Plenary paper

Expression of DNA methyltransferases *DNMT1*, *3A,* and *3B* in normal hematopoiesis and in acute and chronic myelogenous leukemia

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Aberrant hypermethylation of tumor suppressor genes plays an important role in the development of many tumors. Recently identified new DNA methyltransferase (*DNMT*) genes, *DNMT3A* and *DNMT3B*, code for de novo methyltransferases. To determine the roles of *DNMT3A*, *DNMT3B,* as well as *DNMT1,* in the development of leukemia, competitive polymerase chain reaction (PCR) assays were performed and the expression levels of *DNMTs* were measured in normal hematopoiesis, 33 cases of acute myelogenous leukemia (AML), and 17 cases of chronic myelogenous leukemia (CML). All genes were constitutively expressed, although at different levels, in T lymphocytes, monocytes, neutrophils, and normal bone marrow cells. Interestingly, *DNMT3B* was expressed at high levels in CD34$^+$ bone marrow cells but down-regulated in differentiated cells. In AML, 5.3-, 4.4-, and 11.7-fold mean increases were seen in the levels of *DNMT1*, *3A,* and *3B*, respectively, compared with the control bone marrow cells. Although CML cells in the chronic phase did not show significant changes, cells in the acute phase showed 3.2-, 4.5-, and 3.4-fold mean increases in the levels of *DNMT1*, *3A,* and *3B*, respectively. Using methylation-specific PCR, it was observed that the *p15^{INAK4B}* gene, a cell cycle regulator, was methylated in 24 of 33 (72%) cases of AML. Furthermore, AML cells with methylated *p15^{INAK4B}* tended to express higher levels of *DNMT1* and *3B*. In conclusion, *DNMTs* were substantially overexpressed in leukemia cells in a leukemia type- and stage-specific manner. Up-regulated *DNMTs* may contribute to the pathogenesis of leukemia by inducing aberrant regional hypermethylation. (Blood. 2001; 97:1172-1179)

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Introduction

DNA methylation plays an important role in tissue- and stage-specific gene regulation,[1,2] genomic imprinting,[3,4] and X-chromosome inactivation,[5] and has been shown to be essential for normal mammalian development.[6] Recent studies have revealed that both global DNA hypomethylation and regional hypermethylation occur in tumorigenesis.[7-9] Such aberrant DNA methylation is observed in a nonrandom, tumor type-specific manner.[10] In particular, certain types of tumors show regional hypermethylation of CpG islands associated with the promoter regions of tumor suppressor genes, such as *RB*,[11] *VHL*,[12] *p16^{INAK4A}*,[13] and *hMLH1*.[14] Furthermore, the regional hypermethylation is often associated with the inactivation of the tumor suppressor genes.[15] These data suggest that this epigenetic process has a pathogenetic role in the clonal evolution of cancer.[9]

In hematologic malignancies, aberrant DNA hypermethylation is thought to have relevance to leukemogenesis.[16] For example, during the progression of chronic myelogenous leukemia (CML), the *ABL1* promoter of the *BCR-ABL* fusion gene becomes significantly hypermethylated.[17,18] Also, aberrant hypermethylation of the *p15^{INAK4B}* tumor suppressor gene is associated with its inactivation in at least half of the patients with acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML).[19,20] Furthermore, hypermethylation of *p15^{INAK4B}* is observed concomitant with the disease progression in myelodysplastic syndrome (MDS).[21] In addition to these tumor-related genes, a number of other genes are concurrently hypermethylated in AML,[22] suggesting that there might be a dysregulation in the normal DNA methylation mechanism, by which the leukemic cells become predisposed to hypermethylation.

Until recently, only one mammalian DNA methyltransferase, *DNMT1*, had been known, which has a higher maintenance DNA methylase activity rather than a de novo methylase activity in vitro.[23] Recently, new mammalian DNA methyltransferase genes, *DNMT3A* and *DNMT3B*, have been cloned.[24-26] Both the mouse *Dnmt3a* and *3b* (the orthologs of human *DNMTs*) enzymes were shown to methylate hemimethylated and unmethylated DNA with equal efficiencies in vitro.[24] In transgenic *Drosophila melanogaster*, *Dnmt3a* clearly exhibited de novo methylase activity, whereas *Dnmt1* did not.[27] Furthermore, a simultaneous inactivation of both *Dnmt3a* and *Dnmt3b* blocked the de novo methylation activity in embryonic stem cells and embryos,[28] suggesting that these enzymes are the long sought de novo methyltransferases.

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To understand the mechanisms underlying the aberrant, tumor type-specific hypermethylation, it is important to know the role of each DNA methyltransferase in the pathogenetic process. In colon cancer, increased *DNMT1* expression has been demonstrated when compared with normal mucosa.[29] In both colon and lung cancers, *DNMT1* activity increases progressively, along with the advancement of their tumor stage.[30,31] In hematologic malignancies, overexpression of *DNMT1* was shown in 12 leukemia samples including AML, ALL, and MDS.[32] As to *DNMT3A* and *3B,* Robertson and coworkers have recently shown that both genes, as well as *DNMT1,* are up-regulated in some malignancies, such as bladder, colon, kidney, and pancreas tumors, though at different levels.[25] Xie and colleagues have also reported the increased expression of all 3 *DNMTs* in several tumor cell lines.[26]

We have obtained *DNMT3A* and *3B* complementary DNAs (cDNAs) independently from the other laboratories by database search. Then we have set out to study the roles of these enzymes in the pathogenesis of hematologic malignancies. In the present study, we report the expression levels of *DNMT3A, DNMT3B,* and *DNMT1* in normal hematopoiesis, AML, and CML, studied by a competitive polymerase chain reaction (PCR) assay. Furthermore, in AML cases, we have investigated whether the expression levels of *DNMTs* are correlated with aberrant hypermethylation of the *p15^{INAK4B}* tumor suppressor gene.

