Distinct Patterns of Inactivation of p15INK4B and p16INK4A Characterize the Major Types of Hematological Malignancies1

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Abstract

Inactivation of the cyclin-dependent kinase inhibitors p16INK4A and p15INK4B are frequent alterations in neoplasia, often resulting from homozygous deletion or promoter region hypermethylation. We have analyzed both modes of inactivation of p15INK4B and p16INK4A in the major types of adult and pediatric hematological malignancies. Hypermethylation of p15INK4B, without alteration of p16INK4A, was an almost universal finding in adult acute myelogenous leukemia, and occurred very frequently in adult acute lymphocytic leukemia and pediatric acute myelogenous leukemia and acute lymphocytic leukemia. In contrast, neither p15INK4B nor p16INK4A were inactivated in any stage of chronic myelogenous leukemia. Hypermethylation of p16INK4A, often without alterations of p15INK4B, was found in non-Hodgkin's lymphoma and was much more frequent in cases with high-grade than low-grade histology. Enriched normal bone marrow stem cells had no detectable promoter region methylation of these genes, as analyzed by a newly developed PCR method. Remarkably distinct patterns of inactivation of p15INK4B and p16INK4A characterize different types of hematological malignancy, and alterations in these tumor suppressor genes are one of the most common alterations in hematological malignancies.

Introduction

Loss of cell cycle control is a frequent alteration in neoplasia, and changes in the cyclin D/retinoblastoma gene (Rb) pathway are often involved. Rb is fundamentally important for the G1-S transition of the cell cycle and is inactivated in many forms of human cancer. However, within the hematological malignancies, inactivation of Rb is infrequent (1), suggesting that other genes involved in cell cycle control might be altered. The cyclin-dependent kinase inhibitor p16INK4A normally inhibits the phosphorylation of Rb by cyclin D and cyclin-dependent kinases 4 and 6 (2). The p16INK4A gene, located at chromosome 9p21, is lost through homozygous deletion in many malignancies (3, 4), including hematological malignancies (4). Homozygous deletions usually include the adjacent gene p15INK4B, which encodes another cyclin-dependent kinase inhibitor (5). Most evidence has suggested that p16INK4A is the target of inactivation at 9p21, because inactivating point mutations of p16INK4A, although infrequent, are found in some tumors (3, 6) and in the germ line of patients with familial melanoma (4, 7), whereas point mutations have been rare in hematological malignancies (20), suggesting that inactivation of p16INK4A is primarily limited to ALL3 and non-Hodgkin's lymphoma (10, 13). Although ALL has among the highest reported rates of homozygous deletion at 9p21 in primary neoplasia (6, 9-11, 14), assigning a tumor suppressor role to p16INK4A alone in these malignancies is difficult, because most cases include both p15INK4B and p16INK4A in the deleted region (15). A subset of malignancies, including ALL, have selective homozygous deletion of p16INK4A (16, 17), but these malignancies generally represent only a minority of those with homozygous deletion.

Our recent findings demonstrate that inactivation of p15INK4B and p16INK4A is a much more frequent event in neoplasia than indicated solely by the presence of homozygous deletions. We and others have observed that aberrant methylation of 5' CpG islands in these genes represents an alternative mechanism to homozygous deletion and coding region mutations for inactivation (18-22). For each gene, this hypermethylation is found in neoplastic cells and is associated with transcriptional loss that was reversed by treatment with the DNA-demethylating agent 5-aza-2'-deoxycytidine (18-20, 22). Unlike homozygous deletions, inactivation of these genes through promoter region hypermethylation is selective, typically limited to only p15INK4B or p16INK4A (20), and usually limited to only p16INK4A in solid tumors (20). However, in a small series of acute leukemias, selective hypermethylation of p15INK4B, without hypermethylation of p16INK4A, was observed (20), suggesting that inactivation of p15INK4B might be an important event in the development of hematological malignancies. To further define the importance of both p15INK4B and p16INK4A in the development of hematological malignancies, we examined the inactivation of these genes in a large number of primary leukemias and lymphomas. Our study demonstrates that by examining both homozygous deletion and hypermethylation as inactivating events, important selective roles for p15INK4B and p16INK4A can be defined for subtypes of hematological malignancy, and that one, and in some tumor types, both, genes are virtually always involved.

