Inactivation of HOXA Genes by Hypermethylation in Myeloid and Lymphoid Malignancy is Frequent and Associated with Poor Prognosis

Gordon Strathdee,1 Tessla L. Holyoake,2 Alyson Sim,3 Anton Parker,4 David G. Oscier,4 Junia V. Melo,5 Stefan Meyer,6,7 Tim Eden,7 Anne M. Dickinson,8 Joanne C. Mountford,2 Heather G. Jorgensen,2 Richard Soutar,3 and Robert Brown1

Abstract  Purpose: The HOX genes comprise a large family of homeodomain-containing transcription factors, present in four separate clusters, which are key regulators of embryonic development, hematopoietic differentiation, and leukemogenesis. We aimed to study the role of DNA methylation as an inducer of HOX gene silencing in leukemia.  Experimental Design: Three hundred and seventy-eight samples of myeloid and lymphoid leukemia were quantitatively analyzed (by COBRA analysis and pyrosequencing of bisulfite-modified DNA) for methylation of eight HOXA and HOXB cluster genes. The biological significance of the methylation identified was studied by expression analysis and through re-expression of HOXA5 in a chronic myeloid leukemia (CML) blast crisis cell line model.  Results: Here, we identify frequent hypermethylation and gene inactivation of HOXA and HOXB cluster genes in leukemia. In particular, hypermethylation of HOXA4 and HOXA5 was frequently observed (26-79%) in all types of leukemias studied. HOXA6 hypermethylation was predominantly restricted to lymphoid malignancies, whereas hypermethylation of other HOXA and HOXB genes was only observed in childhood leukemia. HOX gene methylation exhibited clear correlations with important clinical variables, most notably in CML, in which hypermethylation of both HOXA5 (P = 0.00002) and HOXA4 (P = 0.006) was strongly correlated with progression to blast crisis. Furthermore, re-expression of HOXA5 in CML blast crisis cells resulted in the induction of markers of granulocytic differentiation.  Conclusion: We propose that in addition to the oncogenic role of some HOX family members, other HOX genes are frequent targets for gene inactivation and normally play suppressor roles in leukemia development.

The human HOX genes are a large gene family, consisting of 39 members, which are located in four gene clusters (HOXA-HOXD). They each encode homeodomain-containing transcription factors which are known to be key regulators of embryonic development (1). The HOX genes are also expressed in adult cells, in which they play important roles in the control of cellular differentiation (1). Expression of many of the HOX genes, primarily from the HOXA and HOXB clusters, has been identified in CD34+ hematopoietic progenitor cells, and transgenic mouse models have implicated several family members in the control of normal hematopoiesis (2). For example, overexpression of Hoxa5 in CD34+ progenitor cells induced increased levels of granulocytic/monocytic differentiation and inhibited erythroid/megakaryocytic differentiation (3). In addition, inhibition of Hoxa5, using antisense oligonucleotides, inhibited granulocytic/monocytic differentiation but increased erythroid/megakaryocytic differentiation (4), suggesting that Hoxa5 is a key regulator of myeloid differentiation.

Authors' Affiliations: 1Centre for Oncology and Applied Pharmacology, Cancer Research U.K., Beatson Laboratories and 2Division of Cancer Sciences and Molecular Pathology, University of Glasgow, 3Department of Haematology, Western Infirmary, Glasgow, United Kingdom; 4Department of Haematology, Royal Bournemouth Hospital, Bournemouth, United Kingdom; 5Department of Haematology, Imperial College London, Hammersmith Hospital, London, United Kingdom; 6Stem Cell and Leukaemia Proteomics Laboratory, Paterson Institute of Cancer Research and 7Paediatric and Adolescent Oncology Unit, University of Manchester and Christie Hospital NHS Trust, Manchester, United Kingdom; and 8Haematological Sciences, School of Clinical and Laboratory Sciences, The Medical School, Newcastle-upon-Tyne, United Kingdom

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Current address for G. Strathdee: Life Knowledge Park, Institute of Human Genetics, Newcastle University, Newcastle-upon-Tyne, NE1 3BZ, United Kingdom.