Materials and methods

Cloning of the human DNMT3A and 3B cDNAs

We performed a TBLASTN search[33] of the dbEST database using modified mouse Dnmt1 motif IV sequences as queries and identified 3 matching mouse EST sequences (AA177277, AA116694, and AA1550177) and 4 matching human EST sequences (W76111, N88352, AA361360, and AA216697). Two of the human EST clones deposited by the I.M.A.G.E. Consortium were obtained from Research Genetics and sequenced in their entirety. These sequences were identical to the corresponding part of the DNMT3A and DNMT3B cDNAs discovered by Robertson and colleagues[25] and Xie and coworkers.[26] PCR primers (Table 1) were prepared to amplify part of the catalytic domain of each DNMT3 cDNA. They were

designed not to amplify genomic fragments, considering the exon-intron organization.

Clinical samples and cell lines

Heparinized peripheral blood and bone marrow cells were collected from patients with AML and CML, and from healthy donors after obtaining their informed consent. Immediately after harvest, mononuclear cells (MNCs) were isolated by sedimentation on Ficoll-Hypaque gradients. More than 90% of the MNC populations from AML and acute phase CML were leukemic blasts. Granulocyte fractions were collected from normal peripheral blood with Polymorphperp (Nycomed Pharma, Oslo, Norway). Plastic-adherent cells from normal MNCs were used as the monocyte fraction. Peripheral lymphocytes were collected as nonadherent cells from normal MNCs, and T lymphocytes were enriched by negative selection, using Dynabeads M-450 CD19 (Dynal, Oslo, Norway). Purified T lymphocytes were stimulated with 2.5 µg/mL phytohemagglutinin (PHA; Sigma Chemical Co, St Louis, MO) for 72 hours and used as the activated T cells. CD34$^+$ cells were isolated from normal bone marrow cells by positive selection, using Dynabeads M-450 CD34 (Dynal),[34] and a flow cytometric analysis confirmed that more than 95% of the cells were CD34$^+$. In addition to these cells, an Epstein-Barr virus (EBV)-transformed B-lymphocyte cell line (LCL), and 3 human leukemic cell lines (HL60, K562, and KU812) maintained under the standard conditions were used in this study.

In vitro assay for hematopoietic progenitors

Clonogenic progenitor assays were performed using the methylcellulose culture system.[35] Five hundred CD34$^+$ cells were cultured in 1 mL Iscove modified Dulbecco medium (GIBCO, Grand Island, NY) supplemented with 30% fetal calf serum (ICN Biochemicals, Osaka, Japan), 50 ng recombinant human interleukin-3 (rhIL-3), 50 ng recombinant human stem cell factor (rhSCF), 10 ng rhIL-6, 10 ng recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), 10 ng recombinant human granulocyte CSF (rhG-CSF), 3 U recombinant human erythropoietin (all cytokines were from Kirin Brewery Co, Tokyo, Japan), 5×10^{-5} M 2-mercaptoethanol, and 0.88% methylcellulose in 35-mm Nunc 171099 culture dishes (Nunc Inc, Naperville, IL) at 37°C under a humidified atmosphere with 5% CO_2. After 14 days of culture, individual colonies were picked up using finely drawn-out Pasteur pipettes and processed for the reverse transcriptase-PCR (RT-PCR) analysis.

Table 1. Oligonucleotide primers

	Oligonucleotide sequence	Nucleotide position* (exon number)	Target size (bp)	Competitor size (bp)
5′ GAPDH primer	5′-AAGGCTGAGAACGGGAAGCTTGTCATCAAT-3′	180-210 (exon 4)	500	710
3′ GAPDH primer	5′-TTCCCGTTCAGCTCAGGGATGACCTTGCCC-3′	651-680 (exon 8)		
5′ PCNA primer	5′-TCCATCCTCAAGAAGGTGTTGGAG-3′	27-50 (exon 1)	664	782
3′ PCNA primer	5′-CAGACATACTGAGTGTCACCGTTG-3′	668-691 (exon 5)		
5′ DNMT1 primer	5′-ACCGCTTCTACTTCCTCGAGGCCTA-3′	3242-3266 (exon 28)	335	460
3′ DNMT1 primer	5′-GTTGCAGTCCTCTGTGAACACTGTGG-3′	3551-3576 (exon 30)		
5′ DNMT3A primer	5′-CACACAGAAGCATATCCAGGAGTG-3′	2070-2093†	551	668
3′ DNMT3A primer	5′-AGTGGACTGGGAAACCAAATACCC-3′	2597-2620		
5′ DNMT3B primer	5′-AATGTGAATCCAGCCAGGAAAGGC-3′	1942-1965 (exon 17)	190	331
3′ DNMT3B primer	5′-ACTGGATTACACTCCAGGAACCGT-3′	2109-2132 (exons 18/19)		
5′ p15-M primer‡	5′-GCGTTCGTATTTTGCGGTT-3′		148	
3′ p15-M primer	5′-CGTACAATAACCGAACGACCGA-3′			
5′ p15-U primer§	5′-TGTGATGTGTTTGTATTTTGTGGTT-3′		154	
3′ p15-U primer	5′-CCATACAATAACCAAACAACCAA-3′			

The 5′ and 3′ primers of a pair for standard and competitive RT-PCR were designed for sequences in different exons so that they amplify only cDNA and not genomic DNA. Target size refers to the size of the PCR products from the gene transcripts and competitor size refers to the size of the PCR products from the competitor DNA. For methylation-specific PCR, primers designed by Herman and coworkers[39] were used.

*The nucleotide sequence positions are relative to the ATG start.

†The exon-intron organization has not been determined.

‡M primer, a primer specific for methylated DNA.

§U primer, a primer specific for unmethylated DNA.