Materials and Methods

Samples and DNA Preparation. High molecular weight DNA was isolated from primary leukemia and lymphoma as described previously (19). Classification of leukemia or lymphoma phenotype was based on the clinical diagnosis. B precursor versus T precursor ALL was determined by surface markers (B with positive staining for CD19, CD10, and HLA-DR and negative for CD4, CD7, and CD34). NHL was divided into low grade (follicular in seven cases, follicular and diffuse in four cases) or high grade (diffuse large cell lymphoma in four cases, Ki-1 in one case, and immunoblastic in one case). Burkitt's lymphoma samples were sporadic (3) and endemic (5) tumors. Fifteen of the 175 samples included in Table 1 were reported previously (20).

Southern Hybridization. Five µg of genomic DNA from cancer cell lines, normal tissues, and primary neoplasia were isolated and digested overnight.
with excess restriction endonuclease (HindIII and EagI) and hybridized as described (18, 20). All blots were first hybridized to the p15INK4B exon 1 probe and then stripped and reprobed with the p16INK4A exon 1 probe.

MSP. Bisulfite treatment of DNA and MSP were performed using primer pairs for p15INK4B (p15U and p15M) and p16INK4A (p16U and p16M) as described (23). DNA isolated from 50,000 bone marrow cells, fractionated as described below, was used. Controls without DNA and positive controls for U and M reactions were performed for each set of PCR. Ten µl of each PCR reaction was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Stem Cell Isolation. Human bone marrow cells were obtained from normal volunteers, and the small-sized cells containing enriched populations of hematopoietic stem/progenitor cells were collected at 25 ml/min (FR25) as described (24). These cells were subjected to staining with an antibody to the CD34 molecule to further enrich for stem/progenitor cells as described (24). These cells were then methylated at the 5' CpG island of the p15INK4B gene, but the remaining 70 AML samples with p15INK4B methylation were not methylated at the 5' CpG island of p16INK4A.

In contrast to the results in acute myelogenous leukemias, we found no alterations of either p15INK4B or p16INK4A in any patient at any stage of CML. There were no homozygous deletions or cases with hypermethylation in 21 samples from 20 patients with CML (11 in chronic phase, 2 in accelerated phase, and 8 in blast crisis; 1 patient had samples from both accelerated phase and blast crisis). We did detect, in many cases of CML, altered size of a cross-hybridizing fragment to the p15INK4B probe, which does not represent p15 (20). The significance of this finding is presently unknown.

Results

Selective Hypermethylation of p15INK4B Characterizes AML but not CML. Restriction of genomic DNA from primary malignancies with the flanking enzyme HindIII, plus the methylation-sensitive enzyme EagI, upon Southern hybridization with either a p15INK4B exon 1 or p16INK4A exon 1 probe, monitors the methylation status of CpG islands in the 5' regions of these genes, as well as detecting homozygous deletion (20). As shown previously by this technique, methylation of these sites does not occur in normal tissue and is associated with transcriptional loss of the associated gene in neoplasms (18–20). We observed no homozygous deletions of p15INK4B or p16INK4A in any of 87 cases of AML examined, confirming the results of others that homozygous deletion of these genes is an infrequent event in AML (10–13). However, we found that hypermethylation of p15INK4B occurred in 88% of adults and 67% of children with AML (Fig. 1; summarized in Table I). In one of these cases, a pediatric patient, the p16INK4A gene was also methylated, but the remaining 70 AML samples with p15INK4B methylation were not methylated at the 5' CpG island of p16INK4A.

Both p15INK4B and p16INK4A Are Frequently Inactivated in ALL. In adult ALL, hypermethylation of p15INK4B was evident in five of seven cases, but there were no homozygous deletions of either