Requests for reprints: Gordon Strathdee, Life Knowledge Park, Institute of Human Genetics, Newcastle University, Newcastle-upon-Tyne, NE1 3BZ, United Kingdom. Phone: 44-191-241-8829; Fax: 44-191-241-8810; E-mail: G.R.Strathdee@newcastle.ac.uk.

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Inappropriate expression of HOX genes has also been implicated in the development of hematopoietic malignancies (5). The HOXA9 gene is one of the targets (along with NUP98) in the acute myeloid leukemia (AML) - associated chromosome translocation t(7;11)(p15;pl5) (refs. 6, 7), and HOXA9 overexpression has been shown to induce AML in mouse models (8). Similarly, several other HOX genes located in the 5’ end of clusters (paralogues 9-13) have also been identified as partners with NUP98 in leukemia-associated translocations (9). Overexpression of four other family members (Hoxa7, Hoxa10, Hoxb3, and Hoxb8) have all been correlated with defective hematopoiesis and myeloproliferative disease/AML in mouse models (5). In addition, the key regulator of HOX gene expression, MLL, is involved in multiple AML and acute lymphoblastic leukemia (ALL) – associated translocations (10) and simultaneous overexpression of multiple HOX genes, identified by microarray analysis, has been correlated with specific subtypes of AML (11) and ALL (12).

Alterations in the patterns of DNA methylation are critical in the development of all types of cancer (13). In particular, transcriptional repression due to hypermethylation of promoter-associated CpG islands has been shown to inactivate many genes whose down-regulation is known to be important in tumor development, both in hematologic malignancies and in solid tumors (14, 15). We have recently shown that hypermethylation of the HOXA4 gene is associated with transcriptional repression in chronic lymphocytic leukemia (CLL; ref. 16) and correlates with high-risk of disease. In addition, we have also identified HOX5 as one of a small group of genes whose cell type-specific expression is controlled by DNA methylation in normal cells (17) and have shown increased levels of DNA methylation and consequent gene inactivation in a small number of AML samples (18).

The above findings suggested that DNA methylation may have an important role in aberrant control of HOX gene expression during the development of leukemia. Thus, in contrast to the oncogenic role identified for some HOX genes, most notably HOXA9, other family members may function as suppressors of the malignant phenotype. In this study, we have analyzed the methylation status of eight HOXA and B cluster genes in a large number of chronic and acute, myeloid, and lymphoid malignancies using quantitative methylation analysis. These studies have identified frequent hypermethylation of HOX genes, in particular within the HOXA cluster, in all types of leukemia studied. The patterns of methylation identified in specific types of leukemia were nonrandom and exhibited a number of clear correlations with the clinical characteristics of the patients. Most strikingly, in chronic myeloid leukemia (CML), methylation of both HOXA4 and HOXA5 was strongly associated with progression to blast crisis. Furthermore, re-expression of HOXA5 in blast crisis CML cells resulted in the induction of markers of granulocytic differentiation. These results suggest that inactivation of HOX genes, particularly HOXA4 and HOXA5, plays an important role in the development of leukemia.

Materials and Methods

Patient samples. DNA was isolated from peripheral blood or bone marrow samples obtained from patients with clinically diagnosed leukemia from Glasgow Royal Infirmary, Royal Bournemouth Hospital, Newcastle Royal Victoria Infirmary, Glasgow Western Infirmary, Hammersmith Hospital (London), Royal Liverpool University Hospital (Liverpool, United Kingdom), and Royal Manchester Children’s Hospital. Peripheral blood samples were also obtained from healthy volunteers. Ethical approval was obtained for all samples collected.