Isolation of RNA and RT-PCR analysis

Total RNA was extracted from various cell samples by acid the guanidine/phenol/chloroform method.[36] To isolate RNA from the individually picked up colonies, MS2 phage RNA (Boehringer Mannheim, Mannheim, Germany) was added as a carrier. First-strand cDNA was synthesized from 2 μg total RNA in a 50-μL reaction mixture containing random hexamers as primers by using a cDNA synthesis kit (Stratagene, La Jolla, CA). One μL cDNA mixture was brought into 25 μL 1 × PCR buffer, 10 mM Tris-HCl (pH 9.5), 50 mM KCl, 0.1% Triton X-100), 0.2 mM of each dNTP, 1.5 mM MgCl$_2$, 10 pmol of each primer, and 2.0 U of Taq-DNA polymerase (Promega, Madison, WI). The primers used are listed in Table 1. PCR was performed in a Perkin Elmer GeneAmp PCR System 9600. Each PCR cycle consisted of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The PCR cycle numbers were 26 for GAPDH, 30 for DNMT1 and 3A, and 35 for DNMT3B (Figure 1). Under the conditions used, the cDNAs were exponentially amplified, and thus a semiquantitative estimation of the products was possible (data not shown).

Competitive PCR for quantitative analysis

To develop a competitive PCR assay[37] for each gene, a competitor plasmid containing an extra DNA fragment in the target sequence was prepared. The GAPDH competitor plasmid was prepared by inserting a 210-bp SacII

fragment from λ DNA into the unique SacII site of a 500-bp GAPDH cDNA fragment cloned in a plasmid vector pCR2.1 (Invitrogen, Gronigen, The Netherlands). The PCNA competitor was prepared by cloning a 118-bp HaeIII fragment from φX174 DNA into the StuI site of a 664-bp PCNA cDNA fragment in pCR2.1. The DNMT1 competitor was prepared by inserting a 125-bp HindIII fragment from λ DNA into the HindIII site of a 335-bp DNMT1 cDNA fragment in a modified pBluescriptSK+ vector, from which the HindIII site within the multicloning site had been deleted. The DNMT3A competitor was prepared by inserting a 117-bp BstPI fragment from λ DNA into the BstPI site of a 551-bp DNMT3A cDNA fragment in pCR2.1. The DNMT3B competitor was prepared by cloning a 141-bp TthHB8I fragment from pBR322 DNA into the NspV site of a 190-bp DNMT3B cDNA fragment in pCR2.1. All the above cDNA fragments were amplified from RNA of HL60 cells by RT-PCR.

The copy number of each competitor plasmid was determined and 3- or 4- fold consecutive dilutions were prepared. PCR was performed under the conditions described above. Series of PCR products were electrophoresed on a 1.5% agarose gel, stained with Vistra green (Amersham, Heidelberg, Germany), exposed to a FluorImager (Molecular Dynamics, Sunnyvale, CA) and quantitated using ImageQuant Software version 4.1 (Molecular Dynamics).

To calculate the initial amount of target transcripts, a range of dilutions in which the ratio of the products from the competitor to the products from the target was between 0.2 and 5.0 were taken. Then, log(products from competitor/products from target) was plotted against log(initial molecular amount of added competitor). A regression analysis revealed that this provided a near-linear curve in each case. The initial amount of target DNA was then determined from the equivalence point of the curve, where log(products from competitor/products from target) = 0. Expression levels of each DNMT were displayed as relative values by setting the mean of the levels in normal bone samples as 1, after normalizing the levels of DNMT by those of GAPDH or PCNA in the same sample.

Bisulfite modification and methylation-specific PCR

One μg genomic DNA was denatured in 50 μL 0.2 M NaOH at 37°C for 10 minutes and then added with 30 μL 10 mM hydroquinone (Sigma, St Louis, MO) and 520 μL 3 M sodium bisulfite (pH 5.0) (Sigma). Samples were incubated at 50°C for 16 hours under mineral oil. Modified DNA was purified using the Wizard purification resin and the Vacuum Manifold (Promega) and eluted into 50 μL water. After addition of 5.6 μL of 3 M NaOH (final 0.3 M), samples were let stand at room temperature for 5 minutes for final desulphonation. After ethanol precipitation, samples were dissolved in 50 μL water. Bisulfite treatment of DNA converts all unmethylated, but not methylated, cytosines to uracils.[38]

Based on the sequence differences resulting from this modification, methylation-specific PCR (MSP) was performed for the p15^{INA4B} gene with the primer sets designed by Herman and colleagues (Table 1).[39] PCR was performed in a 50-μL reaction mixture containing 100 ng bisulfite modified DNA, 1 × PCR reaction buffer (Promega), 0.2 mM of each dNTP, 1.5 mM MgCl$_2$, 20 pmol of each primer, and 5% dimethylsulfoxide. Reactions were initiated by a hot start procedure: an initial denaturation at 95°C for 5 minutes, a further incubation at 98°C for 30 seconds, and then an addition of 2.0 U Taq-DNA polymerase (Promega). This was followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. A final extension was done at 72°C for 10 minutes. Products were loaded on a 2% agarose gel, stained with ethidium bromide, and observed under UV illumination.

