RAPID COMMUNICATION

Hypermethylation of the \( p15^{INK4B} \) Gene in Myelodysplastic Syndromes

By Toshiki Uchida, Tomohiro Kinoshita, Hirokazu Nagai, Yohsuke Nakahara, Hidehiko Saito, Tomomitsu Hotta, and Takashi Murate

Previous studies have shown that the cyclin-dependent kinase inhibitor (CDKI) genes \( p15^{INK4B} \) and \( p16^{INK4A} \) are frequently inactivated by genetic alterations in many malignant tumors and that they are candidate tumor-suppressor genes. Although genetic alterations in these genes may be limited to lymphoid malignancies, it has been reported that their inactivation by aberrant methylation of 5' CpG islands may be involved in various hematologic malignancies. In this study, we investigated the \( p15^{INK4B} \) and \( p16^{INK4A} \) genes to clarify their roles in the pathogenesis of myelodysplastic syndrome (MDS). Southern blotting analysis showed no gross genetic alterations in either of these genes. However, hypermethylation of the 5' CpG island of the \( p15^{INK4B} \) gene occurred frequently in patients with MDS (16/32 [50%]). Interestingly, the \( p15^{INK4B} \) gene was frequently methylated in patients with high-risk MDS (refractory anemia with excess blasts [RAEB], RAEB in transformation [RAEB-t], and overt leukemia evolved from MDS; 14/18 [78%]) compared with patients with low-risk MDS (refractory anemia [RA] and refractory anemia with ring sideroblast [RARS]; 1/12 [8%]). Furthermore, methylation status of the \( p15^{INK4B} \) gene was progressed with the development of MDS in most patients examined. In contrast, none of the MDS patients showed apparent hypermethylation of the \( p16^{INK4A} \) gene. These results suggest that hypermethylation of the \( p15^{INK4B} \) gene is involved in the pathogenesis of MDS and is one of the important late events during the development of MDS.

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MATERIALS AND METHODS

Patient profiles and preparation of DNA. We analyzed a total of 32 patients with MDS: 8 with RA, 4 with RARS, 2 with CMMoL, 6 with RAEB, 2 with RAEB-t, and 10 with overt leukemia (OL) evolved from MDS, who were diagnosed at our hospital and affiliated hospitals. Most samples were obtained at the time of initial diagnosis, and additional samples were also obtained from 7 patients at other times. We also analyzed 5 patients with aplastic anemia (AA), 1 patient with panmyelopenia due to liver cirrhosis, and 3 healthy volunteers (HV) as controls. Their samples were mostly obtained by bone marrow (BM) aspiration or from peripheral blood (PB) in healthy volunteers and some cases of OL, after informed consent was obtained. We separated mononuclear cells (MNC) by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. We obtained polymorphonuclear cells (PMN) from 2 patients and T lymphocytes from 1 patient from the PB according to the method described previously. DNA extraction from clinical samples and cell lines (ML1, HL60, and Raji) was performed by the standard procedure.

Southern blotting analysis. Five-microliter aliquots of DNA were digested with 20 U of HindIII (Boehringer Mannheim, Mannheim, Germany) and 10 U of Eco52I (Takara, Kyoto, Japan), which is an isoschizomer of EcoRI, digested with HindIII and methylation-sensitive Eco52I alone, or with HindIII and Eco52I studied, at 37°C for 12 hours, followed by an additional 10 U of Eco52I at 37°C for 12 hours. Digested genomic DNA was separated by electrophoresis through 0.7% agarose gels and blotted onto nylon membranes (Hybond N; Amersham, Amersham, Buckinghamshire, UK). The p15\textsubscript{INK4B} cDNA probe was produced by reverse transcription-polymerase chain reaction (RT-PCR) in a Thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). RT-PCR was performed essentially as described previously as follows: as primers designed to amplify the region covering the entire sequence of the exon 1 of p15\textsubscript{INK4B} (Fig 1). Their sequences were as follows: p15C2, TCCCAAGAGCAATCCAGGGC; and p15C3, GCCTCCGAAAACGGTTGACT. A p16\textsubscript{INK4A} DNA probe, PE1, was produced by PCR using the reported primers. The primers were used for detection of the HindIII and Eco52I double-digested fragments of the p15\textsubscript{INK4B} and p16\textsubscript{INK4A} genomic DNA, respectively. The probe was labeled with [\alpha\textsuperscript{32}P]dCTP by the random primer method and hybridization was performed for 2 hours at 65°C using Rapidhyb buffer (Amersham). After washing at high stringency with 0.1× SSC and 0.1% sodium dodecyl sulfate at 65°C, membranes were exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at -70°C. To exclude the possibility of incomplete digestion, all samples showing methylated patterns were examined at least twice. Subsequently, we measured the intensity of the bands that reflect methylated and unmethylated DNA, respectively, to evaluate the methylation intensity of the p15\textsubscript{INK4B} gene calculated as follows. Methylation Intensity (MI; %) = (Sample 2.8-kb Band × 100)/ (Sample 2.8-kb Band + Sample 2.2-kb Band × Control 2.8-kb Band)