Both childhood ALL and AML samples were obtained from diagnostic bone marrow aspirates and all samples were determined to have >95% blasts by morphologic assessment of bone marrow aspirate films. All CML samples were derived from peripheral blood. All chronic phase CML samples consisted of leukocytes derived from peripheral blood from patients undergoing leukopheresis. These samples were taken at diagnosis from patients with very high white cell counts and will contain >95% BCR/ABL-positive cells. The majority of blast crisis samples were obtained by the isolation of leukocytes directly from peripheral blood samples, although a small number were also isolated from leukopheresis. The blast crisis samples all had between 80% and 99% blasts and essentially no identifiable normal cells. CLL samples were derived from peripheral blood mononuclear cells obtained by Ficoll gradient centrifugation. This should result in at least 80% (and usually >90%) malignant cells in all samples. Adult AML samples were obtained either from bone marrow or peripheral blood samples (22 peripheral blood samples and 61 bone marrow samples). Blast counts were available from 68 of 83 AML patients. Only one sample had a count <50% and the overwhelming majority had >80% blasts (54 of 68 samples). No differences were detected between the methylation frequencies of samples derived from peripheral blood or bone marrow.

For the CML chronic phase series, patients at high risk (for progression to blast crisis) were defined as those having one or more of the following high: Hasford/ Sokal score (19, 20), incomplete response to imatinib (21), or the presence of deletions on the short arm of chromosome 9 (22). Incomplete response to imatinib was defined as less than complete cytogenetic response by 12 months after initiation of therapy, loss of complete cytogenetic response, or development of imatinib resistance. For AML, patients were separated into good prognosis ([t(8;21), t(15;17) and inv(16)] or intermediate prognosis (normal karyotype; ref. 23), based on cytogenetic analysis. For CLL, the immunoglobulin variable heavy chain mutational status was known for all cases.

Tissue culture and cells. The LAMA84 CML cell line was maintained in RPMI with 2 mmol/L of glutamine and 10% FCS in 95% air/5% CO2 at 37°C.

COBRA analysis. COBRA analysis was done largely as described before (24). One microgram of genomic DNA was modified with sodium bisulfite using the CpGenome modification kit (Chemicon International) as per the manufacturer’s instructions. All samples were resuspended in 40 μL of Tris-EDTA and 1 μL of this was used for subsequent PCR reactions. The samples were amplified in 25 μL volumes containing 1× manufacturer’s buffer, 1 unit of FastStart taq polymerase (Roche), 1 to 4 mmol/L of MgCl2, 10 mmol/L of deoxynucleotide triphosphates, and 75 ng of each primer. PCR was done with one cycle of 95°C for 6 min, 35 cycles of 95°C for 30 s, 58°C to 63°C for 30 s, and 72°C for 30 s, followed by one cycle of 72°C for 5 min. All PCR reactions were carried out on a PTC-225 DNA engine tetrad (MJ Research). Following amplification, the PCR products were digested with the appropriate restriction enzymes, specific for the methylated sequence after sodium bisulfite modification. Digested PCR products were separated on 1.5% or 2% agarose gels and visualized by ethidium bromide staining on GeneSnap gel documentation system (Syngene). Quantitation of methylation levels was carried out by measurement of band intensities using the GeneTools system (Syngene) and methylation levels in a particular sample were calculated correcting for the smaller size (and thus lower ethidium bromide binding capacity) of the methylated band. In vitro methylated DNA (Chemicon International) was diluted into DNA extracted from normal peripheral blood to produce standards (100%, 66%, 33%, and 0%) of known methylation status for all COBRA assays.
Hypermethylation of HOXA4 and HOXA5 correlates with blast crisis in CML. We have previously identified frequent methylation of HOXA4 (16) in CML and hypermethylation of HOXA5 in a small series of patients with AML (18), suggesting that genes in the central portion of the HOX clusters may be targets for aberrant methylation in leukemia. To investigate this possibility in CML, we performed methylation analysis of HOXA and B cluster genes (HOXA4, A5, A6, A7, B4, B5, B6, and B7) in 44 samples of chronic phase CML. The analysis was done using the COBRA assay to assess methylation levels in the proximal promoter/first exon of each gene (examples in Fig. 1). Multiple restriction digests were used to ensure at least 4 (up to 11) separate CpG sites were analyzed for each loci. Also, known methylation standards of 100%, 66%, 33%, and 0% methylation were assessed to ensure appropriate quantitation of all assays. No methylation was detected in normal peripheral
blood for any of the loci, except the \textit{HOXA5} gene. As previously reported (18), \textit{HOXA5} exhibited methylation of 50\% of alleles in normal peripheral blood and, to account for this, hypermethylation of \textit{HOXA5} was defined as >80\% methylation at the majority of CpG sites tested. Using this approach, both the \textit{HOXA5} gene (34\%) and the neighboring \textit{HOXA4} gene (59\%) were found to be frequently hypermethylated in CML chronic phase samples (Fig. 1; Table 1). In contrast, no methylation was detected at the other \textit{HOX}A and \textit{HOX}B cluster genes analyzed.