Statistical analysis

To compare the DNMT levels between different sample categories, the Kruskal-Wallis test with the Bonferroni method for multiple comparisons was used. The strength of the association between the expression levels of each DNMT in different sample categories was calculated by the Spearman rank-correlation coefficient. The Mann-Whitney U test was used to compare the expression levels of DNMTs between 2 sample groups with or without p15^{INAK4B} methylation.
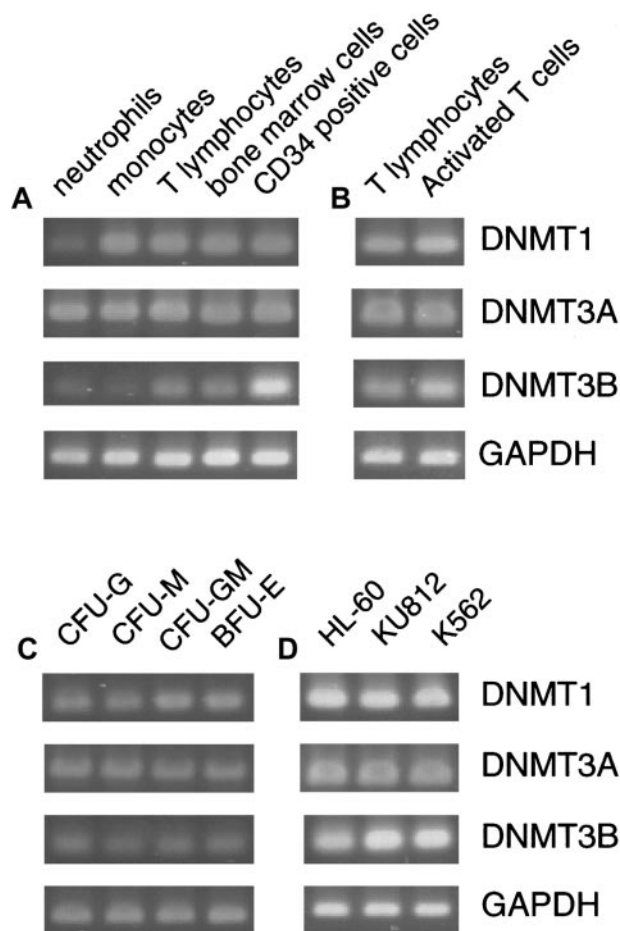

Figure 1. Expression of DNMTs in normal hematopoiesis and leukemia cell lines. Expression of DNMTs was assayed by a standard RT-PCR method in normal peripheral cells and bone marrow cells (A), resting and PHA-activated T cells (B), various colonies formed in hematopoietic progenitor assays (C), and 3 leukemia cell lines (D). With the cells other than the hematopoietic progenitor colonies, PCR amplification was performed for 26 cycles for GAPDH (a housekeeping control), 30 cycles for DNMT1 and 3A, and 35 cycles for DNMT3B (A,B,D). To amplify transcripts from the picked hematopoietic colonies, PCR was performed for 35 cycles for DNMT1, 3A, and GAPDH, and 40 cycles for DNMT3B.

Results

Expression of *DNMTs* in normal hematopoiesis and leukemia cell lines

We first examined whether the 3 *DNMT* genes were expressed in normal hematopoiesis. By a standard RT-PCR method, transcripts from all *DNMTs* were detected in peripheral neutrophils, monocytes, T lymphocytes, total bone marrow cells, CD34^{+} bone marrow cells, and various colonies derived from the CD34^{+} cells (Figure 1A-C). However, *DNMT3B* was expressed at levels lower than those of the other 2 genes, because 5 or more additional PCR cycles were required to obtain band signals comparable to those of *DNMT1* and *3A. DNMT3B* expression was especially low in differentiated cell populations such as neutrophils, monocytes (Figure 1A), and colonies of both the myeloid and erythroid lineages (Figure 1C). In contrast, total bone marrow cells and CD34^{+} cells expressed *DNMT3B* at somewhat higher levels (Figure 1A). We also examined 3 established leukemia cell lines HL60, KU812, and K562, by a standard PCR method and found that all 3 genes are respectively expressed at levels higher than those in normal hematopoiesis (Figure 1D). This suggests that overexpression of *DNMTs* might have some relevance to leukemic transformation (discussed later).

To obtain more accurate information on *DNMT* expression in normal hematopoiesis, we adopted the competitive PCR method using a synthetic mimic DNA as an internal control. Competitive PCR is an effective method to address an expression level of a certain gene[37], however, it has been reported that there could be some pitfalls for making an absolute estimation of targets.[40,41] In this study, we demonstrated the results as fold-increase or decrease relative to the mean value of the same gene in control bone marrow cells after the normalization of the level of expression of each *DNMT* in a particular sample by *GAPDH* (a housekeeping control) or *PCNA* (a cell proliferation marker) in that sample (Figures 2 and 3). Examples showing actual PCR-amplified bands from both
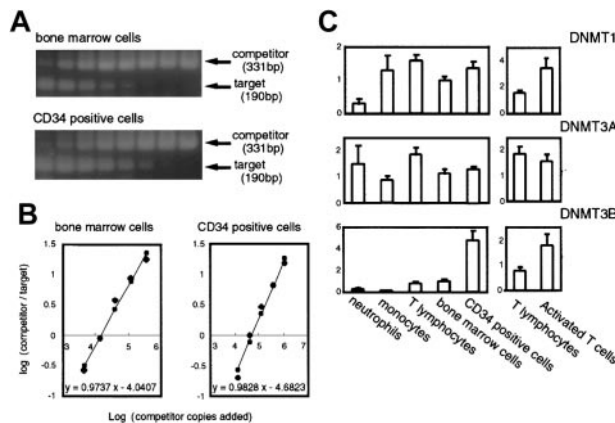

Figure 2. Expression levels of *DNMTs* in normal hematopoietic cells measured by competitive PCR. (A) Examples of the competitive PCR assay for *DNMT3B*. Phosphorimages of 2 agarose gels stained with Vistra green are shown. The lanes contained the products amplified from a constant amount of the cDNA mixture, and the competitor DNA prepared in successive 3-fold dilutions. (B) Graphs show the regression analysis of the results obtained in panel A. The initial amount of target DNA was estimated by the point where log(products from competitor/products from target) = 0. (C) Levels of the *DNMT* transcripts in normal hematopoietic cells. The number of samples are neutrophils, n = 3; monocytes, n = 3; peripheral T cells, n = 3; PHA-activated T cells, n = 3; bone marrow cells, n = 6; CD34^{+} cells, n = 5. The levels of *DNMTs* are displayed as relative values calculated such that the mean of the normal bone marrow cells would equal a value of 1 after correcting the variations by the levels of *GAPDH*.

competitors and targets (Figure 2A) and graphs used to measure the expression levels of the transcripts (Figure 2B) are shown.