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>HD&quot;</th>
<th>Meth&quot;</th>
<th>p15 or p16</th>
<th>p15 and p16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult AML (60)</td>
<td>0</td>
<td>53 (88%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pediatric AML (27)</td>
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<td>18 (67%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adult ALL (7)</td>
<td>0</td>
<td>5 (71%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ped B precursor AML (23)</td>
<td>4 (17%)</td>
<td>11 (48%)</td>
<td>3 (13%)</td>
<td>0</td>
</tr>
<tr>
<td>Ped T precursor AML (12)</td>
<td>4 (33%)</td>
<td>6 (50%)</td>
<td>9 (75%)</td>
<td>12 (100%)</td>
</tr>
<tr>
<td>Adult CML (21)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NHL-low grade (11)</td>
<td>0</td>
<td>1 (9%)</td>
<td>1 (99%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>NHL-high grade (6)</td>
<td>0</td>
<td>1 (100%)</td>
<td>5 (83%)</td>
<td>5 (83%)</td>
</tr>
<tr>
<td>Burkitt’s lymphoma (8)</td>
<td>0</td>
<td>4 (50%)</td>
<td>6 (75%)</td>
<td>7 (88%)</td>
</tr>
</tbody>
</table>

"HD, homozygous deletion; Meth, hypermethylated in the 5' promoter region.

Table I Inactivation of p15INK4B and p16INK4A in 175 cases of primary hematological malignancy, according to clinical diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>p15INK4B</th>
<th>p16INK4A</th>
<th>Inactivation</th>
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<tbody>
<tr>
<td>AML</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B-ALL</td>
<td>4</td>
<td>0</td>
<td>67%</td>
</tr>
<tr>
<td>T-ALL</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>0</td>
<td>0</td>
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</table>

Fig. 1. Southern Analysis of p15INK4B (a) and p16INK4A (b) in primary hematological malignancies. The numbered lanes in a and b correspond to DNA from the same sample. The clinical diagnosis of malignancy is shown above. Among the lymphoma samples, Lane 27 is a Burkitt's lymphoma. Lane 1 is DNA digested with HindIII alone, whereas all other lanes are digested with both HindIII and EagI. In a, the unmethylated pattern of p15INK4B is observed in Lanes 2, 6, 12, and 19–26 (presence of 2.2- and 0.5-kb bands), as seen previously for normal tissue (20). Hypermethylation of p15INK4B is observed in Lanes 3, 5, 7, 8, 11, 14, 15, 18, and 27 (retention of the 2.8-kb band). Homozygous deletion of p15INK4B is seen in Lanes 13, 16, and 17. In b, a cross-hybridizing fragment recognized by the p15INK4B exon 1 probe (20). The normal unmethylated pattern of p16INK4A is observed in Lanes 2–12, 14, 16, 19–22, 23, and 24 (presence of 3.9, 3.1, and 0.55-kb bands). Hypermethylation of p16INK4A (retention of 6.0-kb band) is observed in Lanes 25, 26, and 27. Homozygous deletion of p16INK4A is seen in Lanes 15, 17, and 18. Lane 13 reveals a rearrangement of p15INK4B exon 1, which occurs with loss of p15INK4B in this tumor, suggesting that the homozygous deletion of p15INK4B approaches the p16INK4A gene promoter region.
p16\(^{NK4A}\) or p15. Previous reports have documented homozygous deletion in p15\(^{NK4B}\) and p16\(^{NK4A}\) and have, in general, suggested more frequent homozygous deletion in T-precursor ALL than in B-precursor ALL (6, 9, 14, 16, 17). Therefore, we examined a larger number of pediatric ALL samples to allow us to determine whether the pattern of inactivation of p15\(^{NK4B}\) and p16\(^{NK4A}\) differed between the B-precursor and T-precursor phenotypes of ALL. In B-precursor ALL, homozygous deletion of p15\(^{NK4B}\) occurred in 17% of patients, whereas hypermethylation of p15\(^{NK4B}\) was found in another 48%. Thus, p15\(^{NK4B}\) is inactivated in 65% of the B-precursor ALL samples, an incidence very similar to that found in pediatric AML. In the 11 pediatric B-precursor ALL samples with hypermethylation of p15\(^{NK4B}\), the p16\(^{NK4A}\) gene was not affected by either homozygous deletion or hypermethylation in any case.