The presence of methylation of \textit{HOXA5} in normal cells means that accurate quantitation of the methylation levels in leukemia samples is crucial to definitively identify samples with increased methylation levels. Thus, to confirm that the accuracy of the methylation levels identified at the \textit{HOXA5} gene by COBRA analysis, all 44 CML chronic phase samples were further analyzed by pyrosequencing. The accuracy of this method in quantitating DNA methylation levels has previously been shown (25, 26). This analysis produced essentially identical results to the COBRA analysis, confirming the hypermethylation of all samples identified as hypermethylated by COBRA analysis and lower levels of methylation in all samples without hypermethylation (as determined by COBRA analysis; Supplementary Table S2). Furthermore, the strong agreement of the COBRA analysis with the pyrosequencing data also shows the accuracy of quantitation using the COBRA method.

CML is a triphasic disorder, normally presenting in the chronic phase of the disease, in which differentiation is relatively normal and finally progressing to blast crisis, in which normal differentiation is arrested (27). As \textit{HOX} genes are key regulators of hematopoietic differentiation, we hypothesized that alterations in \textit{HOX} gene methylation may be associated with progression to blast crisis. Samples from patients with the chronic phase of the disease were separated into high- and low-risk (of transformation to blast crisis) groups based on known prognostic markers (as defined in Materials and Methods) and compared with \textit{HOXA4} and \textit{HOXA5} methylation status. Hypermethylation of either gene individually or co-hypermethylation of both genes were clearly associated with the high-risk group of patients (Table 2), suggesting that methylation of \textit{HOXA4} and \textit{HOXA5} is associated with an increased risk of progression to blast crisis.

To further assess the potential link between \textit{HOXA4} and \textit{HOXA5} methylation and CML blast crisis, 23 blast crisis samples were analyzed for hypermethylation as above. This analysis identified exceptionally high levels of hypermethylation of both genes in blast crisis samples (91\% for \textit{HOXA4} and 87\% for \textit{HOXA5}) and both \textit{HOXA5} and \textit{HOXA4} exhibited

### Table 1. Hypermethylation frequencies of \textit{HOX} genes in leukemia

<table>
<thead>
<tr>
<th>Leukemia</th>
<th>\textit{HOXA4}</th>
<th>\textit{HOXA5}</th>
<th>\textit{HOXA6}</th>
<th>\textit{HOXA7}</th>
<th>\textit{HOXB4}</th>
<th>\textit{HOXB5}</th>
<th>\textit{HOXB6}</th>
<th>\textit{HOXB7}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML, n = 44 (%)</td>
<td>59</td>
<td>34</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Blast crisis-CML, n = 23 (%)</td>
<td>91</td>
<td>87</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AML, n = 83 (%)</td>
<td>64</td>
<td>59</td>
<td>34</td>
<td>13</td>
<td>21</td>
<td>13</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>CLL, n = 151 (%)</td>
<td>38</td>
<td>77</td>
<td>19</td>
<td>7</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Childhood ALL, n = 48 (%)</td>
<td>69</td>
<td>39</td>
<td>14</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Childhood AML, n = 28 (%)</td>
<td>54</td>
<td>39</td>
<td>14</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: Number (n) of samples analyzed for each gene at which methylation was detected in the first 20 samples (except for \textit{HOXA6} in CLL, where n = 61). For loci in which no methylation was detected in the first 20 samples, no further samples were analyzed (except \textit{HOXB5} in childhood ALL, where n = 48, and in blast crisis CML, where n = 23 for all loci).