As shown in Figure 2C, expression of *DNMT1* was rather uniform in various cell types except neutrophils, where its expression was relatively weak. *DNMT3A* was expressed at higher levels in T lymphocytes and neutrophils than in other cells although the differences were not so distinct (Figure 2C). It was characteristic, however, that CD34^{+} cells expressed *DNMT3B* at a level about 5-fold higher than that in normal bone marrow cells (Figures 2C and 1A). Furthermore, in contrast to the CD34^{+} cells, differentiated cells such as neutrophils and monocytes showed very low levels of *DNMT3B* expression (Figure 2C), indicating that this gene is dramatically down-regulated on hematopoietic cell differentiation, at least in some lineages. In addition, on activation with PHA, T lymphocytes showed a 2-fold increase in expression of *DNMT1* and *3B* (Figures 2C and 1B). Thus, all 3 *DNMTs* were expressed at detectable levels in normal hematopoietic cells, and their expression levels varied among cell types and differentiation conditions.

DNMTs were overexpressed in AML

To know the possible role of *DNMTs* in leukemogenesis, we analyzed their expression levels in 33 AML cases by competitive PCR. When normalized by the *GAPDH* levels, we observed 5.3-, 4.4-, and 11.7-fold mean increases in *DNMT1, 3A,* and *3B* levels, respectively, compared with their levels in normal bone marrow cells (Figures 3A-C and 4A-C), although the exact level varied from case to case (Figure 3A-C). These increases in the mean expression levels of *DNMTs* were statistically significant (Figure 4A-C). It is noteworthy that among the 3 *DNMT* genes, *DNMT3B* showed the largest fold increase (11.7-fold; Figure 4C). However, when the mean *DNMT3B* level in AML cells was compared with that in CD34^{+} cells, only 2.4-fold mean increase was observed (Figure 4C). It is also interesting that all 4 M3 AML patients expressed lower levels of all *DNMTs* compared with the other subtypes (Figure 3A-C) although the number of cases studied was rather small.

Several reports pointed out that, in colon cancer, *DNMTs* appear to be overexpressed when the levels are normalized by those of a housekeeping gene but not overexpressed when corrected by those of cell proliferation marker genes.[42,43] To exclude the possibility that the observed overexpression was due to accelerated cell proliferation of the AML cells, we corrected the levels of *DNMTs* by those of a cell proliferation marker *PCNA* (Figures 3D-F and 4D-F). As a result, the overexpression of *DNMTs* in 3 leukemia cell lines was clearly canceled (Figure 3D-F). However, the overexpression of all *DNMTs* in AML was not affected, or became even more evident, by this treatment (Figures 3D-F and 4D-F).

Because the extent of overexpression varied among the AML cases, we next investigated whether the 3 *DNMT* genes are overexpressed coordinately or independently. As shown in Figure 5, analysis of the expression levels in AML cases showed coordinate overexpression of *DNMTs*. When the values obtained by *GAPDH* correction were used, moderate correlations between *DNMT1* and *3B* ($r = 0.464$, $P = .0065$) and between *DNMT3A* and *3B* ($r = 0.568$, $P = .0006$) were observed (Figure 5B,C). Furthermore, when the values by *PCNA* correction were used, we observed strong correlations between *DNMT1* and *3A* ($r = 0.737$, $P < .0001$), *DNMT1* and *3B* ($r = 0.8339$, $P < .0001$), and *DNMT3A* and *3B* ($r = 0.794$, $P < .0001$) (Figure 5D-F).
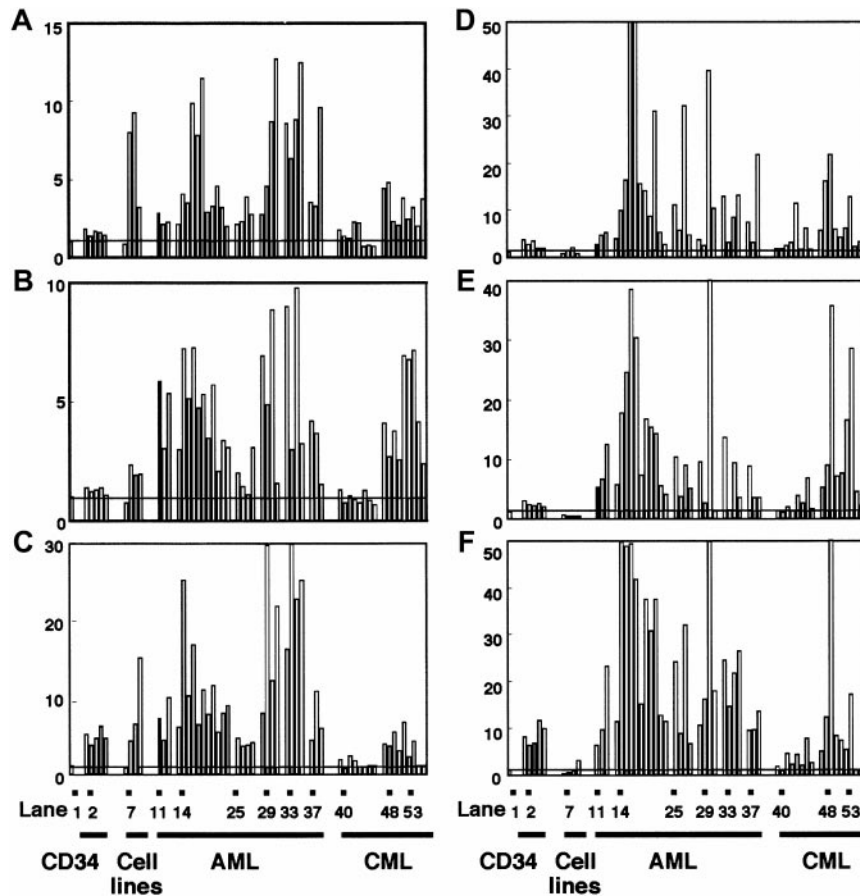
Figure 3. Expression levels of *DNMTs* in normal cells and individual leukemia cases measured by competitive PCR. Expression levels of *DNMT1* (A,D), *3A* (B,E), and *3B* (C,F) in CD34+ cells, leukemia cell lines, AML, and CML were assayed by competitive PCR. The levels of *DNMTs* were displayed as in Figure 2, but normalization was done by using the levels of either *GAPDH* (A-C) or *PCNA* (D-F). Lane 1 shows the mean value of the levels in normal bone marrow cells; lanes 2-6, CD34+ cells; lane 7, EBV-transformed B-cell line; lane 8, HL60; lane 9, KU812; lane 10, K562; lane 11, AML (FAB subtype M0); lanes 12 and 13, AML (M1); lanes 14-24, AML (M2); lanes 25-28, AML (M3); lanes 29-31, AML (M4); lane 32, AML (M5); lanes 33 and 34, AML (M6); lanes 35 and 36, AML (M7); lanes 37-39, AML (mixed lineage); lanes 40-47, CML (chronic phase); lanes 48-52, CML (myeloid crisis); lanes 53-56, CML (lymphoid crisis).