The pattern of inactivation for p15\(^{NK4B}\) and p16\(^{NK4A}\) in T-precursor ALL was quite different from that in B-precursor ALL. Inactivation of either p15\(^{NK4B}\) or p16\(^{NK4A}\), and most frequently both, was found in all 12 of the pediatric T-precursor samples examined. The analysis of T-precursor ALL confirmed the higher rate of homozygous deletion of p16\(^{NK4A}\) in this subtype (75%) observed by others (6, 14, 16, 17). Somewhat surprisingly, these homozygous deletions of p16\(^{NK4A}\) included the p15\(^{NK4B}\) gene in only two of nine T-precursor ALL. However, of the seven leukemias with homozygous deletion limited to p16\(^{NK4A}\), five were hypermethylated at the p15\(^{NK4B}\) gene (Fig. 1; Table 1). This unusual pattern of dual inactivation of p15\(^{NK4B}\) and p16\(^{NK4A}\) occurred in two of two patients with the clinical diagnosis of lymphoblastic lymphoma. The only time we have previously observed homozygous deletion of p16\(^{NK4A}\) and hypermethylation of p15\(^{NK4B}\) was in leukemic cell lines also of T-precursor origin (A3.01, Molt3, and ML-1; Ref. 20), suggesting that this unusual pattern of inactivation of both p15\(^{NK4B}\) and p16\(^{NK4A}\) may be unique to T-precursor ALL.

p16\(^{NK4A}\) is Frequently Hypermethylated in NHL. We examined inactivation of p15\(^{NK4B}\) and p16\(^{NK4A}\) in another group of hematological malignancies, the NHLs. Although inactivation of these cyclin-dependent kinase inhibitors was found, the pattern was strikingly different from that in acute leukemia. In 25 cases of NHL, we found inactivation of p15\(^{NK4B}\) by homozygous deletion in only one sample (4%), but frequent inactivation of p16\(^{NK4A}\) by hypermethylation (12 samples, 48%) and less frequent hypermethylation of p15\(^{NK4B}\) (28%). Most importantly, in analyzing different types of non-Burkitt’s NHL, we found that low-grade NHL cases had frequent hypermethylation of p16\(^{NK4A}\) (9%), whereas high-grade NHL cases had frequent hypermethylation (83%) of this gene (Fig. 1; Table 1, different at P < 0.005 by Fisher’s exact test). The association of hypermethylation of p16\(^{NK4A}\) with more aggressive phenotype is also evident if these lymphomas are classified according to the working formulation [low grade, 0 of 7; intermediate grade, 4 of 8 (50%); high grade, 2 of 2 (100%)].

Another distinct pattern of p15\(^{NK4B}\) and p16\(^{NK4A}\) inactivation was found in lymphomas of the Burkitt’s type. A high frequency of inactivation of both genes, including one homozygous deletion of p15\(^{NK4B}\) alone, two samples with selective hypermethylation of p16\(^{NK4A}\), and four examples of hypermethylation of both p15\(^{NK4B}\) and p16\(^{NK4A}\). We have previously found hypermethylation of both p15\(^{NK4B}\) and p16\(^{NK4A}\) to be very unusual in a primary malignancy. Interestingly, one of the previous examples of this pattern was the Burkitt’s lymphoma cell line Raji (20).

Hypermethylation of p15\(^{NK4B}\) and p16\(^{NK4A}\) is a De Novo Event in Hematological Malignancies. One important consideration, for both the biology of neoplasms and considerations for clinical marker studies, is whether the promoter region hypermethylation of p16\(^{NK4A}\) and p15\(^{NK4B}\) is an absolutely de novo event in tumor progression. Although homozygous deletions or point mutations of a gene should not be found in any normal cell, the changes in methylation could theoretically occur. If present in a small normal cell population, such as a stem/progenitor cell, from which a malignancy may arise, this would not be detected by the Southern analysis usually employed for assay of methylation status.