### Table 2. Correlations between \textit{HOX} gene hypermethylation and patient outcome

<table>
<thead>
<tr>
<th>Leukemia</th>
<th>Gene(s)</th>
<th>% Methylated*</th>
<th>(P)</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML</td>
<td>\textit{HOXA5}</td>
<td>87% (20/23)</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blast crisis vs.</td>
<td>33% (15/45)</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chronic phase</td>
<td>57% (8/14)</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High risk for</td>
<td>6% (1/16)</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>progression* vs.</td>
<td>91% (21/23)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low risk for</td>
<td>86% (27/45)</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>progression</td>
<td>44% (7/16)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>\textit{HOXA4}</td>
<td>83% (5/6)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A5 comethylation</td>
<td>10% (2/21)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>\textit{HOXA4}</td>
<td>69% (22/32)</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A5 comethylation</td>
<td>14% (3/21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal karyotype</td>
<td>57% (25/44)</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot;Favorable&quot; cyto genetics</td>
<td>30% (32/107)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>\textit{HOXA4}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgVh unmutated vs.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgVh mutated</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: IgVh, immunoglobulin variable heavy chain.

*Percentage of samples methylated at the indicated gene (no. of methylated samples/total no. of samples).

1\(P\) values determined using the Fisher exact test.

*Risk for progression (from chronic phase to blast crisis) in CML as defined in Materials and Methods.

"Favorable" cyto genetics" in AML samples include t(8;21), t(15;17), and inv(16).
Hypermethylation of HOXA cluster genes is common in both myeloid and lymphoid malignancies. To further assess the role of methylation in the aberrant control of HOX gene expression in leukemia, we extended the above analysis to determine the frequency of HOX gene methylation in samples of CLL, AML, childhood ALL, and childhood AML. This analysis was carried out using COBRA assays as described for the CML samples above. Methylation analysis of the eight HOXA and HOXB cluster genes was carried out initially on a panel of 80 samples, consisting of 20 samples each of the four types of leukemia. The use of 20 samples from each leukemia type yielded a >95% chance of identifying genes methylated with a frequency of ≥15%. In leukemia types in which a particular gene was identified as methylated in the original panel, the analysis was expanded to larger sample sets (see Table 1) to more clearly define the level of hypermethylation and to allow a comparison of methylation status to clinical characteristics. As in the CML samples, this analysis identified frequent hypermethylation of both the HOXA4 and HOXA5 genes in all types of leukemia analyzed. The relative frequencies of hypermethylation were, however, disease-specific, with HOXA4 hypermethylation levels being greater than HOXA5 in myeloid malignancies, whereas the opposite was true for lymphoid malignancies (Table 1). Furthermore, hypermethylation of the HOXA6 gene was frequently identified in both adult CLL and childhood ALL, but was rare in the myeloid malignancies (Table 1). The other five HOX genes analyzed exhibited no evidence of methylation in adult leukemia. In contrast, all the HOX genes, except HOXB5, exhibited hypermethylation in the childhood ALL and AML samples (Table 1). In addition, although no clear comethylation of HOX genes was seen in adult leukemias, there were very strong correlations between methylation of different HOX genes in the childhood samples, even between HOX genes from different clusters (Table 3).

Hypermethylation of gene promoter–associated CpG islands has been shown to lead to inactivation of gene expression (28). We have previously shown that hypermethylation of both HOXA4 (16) and HOXA5 (18) is associated with the loss of expression in primary leukemia samples. To confirm that promoter hypermethylation of the other frequently hypermethylated HOX gene, HOXA6, was associated with the loss of expression, quantitative RT-PCR analysis was used to assess expression in primary CLL samples. As shown in Fig. 3,

![Image](image_url)

**Fig. 2.** Methylation of HOXA5 and HOXA4 in blast crisis CML patient samples. Examples of COBRA analysis of the blast crisis samples for HOXA5 (top) and HOXA4 (bottom). Top, all examples are of myeloid blast crisis (HOXA5). Bottom, both types of blast crisis samples are included (HOXA4); first lane, normal peripheral blood (PBL); final lane, 100% in vitro – methylated (IVM) control. Positions of unmethylated (undigested) and methylated (digested) bands; presence (+) or absence (−) of hypermethylation is indicated below each sample.