Expression of *DNMTs* in CML

Chronic myelogenous leukemia is distinct from AML with its phasic clinical course that reflects the clonal evolution of leukemia cells. We investigated the expression levels of *DNMTs* in CML cells in each clinical phase. In contrast to AML, CML cells in the chronic phase expressed *DNMTs* at levels almost equal to those in normal bone marrow cells (Figures 3A-C and 4A-C). CML cells in the acute phase showed 3.2-, 4.5-, and 3.4-fold mean increases in the expression levels of *DNMT1*, *3A,* and *3B*, respectively, compared with their levels in normal bone marrow cells (Figures 3A-C and 4A-C). As in AML, the overexpression was not abolished even when the expression levels were corrected by *PCNA* (Figures 3D-F and 4D-F). It is of interest that the overexpression of *DNMT3B* was not evident in acute phase CML when the mean expression level was compared to that in CD34+ cells (Figures 3C and 4C).

Methylation status of *p15INAK4B* and its correlation with *DNMT* overexpression in AML

It has been reported that a tumor suppressor gene, *p15INA4B*, is frequently methylated and silenced in AML.[19,20] Under the physiologic condition, expression of *p15INA4B* is induced by transforming growth factor-β (TGF-β)[44] and its protein product inhibits cell cycle progression.[45] To explore the possible role of the *DNMT* overexpression in *p15INA4B* methylation, we investigated the methylation status of the promoter region of this gene in our AML samples. By using methylation-specific PCR (Figure 6 represents the results),[39] we observed that *p15INAK4B* was methylated in 24 (72%) of the 33 cases of AML (Table 2).

Interestingly, the AML cases with methylated *p15INAK4B* had a tendency to express higher levels of *DNMT1*, *3B*, and potentially *3A*, compared to the cases with unmethylated *p15INAK4B* (Figure 7). Although a statistically significant result was obtained only for *DNMT1* ($P = .0147$), our findings suggests that the overexpressed *DNMTs* play a role in aberrant regional hypermethylation observed in AML.

Discussion

In the present paper, we have described the expression levels of the 3 *DNMTs* in normal hematopoiesis and leukemia cells. We first found that all *DNMTs* are expressed at detectable levels in normal hematopoietic cells. However, there are some gene-specific features such as the down-regulation of *DNMT3B* on differentiation of hematopoietic progenitor cells and the induction of both *DNMT1* and *3B* on activation of peripheral T cells. These findings are of interest from the viewpoint of hematopoietic cell differentiation because it has been reported that some hematopoietic cell-specific genes, such as those coding for myeloperoxidase,[46] globin,[47] c-fms,[48] and G-CSF receptor,[49] are regulated by methylation in a lineage- and differentiation-dependent manner. In addition, it is known that differentiation of naive T cells into Th1 or Th2 cells is accompanied by changes in methylation at the genes coding for cytokines such as interferon-γ, IL-4, and IL-13.[50] Furthermore, the importance of *DNMT3B* in the hematopoietic system has been also shown in ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome. ICF syndrome is a rare autosomal recessive
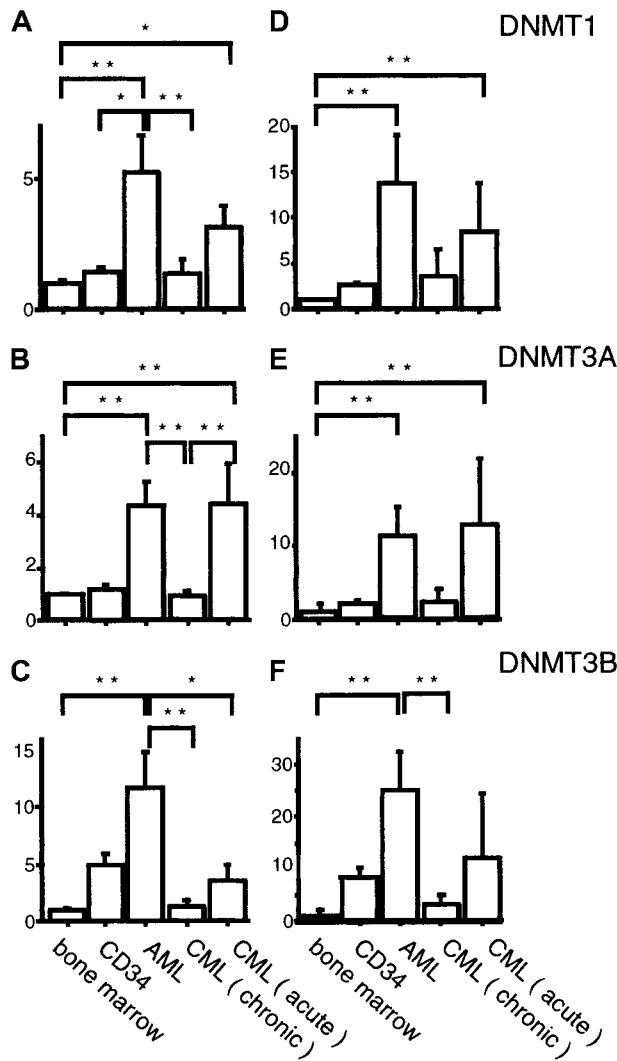
Figure 4. Summary of the expression levels of *DNMTs* in AML and CML measured by competitive PCR. Expression levels of *DNMTs*, corrected by the levels of *GAPDH* (A-C) or *PCNA* (D-F) transcripts are shown. The Kruskal-Wallis test with the Bonferroni method for multiple comparison was used to compare the levels of each *DNMT* in different sample categories. *, ** indicates statistical significance (*, $P < .05$; **, $P < .01$).

