HIC1 Hypermethylation Is a Late Event in Hematopoietic Neoplasms

Jean-Pierre J. Issa, Barbara A. Zehnbauer, Scott H. Kaufmann, Maggie A. Biel, and Stephen B. Baylin

The Oncology Center [J.-P. J. L., B. A. Z., S. H. K., M. A. B., S. B. B.] and the Department of Medicine [S. B. B.]. The Johns Hopkins University School of Medicine, Baltimore, Maryland 21231

Abstract

HIC1, a candidate tumor suppressor gene on 17p13.3, is hypermethylated and silenced in a large number of solid tumors. To determine its potential role in leukemias, we studied its methylation status in normal and neoplastic hematopoietic cells. We found HIC1 to be unmethylated in peripheral blood cells, bone marrow cells, and CD34+ cells. HIC1 was rarely methylated in newly diagnosed acute myelogenous leukemias (10%) but was relatively frequently methylated in newly diagnosed non-Hodgkin’s lymphoma (25%), acute lymphocytic leukemia (ALL; 53%), and chronic-phase chronic myelogenous leukemia (59%). By contrast, HIC1 was hypermethylated in 100% of recurrent ALL and 100% of blast crisis chronic myelogenous leukemia. In two patients with ALL for whom paired diagnosis/relapse samples were available, HIC1 was unmethylated at diagnosis but was highly methylated at relapse after a chemotherapy-induced complete remission. HIC1 methylation, therefore, seems to be a progression event in hematopoietic neoplasms.

Introduction

HIC1, a gene distal to p53 on 17p13.3, encodes a zinc finger transcription factor (1). It was cloned because of the presence of a dense CpG island in this region that undergoes frequent hypermethylation in various solid tumors, including those of the colon, lung, breast, brain, and kidney (2–4). HIC1 is located within 5 kb of the VNTR5 YNZ22, which is frequently reduced to homozygosity in many tumors, including those of the breast (5) and ovary (6), and is a candidate gene for the putative tumor suppressor distal to p533 on 17p because: (a) it is frequently methylated and silenced in solid tumors; (b) it lies close to the area of minimal deletion in this region (6, 7); (c) its expression seems to be upregulated by p53 (1); and (d) transfection of a sense construct encoding HIC1 into cancerous cells resulted in a significant reduction in clonogenic survival, suggesting that its expression might provide a negative growth or survival advantage to tumor cells (1).

Several lines of evidence have suggested a possible role for HIC1 in hematopoietic neoplasms: (a) the distal arm of 17p is often reduced to homozygosity in advanced stages of CML and ALL (8, 9), but p53 mutations are distinctly rare in these neoplasms (9–11); (b) HIC1 belongs to the ZIN family of zinc finger transcription factors, and two other members of this family are rearranged and/or overexpressed in acute leukemias (12, 13); and (c) hypermethylation seems to be a frequent way by which hematopoietic neoplasms inactivate growth regulatory and/or tumor suppressor genes (14). In particular, we have previously reported a high incidence of estrogen receptor (ER) (15) and p15 (16) gene methylation in hematopoietic neoplasms.

To study the role of HIC1 in leukemia, we have determined its methylation status in normal and neoplastic hematopoietic cells. We now report that HIC1 is unmethylated in normal blood and BM cells and is relatively infrequently methylated in acute leukemias at diagnosis or in CML-CP but is frequently methylated in recurrent ALL and CML-BC. Our data suggest that HIC1 methylation is a progression event in hematopoietic neoplasms.

Materials and Methods

Tissue Samples. Normal lymphocytes and BM cells were obtained from healthy donors of BM for allogeneic transplantation. Leukemia samples were obtained from peripheral blood or BM of patients at the time of diagnosis, remission, BMT or relapse. Patients were treated at the Johns Hopkins Oncology Center on a variety of chemotherapy and BMT protocols that were approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Hospital in accordance with the policies of the Department of Health and Human Services. All patients gave informed consent for the use of their tissue samples.

Cell Culture. The human hematopoietic cancer cell lines used in this study were LA28, HL-60, Raji, CEM, Molt3, K562, KG1A, ML-1, and Jurkat. All cell lines were obtained from the American Type Culture Collection and maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Sigma).

Methylation Analysis. DNA was extracted using standard techniques. As in previous studies, Southern blots were used to determine the methylation state of the HIC1 CpG island (2). This island contains a cluster of methylation-sensitive restriction enzymes, including five NotI sites (1). NotI will digest DNA to completion if the two CG sites in its recognition sequence are unmethylated, but it will not cut DNA if any of the two CG sites are methylated. Briefly, 5 μg of genomic DNA were digested with 50 units of EcoRI and 100 units of NotI for 16 h as specified by the manufacturer (New England Biolabs), run on a 1% agarose gel, transferred to a Zetaprobe nylon membrane (Bio-Rad), and probed with the VNTR probe YNZ22 (American Type Culture Collection). The Southern blots were then exposed on a phosphor screen for 2–3 days and developed using a PhosphorImager (Molecular Dynamics). To rule out incomplete digestion with NotI, all of the blots were reprobed with a 5′ fragment of the c-abl gene, which contains two Not1 sites in its 5′ CpG island. In all cases (except for CML; Ref. 17), an expected 5-kb band was the only band present, indicating complete digestion of the DNA with NotI.

