissue distribution and chromosomal localization of the human ID4 gene. DNA Res 5 : 309- 313, 1998
16 Nagai H, Kinoshita T, Suzuki H, Hatano S, Murate T, Saito H : Identification and mapping of novel tumor suppressor loci on 6p in diffuse large B-cell non-Hodgkin's lymphoma. Genes Chromosomes Cancer 25 : 277- 283, 1999
17 Esteller M, Herman JG : Cancer as an epigenetic disease : DNA methylation and chromatin alterations in human tumors. J Pathol 196 : 1- 7, 2002
18 Herman JG, Baylin SB : Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 349 : 2042- 2054, 2003
19 Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG : Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 10 : 687- 692, 2001
20 Baur AS, Shaw P, Burri N, Delacretaz F, Bosman FT, Chaubert P : Frequent methylation silencing of $p15^{INK4b}$ (MTS2) and $p16^{INK4a}$ (MTS1) in B-cell and T-cell lymphomas. Blood 94 : 1773- 1781, 1999