Figure 5. Correlation between the levels of different *DNMTs* in individual AML cases. The levels of *DNMTs* were corrected by *GAPDH* (A-C) or *PCNA* (D-F) and the correlation coefficients were calculated by the Spearman rank-correlation method.

disorder characterized by variable immunologic defects, such as a decreased number of lymphocytes and variable reduction in serum immunoglobulin levels,[51,52] and recently, mutations were identified in *DNMT3B* in some ICF patients.[28,53,54] Although how the defects in *DNMT3B* cause immunologic disorders remains unknown, it has been proposed that a loss of the proper methylation pattern may impair stage-specific gene regulation, resulting in interference with lymphocyte maturation. Therefore, the expression of *DNMTs* in hematopoietic cells is thought to have important roles for the differentiation process of blood cells.

We next found that all *DNMTs* are substantially overexpressed in most cases of AML when compared to normal bone marrow cells. Also, all *DNMTs* are expressed at higher levels in AML cells than in CD34+ cells, although this is statistically significant only for *DNMT1*. In addition, this overexpression observed in AML is not due to the accelerated proliferation of the leukemic cells because the data are basically unaffected even after corrected by the levels of a cell proliferation marker,
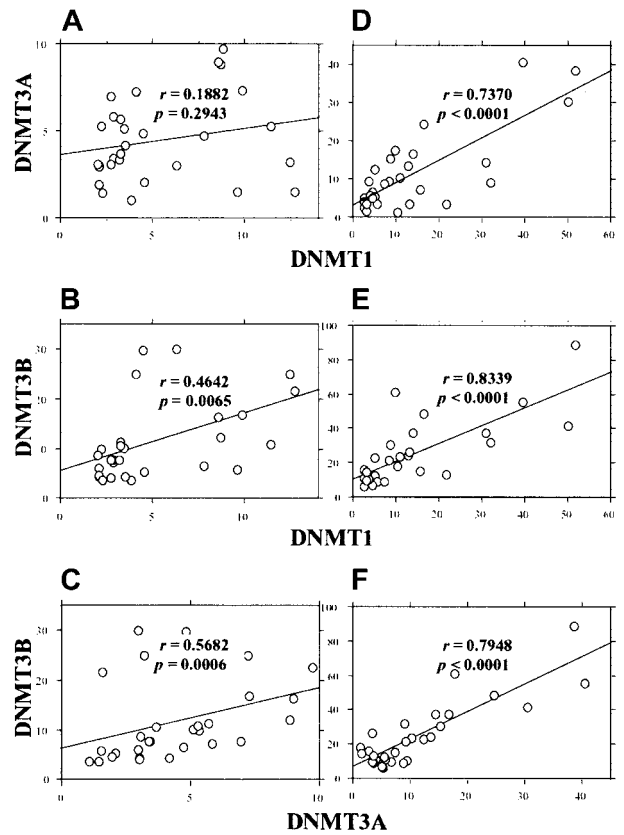
PCNA. Furthermore, *DNMTs* were coordinately, not independently, up-regulated in AML cells. These results suggest that AML cells may possess higher maintenance and de novo methyltransferase activities than normal hematopoietic cells and these methyltransferases may contribute to the leukemogenesis by inducing aberrant methylation. Among the FAB subtypes, however, M3 (4 cases) showed only a minimal increase in the expression levels of *DNMTs*. Although this observation has to be confirmed in other M3 cases, it suggests that the possible roles of *DNMTs* in leukemogenesis vary among subtypes of AML.

In contrast to AML, CML shows phase-dependent expression of *DNMTs*. In the chronic phase, levels of *DNMTs* are not significantly different from those in normal bone marrow cells. However, CML cells in the acute phase expressed higher levels of *DNMTs* than normal bone marrow cells. *DNMT1* and *3A*, but not *3B*, are also expressed at higher levels in acute phase CML cells than in CD34+ cells. Thus, the expression pattern of
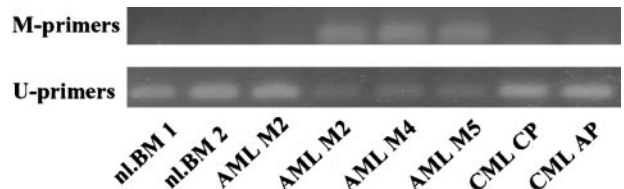

Figure 6. Methylation-specific PCR for the p15^INAK4B promoter in normal bone marrow cells and leukemia samples. Three of the 4 AML cases gave the methylation-specific PCR band, whereas normal bone marrow cells and CML cases showed only the unmethylated DNA-specific band. M-primer indicates primers specific for methylated DNA; U-primers, primers specific for unmethylated DNA.