Results

To determine normal patterns of HIC1 methylation in hematopoietic cells, DNA was digested with EcoRI and the methylation-sensitive restriction enzyme NotI, subjected to agarose gel electrophoresis, and probed with YNZ22, which recognizes a VNTR 3 kb upstream of the HIC1 promoter-associated CpG island. In this analysis, unmethylated DNA yields one or two allelic bands of 4.0–5.0 kb, whereas methylated DNA yields bands ranging from 6–16 kb, depending on the number of NotI sites affected by methylation. We found no
evidence of methylation in peripheral blood leukocyte DNA from 16 healthy individuals ranging from 29–67 years of age (Fig. 1, PBL). In DNA isolated from BM aspirates of 13 other healthy individuals ranging from 18–55 years of age, we likewise found no methylation of the HIC1 gene CpG island NolI sites (Fig. 1, BM). In addition, we studied one sample of BM separated into CD34− and CD34+ cell fractions and found both fractions to be completely unmethylated (Fig. 1). Finally, DNA derived from five nonneoplastic LNs also revealed no methylation at the HIC1 locus (data not shown).

Having established that normal hematopoietic cells are unmethylated at the HIC1 CpG island, we next studied its methylation status in a panel of cultured hematopoietic cell lines (Fig. 1, right panel). Of nine cell lines studied, five (LA28, Raji, KG1A, K562, and ML-1) showed complete methylation of this region, as indicated by the lack of any band at 4–5 kb, and the presence of one or several bands of higher molecular weight. Three cell lines (Jurkat, Molt3, and CEM) showed partial methylation as indicated by the coexistence of normal and methylated bands, and one (HL-60) was devoid of methylation at the HIC1 NolI sites.

To determine whether the frequent methylation of HIC1 in cell lines reflects the patterns of HIC1 methylation in vivo, we next studied DNA extracted from various primary hematopoietic neoplasms. Fig. 2 shows examples of HIC1 methylation patterns in primary hematopoietic neoplasms, and Fig. 3 provides a summary of all samples tested. HIC1 methylation was observed in only 6 of 61 newly diagnosed acute myelogenous leukemia (10%), suggesting that it is an infrequent event in this disease. In contrast, HIC1 methylation was present in 9 of 17 cases (53%) of ALL and in 3 of 6 cases (50%) of CML-CP. Finally, in primary non-Hodgkin’s lymphomas, HIC1 was methylated in two of eight cases (25%). Age of the patient at diagnosis, gender, cell lineage in ALL, French-American-British classification in acute myelogenous leukemia, and BC morphology in CML (lymphoid or myeloid) was not related to the prevalence or extent of HIC1 methylation in the population studied. These data suggest that HIC1 methylation is significantly less frequent than ER methylation in all of these same neoplasms (15).

Because HIC1 was hypermethylated in most of the cell lines examined, we next considered the possibility that HIC1 methylation is a late event in the ontogeny of hematopoietic malignancies. To test this hypothesis, we have examined HIC1 methylation in more advanced stages of ALL and CML. In ALL patients whose neoplasms had recurred after a chemotherapy-induced complete remission, HIC1 methylation was observed in eight of eight cases (Fig. 2 and 3). In two such cases for which paired DNA samples were available, the neoplasm was unmethylated at diagnosis but was highly methylated at recurrence (Fig. 4). HIC1 methylation was also significantly more frequent in CML-BC (eight of eight cases, 100%) than in CML-CP (three of six cases, 50%; Figs. 2 and 3). These observations then

Fig. 1. HIC1 methylation in normal hematopoietic cells and hematopoietic cell lines. Shown are examples of Southern blots of DNA derived from the tissue samples or cell lines indicated on the top of each lane. The single or double band at 4–5 kb represents the normal (unmethylated) pattern detected by the VNTR probe YNZ22. Higher molecular weight bands reflect the methylation of one or several NolI sites in the HIC1 CpG island. Left panel, normal tissues; right panel, examples of leukemic cell lines.

Fig. 2. HIC1 methylation in hematopoietic neoplasms. Shown are representative Southern blots of DNA derived from the BM or LNs of patients with the neoplasm indicated on top of each panel. As in Fig. 1, bands of molecular weight higher than 4–5 kb indicate HIC1 methylation, as can be seen in AML (Lane 3), ALL (Lanes 1 and 2), CML-CP (Lanes 2 and 4), CML-BC (Lanes 1–5), and non-Hodgkin’s lymphoma (Lane 3). The coexistence of normal and methylated bands in some lanes (e.g., ALL, Lane 1; CML-CP, Lane 2) may be due to contamination of the samples with nonneoplastic cells or true partial methylation within the cells, as can be seen in some cell lines (Fig. 1).