Table 3. Comethylation of HOX genes in childhood leukemia

<table>
<thead>
<tr>
<th>Meth A4 (48)</th>
<th>Meth A5 (49)</th>
<th>Meth A6 (13)</th>
<th>Meth A7 (8)</th>
<th>Meth B4 (12)</th>
<th>Meth B6 (7)</th>
<th>Meth B7 (15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meth A5</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39 † (0.0012) ‡</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meth A6</td>
<td>13 (0.0032)</td>
<td>13 (0.0065)</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meth A7</td>
<td>8 (0.046)</td>
<td>7 (&gt;0.05)</td>
<td>4 (0.031)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meth B4</td>
<td>9 (&gt;0.05)</td>
<td>11 (&gt;0.05)</td>
<td>5 (0.034)</td>
<td>5 (0.0024)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Meth B6</td>
<td>5 (&gt;0.05)</td>
<td>6 (&gt;0.05)</td>
<td>3 (&gt;0.05)</td>
<td>4 (0.0021)</td>
<td>4 (0.012)</td>
<td>NA</td>
</tr>
<tr>
<td>Meth B7</td>
<td>12 (&gt;0.05)</td>
<td>14 (0.027)</td>
<td>6 (0.022)</td>
<td>6 (0.0007)</td>
<td>10 (3.20E-07)</td>
<td>4 (0.031)</td>
</tr>
</tbody>
</table>

*Number of samples hypermethylated at the above loci (out of a total of 72 childhood ALL and AML samples).
† Number of samples hypermethylated at both loci.
‡ P value, determined by Fisher exact test. Values in boldface were significant after correction for multiple comparisons.
hypermethylation of HOXA6 was also found to be associated with loss of gene expression ($P = 0.0012$, Mann-Whitney U test).

**Hypermethylation of HOXA4 and HOXA5 is associated with AML patients with poorer prognosis.** As shown above, hypermethylation of both HOXA4 and HOXA5 was associated with disease progression in CML. In addition, we have previously shown that hypermethylation of HOXA4 was associated with CLL patients with a poor prognosis (16). To determine if HOX gene methylation was generally associated with patients with a poorer prognosis, the methylation data was also compared with clinical data for adult AML (for the childhood leukemias, the sample numbers were insufficient to allow meaningful comparisons to be made). In AML, cytogenetic abnormalities are one of the most important prognostic indicators (23).

Comparison of HOXA4 andHOXA5 methylation with patient cytogenetics showed clear differences in frequency. Almost all samples analyzed in this study were either from good prognosis cytogenetic groups ([t(8;21), t(15;17), and inv(16)] or exhibited a normal karyotype (associated with an intermediate prognosis; ref. 23). Although methylation of both HOXA4 (38%) and HOXA5 (43%) was observed in patients exhibiting cytogenetics associated with a good prognosis, it was significantly more frequent in patients with a normal karyotype who have a poorer prognosis [78% for both; $P = 0.004$ (HOXA4) and $P = 0.018$ (HOXA5)]. Furthermore, the clear majority of patients with normal karyotype leukemias showed methylation of both genes (69%), whereas this was rare in the good prognosis patients (14%, $P = 0.0002$; Table 2).