PCR-based methylation assay. We examined the same restriction site as that analyzed by Southern blotting. One-microliter aliquots of DNA were digested with 4 U of Eco52I at 37°C for 12 hours, followed by an additional 4 U of Eco52I at 37°C for 12 hours. Fifty-nanogram aliquots of the digested DNA were amplified with primers flanking the restriction sites in either p15\textsubscript{INK4B} or p16\textsubscript{INK4A} genes (Fig 1 and Table 1). PCR conditions were as follows: 27 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes in p15\textsubscript{INK4B}; and 27 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes in p16\textsubscript{INK4A}}, in the presence of 5% dimethylsulfoxide. PCR products were directly loaded onto 3% NuSieve agarose gels (FMC Bioproducts, Rockland, MD), stained with ethidium bromide, and visualized under UV illumination.

PCR-mediated single-strand conformation polymorphism (PCR-SSCP) analysis. Analysis by PCR-SSCP was performed essentially as described previously. We analyzed the whole coding region of the p15\textsubscript{INK4B} gene using the primers shown in Table 1. Thirty-five cycles of PCR at 94°C, 62°C, and 72°C for 0.5, 0.5, and 1 minute, respectively, were performed in the presence of 5% dimethylsulfoxide. The products were diluted 100-fold with 98% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol and heated at 94°C for 4 minutes. Aliquots of 2 μL were then applied to 5% polyacrylamide gels with 5% glycerol. After electrophoresis at room temperature for 2 hours at a constant power of 40 W with vigorous air cooling, the gels were dried on Whatman 3MM paper (Whatman, Maidstone, UK) and exposed to x-ray film for appropriate times at -70°C.

DNA sequencing. Nucleotide sequences of all samples showing mobility shifts and a sample from control B lymphocytes were determined. Briefly, a small area of the gel corresponding to the position of the band was cut out, and single-stranded DNA from the dried gel was eluted into 50 μL of distilled water. These single-stranded DNA samples were sequenced using an ABI PRIZM genetic analyzer (Perkin Elmer, Foster City, CA) according to the manufacturer’s protocol.
Table 1. Nucleotide Sequences of the Primers Used in This Study

<table>
<thead>
<tr>
<th>Method</th>
<th>Target Gene</th>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>PCR-based methylation assay</td>
<td>p15</td>
<td>p15C2</td>
<td>TCCCAGAGAAGCATCCAGGCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p15C4</td>
<td>TCAGCTTCAATTACCCTCCCG</td>
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<td>p16</td>
<td>p16C1</td>
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<tr>
<td></td>
<td></td>
<td>1108R</td>
<td>GGCGCTACGTAATCCCAATTCC</td>
</tr>
<tr>
<td>PCR-SSCP</td>
<td>p15</td>
<td>p15C9</td>
<td>AGGAAGGAGAGATCGGCCGG</td>
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<tr>
<td></td>
<td></td>
<td>p15C8</td>
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<td>p15C6</td>
<td>ATCTCCCATACCTGGGCC</td>
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<td></td>
<td></td>
<td>p15C5</td>
<td>AATAAAGTCGTTGTGGGCG</td>
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RESULTS

Methylation analysis of the p15\textsuperscript{INK4B} and p16\textsuperscript{INK4A} genes.

We analyzed the methylation status of the promoter regions of the p15\textsuperscript{INK4B} and p16\textsuperscript{INK4A} genes by Southern blotting in a total of 53 samples obtained from patients with MDS and other diseases and from healthy volunteers. Methylation status in patients with MDS was also analyzed by PCR-based methylation analysis. Only MDS patients showed hypermethylation of the p15\textsuperscript{INK4B} gene (16/32 [50%]; Figs 2 and 3). Although hypermethylation of the p15\textsuperscript{INK4B} gene was detected in most clinical subtypes of MDS, it was more frequently methylated in the high-risk subtype (RAEB, RAEB- t, and OL) evolved from MDS; 14/18 [78%]) than the low-risk subtype (RA and RARS; 1/12 [8%]; Fig 4). MI of the p15\textsuperscript{INK4B} gene in the high-risk subtype was significantly higher than that of the low-risk subtype as determined by the Mann-Whitney test ($P = .002$).