Fig. 3. Summary of HIC1 methylation in primary hematopoietic neoplasms. For each tissue or neoplasm indicated, DNA was analyzed as in Figs. 1 and 2. The number of samples with HIC1 methylation is represented in graphical form. PBL, BM, and LN samples are from control patients. The number of samples positive for HIC1 methylation as well as the total number of samples studied is indicated on the top of each bar.
suggest that \textit{HIC1} methylation may be a feature of progression in ALL and CML.

\textbf{Discussion}

\textit{HIC1}, a candidate tumor suppressor gene on 17p13.3, is frequently methylated and inactivated in solid tumors (1). Our present data suggests that \textit{HIC1} methylation might also be an important event in hematopoietic neoplasms, particularly in the later stages of these diseases.

\textit{HIC1} does not seem to be methylated in normal hematopoietic precursor cells, suggesting that its methylation is a \textit{de novo} event in leukemias. This is in contrast to some other tissues such as prostate (18), kidney (4), and ovary (19), in which some \textit{HIC1} methylation seems to precede neoplasia and may possibly mark the cells predisposed to neoplasia in these tissues. This also contrasts with methylation of the \textit{ER} gene, which is much more frequent than \textit{HIC1} in hematopoietic neoplasms (15), perhaps reflecting the fact that some methylated \textit{ER} alleles can be detected in normal BM cells as well. Our data then suggest that, in contrast to its role in most solid tumors in which it is an early event, \textit{HIC1} methylation seems to be a late event in leukemogenesis. The mechanism by which \textit{HIC1} becomes methylated during leukemia progression is unclear. It is possible that \textit{HIC1} methylation is a rare random event during cellular replication that is selected for in the later stages of leukemia because of its potential function as a growth suppressor (1). Alternatively, it is possible that \textit{HIC1} methylation itself is induced or accelerated by drug therapy, as has been demonstrated for some genes (20). This is unlikely to be a high frequency event, because patients who achieve a remission after chemotherapy do not have \textit{HIC1} methylation in their normal BM cells. Nevertheless, we cannot exclude this possibility at the present time.

\textit{HIC1} methylation is also relatively disease-specific in acute leukemias. \textit{HIC1} is infrequently methylated in \textit{de novo} AML but is frequently methylated in ALL. This relative specificity for lymphoid neoplasms contrasts with the methylation of other genes in hematopoietic neoplasms; Calcitonin (21), \textit{ER} (15), and \textit{p15} (16) seem to be almost equally methylated in ALL and AML. \textit{C-abl} methylation is restricted to CML (17), but this is most likely a result of the bcr-abl translocation seen in this disease. Only \textit{p16} shows a similar pattern of disease-restricted methylation, being methylated exclusively in lymphomas (16). It remains to be determined whether the cellular specificity of \textit{HIC1} methylation is due to functional distinctions between cell types or tissue-specific differences in chromatin structure on 17p13.3 (as can be seen for fragile sites; Ref. 22), which may differentially predispose \textit{HIC1} to neoplasia-related methylation.

The function of \textit{HIC1} remains to be determined. Its structure, however, suggests that it is a transcription factor (1). The chicken homologue of \textit{HIC1} has been cloned and has been shown to suppress transcription from a crystallin gene promoter reporter gene construct \textit{in vitro} (23). Future experiments should clarify the role of \textit{HIC1} in normal hemopoiesis and the functional impact of loss of \textit{HIC1} expression in leukemias. In view of the presence of \textit{HIC1} methylation in some recurrent leukemias, it would be interesting to explore the impact of \textit{HIC1} expression on chemotherapy resistance and apoptosis.

\textit{HIC1} methylation may have important clinical applications in the field of leukemia therapy. Just like \textit{ER} (15) and \textit{p15} (16), \textit{HIC1} may be useful as a molecular marker of disease activity. In addition, the absence of \textit{HIC1} methylation in normal marrow, including CD34+ cells, suggests the possibility of using a novel and sensitive PCR approach to detect this molecular abnormality as a marker of minimal residual disease (24). Furthermore, the high prevalence of \textit{HIC1} methylation in recurrent ALL and CML-BC raises the possibility that \textit{HIC1} methylation in newly diagnosed ALL and CML-CP may serve as a prognostic factor in these diseases. Finally, \textit{HIC1} methylation may also be a target for pharmacological demethylation using 5-deoxy-azacytidine, an inhibitor of DNA-methyltransferase that is currently in clinical trials for hematopoietic malignancies (25).

\textbf{References}


H/Cl M ETHYLATION IN LEUKEMIA