**Re-expression of HOXA5 in CML blast crisis cells results in the induction of differentiation.** HOXA5 has previously been shown to be an important regulator of normal myeloid differentiation and promotes differentiation down the monocytic/granulocytic pathway (3, 4). The strong correlation identified between hypermethylation of HOXA5 and CML blast crisis led to the hypothesis that loss of HOXA5 expression may play an important role in the arrest of normal differentiation in this phase of the disease. Indeed, we have previously shown that treatment of a CML blast crisis cell line with a DNA methylation inhibitor led to the re-expression of HOXA5 and myeloid differentiation of the cells (18). A major drawback of this approach is that the expression of many other genes will be affected by the DNA methylation inhibitor, and thus, to more directly assess the potential role of HOXA5 in the control of differentiation in CML blast crisis cells, the HOXA5 gene was transiently re-expressed in the hypermethylated LAMA84 CML blast crisis cell line. The HOXA5 cDNA sequence was introduced into the pRES2-enhanced GFP vector, which also provides the expression of GFP, to allow the detection of transfected cells. Cells were fluorescence-activated cell–sorted for GFP expression 24 h posttransfection and assayed at 5 days posttransfection for expression of a panel of markers of differentiation using quantitative RT-PCR (compared with cells transfected with a vector expressing GFP alone). At 5 days posttransfection, HOXAS-transfected cells exhibited clear increases in expression of several markers of myeloid differentiation, including CD13, C/EBPα, and C/EBPε (all associated with differentiation down the granulocytic lineage), but no detectable expression of CD11b (marker of terminal granulocytic differentiation) or CD14 (monocytic differentiation; ref. 29; Fig. 4). Therefore, the pattern of markers expressed following HOXA5 transfection is consistent with differentiation down the granulocytic (as opposed to the monocytic) lineage (29). Although clearly up-regulated, the expression levels of these markers remained low and no clear evidence of morphologic differentiation was seen, consistent with the lack of CD11b expression. This is likely due to the transient nature of HOXA5 re-expression, as well as selective loss of HOXA5-positive cells from the population as shown by the decreased levels of GFP-positive cells at day 5 in the HOXAS/GFP transfectants (1.2% GFP-positive cells) versus the GFP alone transfectants (5.6% GFP-positive cells; Fig. 4B). Thus, re-expression of HOXA5 in CML blast crisis cells resulted in altered expression patterns consistent with the induction of differentiation, and further implicating the loss of HOXA5 expression in the arrest of normal differentiation seen in CML blast crisis.

**Discussion**

The HOX genes play crucial roles in the control of differentiation of adult hematopoietic cells (1). The results presented here show that several members of the HOX gene family, particularly from the HOXA cluster, are frequently inactivated by CpG island hypermethylation in leukemia. For two of these genes, HOXA4 and HOXA5, hypermethylation was seen in all types of leukemias studied, including both myeloid and lymphoid malignancies. Methylation of both genes showed clear associations with clinical characteristics of the different leukemia types. Of particular interest, methylation of both HOXA5 ($P = 0.00002$) and HOXA4 ($P = 0.006$) were significantly more frequent in CML blast crisis (87% and 91%, respectively) than in the chronic phase of the disease (31% and 59%, respectively). Hypermethylation of HOXA5, a known regulator of myeloid differentiation, was invariably observed in myeloid blast crisis samples (15 of 15 samples hypermethylated), suggesting that loss of HOXA5 expression may be crucial in the progression to myeloid blast crisis. Furthermore, hypermethylation of both genes was also significantly more frequent in chronic phase patients at high risk of progression (as determined by previously identified prognostic markers) than in patients at low risk. These results suggested an important role for the loss of HOXA4 and HOXA5 in the progression to blast crisis. Importantly, the observed changes in methylation are not simply a reflection of an increased number of immature cells in
the CML samples, as neither CD34+ nor CD133+ selected normal hematopoietic cells exhibiting increased methylation of either HOXA4 (data not shown) or HOXA5 (18).

This possibility was further supported by results from gene reintroduction studies which showed that re-expression of HOXA5 in LAMA84 CML blast crisis cells induced the expression of several markers associated, in particular, with differentiation down the granulocytic lineage. These results are similar to those previously reported for the re-expression of C/EBPα in CML blast crisis cells, which also resulted in the induction of granulocytic differentiation (30). As C/EBPα was one of the genes induced following HOXA5 re-expression, it is possible that this represents a critical target for HOXA5-induced differentiation. However, because induction of expression of these markers did not occur until 5 days after transfection, this would suggest that neither C/EBPα nor indeed any of the other induced genes, are likely to be direct transcriptional targets for HOXA5, but are probably secondary to the activation or repression of other genes. Clearly, it will be important to do expression studies to allow the identification of specific transcriptional targets of HOXA5 in these cells and the particular pathways activated or repressed by HOXA5 reintroduction.