We also analyzed the methylation status of the promoter region of the p16\textsuperscript{INK4A} gene. However, no apparent hypermethylation of p16\textsuperscript{INK4A} gene was detected in patients with MDS or controls (Fig 3).

We analyzed the methylation status of the PMN cell population in 2 patients in whom the p15\textsuperscript{INK4B} gene was intensively hypermethylated. In patient no. 26, the PB-PMN cell population showed the methylated pattern as well as the BM-MNC population. In patient no. 3, the PMN cell population at complete remission showed the unmethylated pattern. The T-lymphocyte population at her leukemic phase also showed the unmethylated pattern (Fig 5).
Fig 3. PCR-based methylation assay of the p15\textsuperscript{INK4B} and p16\textsuperscript{INK4A} genes. Results of various clinical subtypes are shown: no. 1, RA (patient no. 32); no. 2, RA (patient no. 2); no. 3, RARS (patient no. 31); no. 4, RAEB (patient no. 8); no. 5, OL (patient no. 3); no. 6, OL (patient no. 4); no. 7, OL (patient no. 26). (+), DNA digested by the methylation-sensitive restriction enzyme Eco52I; (−), undigested DNA. DNAs from some clinical samples digested by Eco52I were amplified by the primer set flanking the Eco52I site in the exon 1 of p15\textsuperscript{INK4B} gene, suggesting that the p15\textsuperscript{INK4B} gene is methylated; whereas none of clinical samples were amplified by the primer set for the p16\textsuperscript{INK4A} gene, suggesting that the p16\textsuperscript{INK4A} gene is not methylated. In ML1, the p16\textsuperscript{INK4A} gene was deleted homozygously.\textsuperscript{12}

Sequential analysis of methylation status of the p15\textsuperscript{INK4B} gene in the same patients with MDS. We investigated the methylation status sequentially in 7 of 32 MDS patients (Figs 6 and 7). Two patients showed intensive hypermethylation of the p15\textsuperscript{INK4B} gene since their first presentation (patients no. 7 and 23). One patient with RA at presentation showed apparent hypermethylation of the p15\textsuperscript{INK4B} gene. He progressed to RAEB, but his methylation pattern did not change at that time (patient no. 2). In patient no. 26, who was initially diagnosed as RA and evolved to OL over a period of 23 months, methylation status progressed at leukemic phase. Three patients with OL evolved from RAEB and RAEB-t also showed progressed methylation status of the p15\textsuperscript{INK4B} gene compared with those at their initial presentation (patients no. 15, 29, and 30). Thus, the increase of methylation status of the p15\textsuperscript{INK4B} gene observed in patients corresponded to the progression of MDS.

Analyses of structural alterations of the p15\textsuperscript{INK4B} and the

Fig 4. MI of the p15\textsuperscript{INK4B} gene in each clinical subtype. Each point represents the MI of the p15\textsuperscript{INK4B} gene of one sample, whereas the horizontal lines in each data set represent the mean ± 1 SD.

Fig 5. Methylation status in PMN and T lymphocytes. (A) Methylation status of the p15\textsuperscript{INK4B} gene of BM-MNC at leukemic phase and those of PB-PMN cells at CMMoL phase of patient no. 26 are shown. (B) The methylation status of the p15\textsuperscript{INK4B} gene of various cell populations from patient no. 3 is shown. PB-T lymphocytes at leukemic phase and PB-PMN cells at complete remission showed the unmethylated p15\textsuperscript{INK4B} gene pattern.
HYPERMETHYLATION OF P15 IN MDS

Fig 6. Intensive hypermethylation of the p15INK4B gene associated with the development of MDS. Changes of the methylation status of the p15INK4B gene in patients no. 15, 26, and 30 are shown. Because the 2.8-kb band was intensified or the 2.2-kb band was faint compared with those at initial analysis in these patients, hypermethylation of the p15INK4B gene may have progressed with the development of MDS.

p16INK4A genes. We analyzed the gross structural alterations of the p15INK4B and the p16INK4A genes by Southern blotting. No homozygous deletions were detected in these genes in any of patients or normal volunteers examined. For the p15INK4B gene, we also used PCR-SSCP analysis to detect small alterations. Two of 32 patients with MDS and the HL60 cell line showed mobility shifts on analysis of exon 2 of p15INK4B (data not shown). Subsequent sequence analysis in these 2 patients identified the same nucleotide change due to polymorphism as described previously in HL60 (C to A at position -27 in intron 1 near the 3' acceptor site). No apparent structural alterations were detected in the p15INK4B or p16INK4A genes in MDS in this study, as described previously in myeloid malignancies. 