DNMTs in CML was different from that of AML, suggesting that there might be different roles of *DNMTs* for leukemogensis. The near-normal expression of *DNMTs* in chronic phase is compatible with the fact that the increased cell population in this phase is seemingly mature myeloid cells with essentially normal morphology and function. These results also suggest that *DNMTs* are not involved in the onset or maintenance of the chronic phase but could be associated with blast crisis. Because the *ABL1* promoter nested within the *BCR-ABL* fusion gene has been demonstrated to be methylated with disease progression,[17,18] it is conceivable that the overexpressed *DNMTs* methylate the *ABL1* promoter region, and this in turn leads to clonal evolution. In the future study, it is important to know whether *DNMTs* are overexpressed prior to blastic crisis in progenitor cells of CML.

The overexpressed *DNMTs* could be involved in the development of leukemia by inducing hypermethylation of tumor suppressor genes. We focused on *p15INAK4b* because this gene frequently becomes inactivated with disease progression in acute leukemia and MDS by hypermethylation of its 5′ CpG island.[19-21] The protein product of *p15INAK4B* is a cell cycle regulator induced by TGF-β[44] and involved in the inhibition of G_1 phase progression.[45] It has been reported that a loss of sensitivity of leukemia cells to growth inhibition by TGF-β is correlated with the inactivation of *p15INAK4B*.[19] Consistent with the previous reports, this gene was methylated in 23 of the 33 (72%) cases with AML. Furthermore, we observed that the AML cases with hypermethylated *p15INAK4B* had a tendency to show higher levels of *DNMT1* and *3B* although the correlation was statistically significant only for *DNMT1*. These results suggest that up-regulated *DNMTs* could have roles for *p15INAK4B* methylation, although further studies will be required to test this possibility.

We studied the expression levels of *DNMTs* to gain a clue to their roles in aberrant regional hypermethylation in leukemia. Although *DNMTs* were substantially overexpressed in leukemia cells in a leukemia type- and stage-specific manner, the mechanism responsible for aberrant methylation remains to be solved. As for *DNMT1*, it has been proposed that a loss of p21 function in cancer facilitates the formation of DNA-DNMT1-PCNA complexes, resulting in aberrant methylation of CpG island.[55,56] However, the mechanisms of recently identified de novo methyltransferases, *DNMT3A* and *3B*, for aberrant methylation are largely unknown. In the study of *D. melanogaster*, induced expression of both *Dnmt1* and *Dnmt3a* cooperated to establish and maintain methylation, resulting in increased methylation.[27] As we have also observed coordinate overexpression of *DNMTs* in AML, there is a possibility that *DNMTs* act cooperatively in leukemia for the establishment of aberrant hypermethylation. It should also be noted that *DNMT3B*
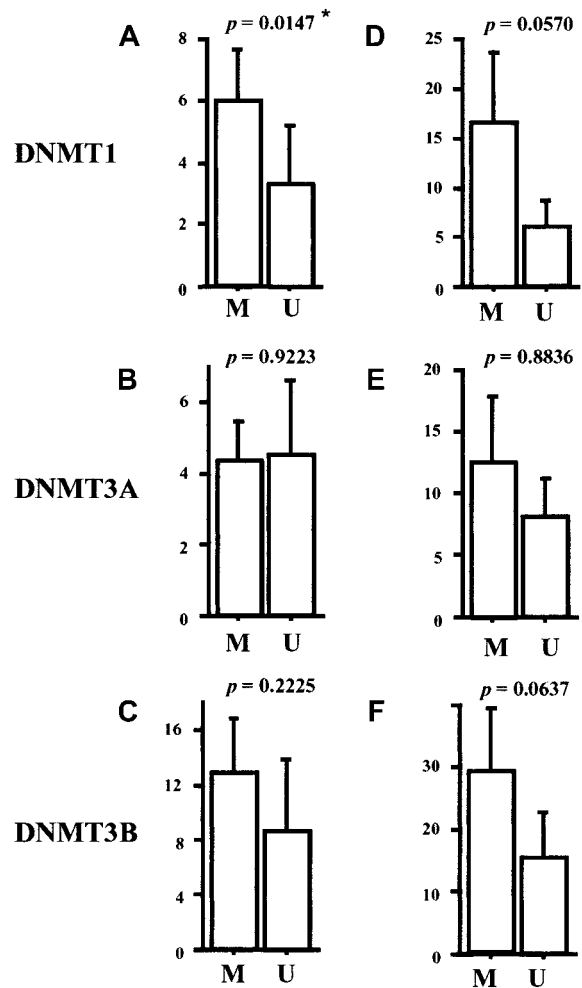

Figure 7. Comparisons of the expression levels of *DNMTs* between the AML groups with or without *p15INAK4B* methylation. The levels of *DNMTs* were corrected by *GAPDH* (A-C) or *PCNA* (D-F). The level of *DNMT1* corrected by *GAPDH* is significantly higher in the group with methylated *p15INAK4B* than in the group with unmethylated *p15INAK4B* (A) (P = .0147). Comparisons were made by the Mann-Whitney *U* test. M indicates methylated cases; U, unmethylated cases.

has at least 4 alternatively spliced transcripts, which are expressed in a tissue-specific manner.[25] Because 2 of the splice variants lack the highly conserved sequence motifs in the methyltransferase catalytic domain, it will be important to know which splice variants are overexpressed in leukemia.

In conclusion, the *DNMT* genes were expressed constitutively in normal hematopoiesis and were overexpressed in some types of leukemia. The overexpressed *DNMTs* with maintenance or de novo methyltransferase activity may contribute to the pathogenesis of leukemia by inducing aberrant regional hypermethylation. Further studies will be needed to clarify the precise pathogenetic roles of *DNMTs* in clonal evolution of leukemia and to develop therapeutic alternatives designed to suppress abnormal hypermethylation.

Table 2. Summary of the methylation status of p15INAK4B in AML

FAB type	No. of patients	No. of methylated cases (%)
M0	1	0 (0)
M1	2	1 (50)
M2	11	8 (72)
M3	4	3 (75)
M4	3	3 (100)
M5	1	1 (100)
M6	2	2 (100)
M7	2	1 (50)
Mix	3	2 (66)
Total	29	21 (72)

Acknowledgment

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