In addition to the frequent targeting of HOXA4 and HOXA5 in CML, hypermethylation of these genes was also frequently observed in AML, CLL, and childhood ALL and AML, suggesting a general role for the inactivation for these HOX genes in the development of human leukemia. In AML, hypermethylation of both HOXA4 and HOXA5 was significantly more frequent in patients with a normal karyotype than in patients exhibiting karyotypic abnormalities associated with good prognosis (78% versus 38%, \( P = 0.0045 \) for HOXA4 and 78% versus 43%, \( P = 0.018 \) for HOXA5). Indeed, hypermethylation of both HOXA4 and HOXA5 in the same sample was seen in the majority of patients with a normal karyotype (69%) but was rare in cases with a good prognosis (14%). Interestingly, patients with normal karyotypes have also previously been shown to exhibit overexpression of multiple HOX cluster genes, including HOXA4 and HOXA5, using microarray studies (11). Although this may at first seem contradictory, our previous analysis showed that hypermethylation of HOXA5 was associated with the loss of protein expression in myeloid leukemia cell lines and primary samples, even in the presence of a HOXA5-containing transcript (18), which is derived from upstream of the HOXA5 promoter and is presumably non-coding. This would be consistent with a model in which up-regulation of HOXA cluster expression, likely targeting the known oncogenic HOX genes, HOXA7 and HOXA9 (31), would lead to a much higher selective pressure for the inactivation of HOX genes that functioned to induce differentiation or inhibit leukemic cell growth (HOXA4 and HOXA5). In agreement with this, recent studies of MLL-AF10–dependent mouse models of leukemogenesis have shown that although the presence of the fusion protein is associated with up-regulation of a Hoxa5-containing transcript (32), transformation by the fusion protein is more efficient in bone marrow from Hoxa5-deficient mice, indicating that Hoxa5 antagonizes MLL-AF10–dependent transformation (33). Interestingly, the same authors also showed that transformation by the CALM-AF10 fusion protein, which is associated with a more specific up-regulation of Hoxa5, was suppressed in the absence of Hoxa5 (33), indicating that the roles of the HOX proteins are likely to be context-dependent. However, the high frequency of HOXA4 and HOXA5 hypermethylation observed in these studies suggests that their predominant role will be inhibitory to leukemogenesis.

Concordant methylation of genes has been described in both hematologic and solid malignancies, and involves a nonrandom distribution of methylation in which a subset of samples exhibit much higher levels of CpG island hypermethylation, or the so-called CpG island methylator phenotype (34). However, despite their close physical proximity, there is no clear evidence for comethylation of the HOX genes in adult leukemias. In contrast to the adult leukemias, hypermethylation of HOX genes exhibited very clear positive correlations in childhood leukemia (Table 3). Furthermore, methylation of HOX genes was also considerably more widespread in the childhood samples than in the adult samples analyzed in this study (Table 1), although analysis of adult ALL samples will be required to confirm whether this is restricted to childhood leukemia. As other reports have suggested that CpG island methylation is, if anything, less frequent in childhood (versus adult) leukemia (35), the high levels of HOX loci methylation suggests that tumor suppressor activity may be more widespread in HOX genes in early life (resulting in selective pressure for inactivation of multiple HOX loci), but is restricted to a small subset of HOX genes in the adult. Alternatively, differences in the mechanisms of gene regulation in early versus adult
hematopoiesis may make HOX loci more susceptible to hypermethylation during childhood leukemogenesis.

Overall, these results suggest an important role for DNA methylation, and consequent loss of expression, of multiple HOX genes in both myeloid and lymphoid leukemia. Furthermore, these studies identify a novel role for HOX genes in both myeloid and lymphoid leukemia.

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