DISCUSSION

MDS is a heterogeneous disease characterized by chronic cytopenia, BM hyperplasia, and dysmyelopoietic abnormalities among BM precursors. Patients with MDS often undergo leukemic transformation and die over a short period because of resistance to chemotherapy and various lethal complications. Although chromosomal abnormalities have been reported in approximately 50% of patients with MDS, of the enormous number of possible target genes, only the ras and fms genes have been well investigated. The mechanisms responsible for the development of MDS have not been evaluated in detail. 

A lot of studies have confirmed that the p16INK4A gene is a really a tumor-suppressor gene. However, they also raised the doubts regarding whether the p15INK4B gene is a target in tumorigenesis, because sole deletions and point mutations of the p15INK4B gene were rare. In this study, we investigated the p15INK4B and p16INK4A genes in MDS patients and detected frequent aberrant hypermethylation of the p15INK4B gene in half of the patients examined. We showed the importance of the p15INK4B gene in the pathogenesis of MDS.

A recent study indicated that the inactivation patterns of the p15INK4B and p16INK4A genes are characteristic in different hematologic malignancies. In most hematologic malignancies, either or both of these genes may be inactivated. Our results suggested that the inactivation pattern in MDS is the same as that of AML. Myeloid clonal disorders, with the exception of CML, may be characterized by this distinct pattern, ie, inactivation of p15INK4B gene by hypermethylation and intact p16INK4A gene.

Multistep pathogenesis is the most likely mechanism of the disease progression of MDS. Because it was shown that MDS patients with complex chromosomal abnormalities tend to evolve to AML and have poor prognosis, the accumulation of alterations in oncogenes and tumor-suppressor genes may cause development of MDS. Because the level of transcriptional repression is clearly dependent on methylation density and increasing methylation of tumor-suppressor gene CpG islands may promote the process of progression, we compared the frequency of the p15INK4B gene methylation in each clinical subtype and also compared the methylation intensity at the first presentation with those at the terminal stage in the same patient.
methyltransferase and CcH3-sensitive PCR have been reported as useful methods for detection of methylation.\textsuperscript{11,31,32} We evaluated the correlation between changes in methylation status and the development of MDS according to the results of Southern blotting. A previous report evaluated the methylation status by the density ratio of methylated over unmethylated DNA.\textsuperscript{13} In the present study, we calculated the ratio with a minor modification because the p15 probe had a tendency to hybridize more weakly to the 2.2-kb DNA than to the 2.8-kb DNA, as also shown previously.\textsuperscript{12}

As shown in Figs 2 and 3, hypermethylation of the \textit{p15}\textsuperscript{INK4B} gene was more frequent in the high-risk group than in the low-risk group. Furthermore, methylation status of the \textit{p15}\textsuperscript{INK4B} gene progressed with the development of MDS in most patients examined (Figs 6 and 7). These results suggest that hypermethylation of the \textit{p15}\textsuperscript{INK4B} gene adds the growth advantage to MDS, resulting in leukemic transformation.

We also evaluated the methylation status of other cell populations in patients whose MNCs showed hypermethylation of the \textit{p15}\textsuperscript{INK4B} gene (Fig 5). In patient no. 26, PB-PMN cells at CMMoL phase showed the methylated pattern as well as BM-MNCs at the leukemic phase, which evolved over a period of 1 month. In patient no. 3, T lymphocytes in PB at the leukemic phase and PB-PMN cells at the CR phase showed the unmethylated pattern, in contrast to BM-MNCs at the leukemic phase. Because it was reported that the clonality of MDS may not be involved in T lymphocytes and that most MDS patients may achieve a polyclonal remission,\textsuperscript{34,35} our results suggest that hypermethylation of the \textit{p15}\textsuperscript{INK4B} gene may be involved in a clonal population, but not in a polyclonal (normal) population. It may occur de novo during the process of disease progression of MDS.

Survival in patients with MDS paralleled the likelihood of development of leukemia.\textsuperscript{19} Even in RA, approximately 10% of patients evolved to OL and had poor prognosis. So, it is important to determine the prognostic index for leukemic transformation. In present study, we analyzed 10 samples obtained at RA state. Two of them progressed to RAEB and OL in a short period and died (patients no. 2 and 26; they were included in RAEB and OL subtypes, respectively). These 2 patients showed apparent hypermethylation of the \textit{p15}\textsuperscript{INK4B} gene since RA state (Fig 2B, lane 6, and Fig 6, respectively). In contrast, the remaining 8 patients showed intact \textit{p15}\textsuperscript{INK4B} gene, except for only 1 patient (patient no. 16), and retained the stable clinical status. These results indicate that progression of MDS is rare in RA patients with intact \textit{p15}\textsuperscript{INK4B} gene, and hypermethylation of the \textit{p15}\textsuperscript{INK4B} gene may be a useful prognostic marker in RA.

\textbf{ACKNOWLEDGMENT}

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