To address this issue directly, we used a newly developed method, MSP (23), to examine stem cells from normal bone marrow. MSP is well suited for this type of study because it can detect one methylated allele in 1000 unmethylated alleles (23). In a number of primary leukemia and lymphoma samples, the pattern of hypermethylation seen by Southern analysis was reproduced by MSP (Ref. 23 and data not shown), confirming that MSP can detect aberrant methylation in primary malignancies. We examined four samples of normal bone marrow fractionated on the basis of CD34 expression, which characterizes early hematopoietic progenitor cells (25). Neither CD34– nor CD34+ cells had any evidence of methylation of the p15\(^{NK4B}\) gene (data not shown). To provide an even more sensitive method for detection of aberrantly methylated p15\(^{NK4B}\) alleles, we examined three additional marrows from normal donors that were not only CD34 selected but also were fractionated by cell elutriation to obtain more highly enriched stem cell populations (24). Even for these samples, we found no evidence of aberrant methylation of p15\(^{NK4B}\) in any fraction (Fig. 2). These samples were similarly negative for p16\(^{NK4A}\) hypermethylation (data not shown).
genes are not methylated in normal hematopoietic cells, exons. Hypermethylation of exon I is shown by a large M intersecting the arrow marking of most types of acute leukemias, including adult and pediatric AML. Certainly not be expected for any normal cell population. Likewise in the @J5lNK4B or @J6lNK4A genes by homozygous deletion would be frequent in two subtypes of hematological malignancies, the absence of this change from any normal hematopoietic cell, suggesting it is a frequent event observed in neoplasia, the absence of this change from any normal stage in the parent cell population is important. Inactivation of the @J5lNK4B or @J6lNK4A genes by homozygous deletion would certainly not be expected for any normal cell population. Likewise in normal cells, many genes contain promoter region CpG islands that are not methylated, allowing a transcription-ready state. Consistent with this, we now show that the CpG islands of the @J5lNK4B and @J6lNK4A genes are not methylated in normal hematopoietic cells, including highly enriched populations of the stem/progenitor cells that are the likely cells of origin for transformation in the development of hematological malignancy. Thus, the methylation events that have been shown previously to correlate with transcriptional inactivation for these genes (18–20, 22) arise de novo during hematopoietic transformation or progression.

The selective or dual inactivation of @J5lNK4B and/or @J6lNK4A genes in the hematological disorders studied, the absence of inactivation events for either gene would also be revealing for pathogenesis. This is exemplified by our findings for CML, where @J5lNK4B or @J6lNK4A inactivation was not observed, even for patients in blast crisis. This finding emphasizes the different cellular events operative in CML versus AML throughout initiation and tumor progression. Previous studies have suggested that a subset of lymphoid blast crisis of CML may demonstrate homozygous deletion of the 9p21 region (30). The blast crisis samples in our present study are predominantly of the myeloid type, and further studies of cases of CML with lymphoid properties are now warranted. However, failure to find methylation of the @J5lNK4A gene in patients presenting with apparently de novo acute leukemia may be useful in identifying those who have progressed through CML. In support of this, one of the adult ALL samples without @J6lNK4A hypermethylation was Philadelphia chromosome positive.

Our present findings have clinical as well as biological significance. The frequent occurrence of @J5lNK4B or @J6lNK4A changes in hematological neoplasms makes these alterations attractive as diagnostic and prognostic markers. This is especially so for the hypermethylation changes, because the sensitive PCR-based technique used did not detect this alteration in any normal hematopoietic cell, suggesting it could be used to monitor residual disease or the reappearance of disease after therapy. Our observations are potentially important for
future therapeutic approaches in these diseases. Transcriptional inactivation associated with aberrant promoter region hypermethylation is at least partially reversible with demethylating agents such as 5-azacytidine and 5-aza-2’-deoxycytidine (18-20, 22). Although these agents have been used in clinical trials for acute leukemias and myelodysplasia, the specific mechanisms underlying beneficial results have not been explored. The extremely frequent methylation of \( p15^{INK4B} \) and/or \( p16^{INK4A} \) in leukemias and lymphomas make reactivation of expression of these cell cycle-controlling genes an inviting therapeutic opportunity. Future studies of 5-azacytidine and new agents have been used in clinical trials for acute leukemias and myelodysplasia, the specific mechanisms underlying beneficial results have not been explored. The extremely frequent methylation of \( p15^{INK4B} \) and/or \( p16^{INK4A} \) in leukemias and lymphomas make reactivation of expression of these cell cycle-controlling genes an inviting therapeutic opportunity. Future studies of 5-azacytidine and new agents have been used in clinical trials for acute leukemias and myelodysplasia, the specific mechanisms underlying beneficial results have not been explored. The extremely frequent methylation of \( p15^{INK4B} \) and/or \( p16^{INK4A} \) in leukemias and lymphomas make reactivation of expression of these cell cycle-controlling genes an inviting therapeutic opportunity. Future studies of 5-azacytidine and new strategies for demethylation as a treatment for hematological malignancies should include careful monitoring of the \( p15^{INK4B} \) and \( p16^{INK4A} \) genes as a guide to therapeutic efficacy.

Acknowledgments

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References

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