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.

**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. 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*, *BCL2*, and *BCL6*, 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.

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. Recently, a global assessment of DNA methylation in a mouse model of T/natural killer acute lymphoblastic leukemia was reported. 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. *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. Promoter methylation and transcriptional silencing of the *ID4* gene has been reported in gastric cancers, colorectal cancers, and breast cancers. In addition, the frequency of *ID4* hypermethylation correlates with lymph node metastasis in breast cancer; therefore, *ID4* silencing might prove useful as a genetic marker to predict early metastasis. 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.

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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’-TGATTTCCGAGGACAATCAA-3’; exon2 reverse, id4EX2R, 5’-GGAGTTTGCTCTCAAGAAACG-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 10μl of H2O. 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 20μl 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).

**Combined bisulfite restriction analysis (COBRA)**

We modified 1μg 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’-ATTGGTTGGTTATTTTAGA-3’; reverse, id4-hBSR, 5’-GGAGTTTGCTCTCAGAAACG-3’ (Fig. 1). Dr. Christoph Plass (The Ohio State University) kindly informed us regarding use of these sequences for COBRA. The PCR amplification was done in a 20μl reaction volume with 2μl 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 BstUI (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-
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 \textit{ID4} gene was methylated and might be inactivated in almost all malignant lymphomas analyzed here and that the frequency of DNA methylation of the \textit{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 \textit{ID4} gene, and showed that \textit{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\textsuperscript{17-19}. In malignant lymphoma, the \textit{p16\textsubscript{INK4a}} and \textit{p15\textsubscript{INK4b}} tumor suppressor genes, which encode cyclin dependent kinase (CDK) inhibitors, have been reported to be frequently methylated\textsuperscript{19}. In addition, we have observed gene inactivation and DNA hypermethylation of \textit{p57\textsubscript{KIP2}}, another type of CDK inhibitor, in B cell malignancies at high frequency\textsuperscript{9}. Therefore, TSG inactivation by DNA methylation is a major mechanism of lymphoma development and progression. \textit{ID4} has recently been identified as a putative TSG, and its expression is silenced by promoter methylation in human leukemia\textsuperscript{10}. Decreased levels of \textit{ID4} mRNA expression in other tumor types have been tightly correlated to promoter methylation\textsuperscript{12-14}. In colorectal cancer, the frequency of hypermethylation of the \textit{ID4} gene has been reported to be higher in primary and metastatic cancer than adenoma, and silencing of \textit{ID4} gene expression is thought to be associated with unfavorable prognosis\textsuperscript{13}. Here we have investigated \textit{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 \textit{ID4} gene may be inactivated by DNA hypermethylation and is a candidate tumor suppressor in malignant lymphoma. The frequency of hypermethylation of the \textit{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 \textit{ID4} gene in this patient might not have altered after disease progression.

We observed methylation of the \textit{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 \textit{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 \textit{ID4} gene methylation remained to be examined.

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

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