

Quantitative HOX expression in chromosomally defined subsets of acute myelogenous leukemia

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We used a degenerate RT-PCR screen and subsequent real-time quantitative RT-PCR assays to examine the expression of HOX and TALE-family genes in 34 cases of chromosomally defined AML for which outcome data were available. AMLs with favorable cytogenetic features were associated with low overall HOX gene expression whereas poor prognostic cases had high levels. Characteristically, multiple HOXA family members including HOXA3–HOXA10 were jointly overexpressed in conjunction with HOXB3, HOXB6, MEIS1 and PBX3. Higher levels of expression were also observed in the FAB subtype, AML-M1. Spearman correlation coefficients indicated that the expression levels for many of these genes were highly inter-related. While we did not detect any significant correlations between HOX expression and complete response rates or age in this limited set of patients, there was a significant correlation between event-free survival and HOXA7 with a trend toward significance for HoxA9, HoxA4 and HoxA5. While patients with elevated HOX expression did worse, there were notable exceptions. Thus, although HOX overexpression and clinical resistance to chemotherapy often coincide, they are not inextricably linked. Our results indicate that quantitative HOX analysis has the potential to add new information to the management of patients with AML, especially where characteristic chromosomal alterations are lacking.

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Introduction

Homeodomain containing genes encode a set of master transcription factors that act during development to control pattern formation, differentiation and proliferation. Mammals possess at least 39 class I HOX genes grouped into four major clusters (HOXA–D) on four different chromosomes. The homeodomain, a highly conserved 61-amino acid helix–turn–helix DNA binding motif, is the common element defining this gene family.¹ During development, HOX expression occurs temporally in accordance with both the position of a gene within its cluster and in a rostral–caudal manner.² This same pattern can be recapitulated during retinoic acid-induced differentiation of embryonal carcinoma cell lines.³ HOX genes in equivalent positions of different clusters (paralogs) are more closely related than adjacent genes in the same cluster. Moreover, evidence from chimeric gene experiments indicates that, at least in some cases, paralogous HOX genes are functionally

equivalent and that it is the expression level of the paralogous group as a whole which is critical for correct development.⁴

In cancer, deregulation of HOX gene expression and HOX alterations have been most convincingly demonstrated in leukemia. For example, various AML translocations fuse the nucleoporin domain of NUP98 to the homeobox of a major or divergent HOX protein.^{5–7} Similarly, the T cell ALL t(10;14) chromosomal translocation results in overexpression of the homeodomain containing gene, HOX11,⁸ and the t(1;19) translocation fuses E2A with the homeodomain and other regions of PBX1.^{9,10} In an experimental setting, a high proportion of mice transplanted with bone marrow cells overexpressing either Hoxa9, a10, b3 or b8 develop AML.^{11–14} In humans, the Trithorax or MLL/ALL-1 gene, which normally functions to maintain Hox gene expression, is a frequent target of chromosomal rearrangements. Joh *et al*¹⁵ demonstrated that a chimeric MLL-LTG9 protein led to the inhibition of Hoxa7, Hoxb7 and Hoxc9 expression in mouse 32Dcl3 myeloid cells. Recently, Rozovskaia *et al*¹⁶ found that the t(4;11) translocation was associated with increased expression of HOXA9 and MEIS1. Importantly, HOXA9 has been identified in a gene expression array-based screen as the single gene whose expression most correlated with treatment failure in AML.¹⁷

Recently, we examined HOX gene expression in human lung cancer.¹⁸ Interestingly, in both cell lines and direct tumors, the HOXA9 and B9 paralogs and HOXA10 were frequently overexpressed. In contrast, HOXA1 was often down-regulated in concert with WNT7a. Moreover, both in our initial report and from more recent studies, we observed that HOX gene expression in lung tumors tends to fall into two groups; one subset expressing multiple HOX genes at high levels and the other with overall low HOX gene expression. We were intrigued by the potential parallels involving HOX expression patterns in two seemingly diverse types of cancer and by the opportunity to correlate HOX expression with chromosomal alterations. In patients with AML, the presence of a t(8;21), t(15;17) or inv(16) is widely recognized as a favorable cytogenetic subset.¹⁹ In contrast, patients with deletions of chromosome 5 or 7, abnormalities involving 3q, and complex karyotypes define a poor prognostic group.¹⁹ Similarly, alterations of the MLL gene are often associated with a poor prognosis although it appears that t(9;11) translocations may do better than other MLL rearrangements.^{20,21} However, for a substantial number of patients, characteristic chromosomal abnormalities are lacking. These patients and their physicians are left with considerable uncertainty regarding the need for additional therapy.

In this report, we demonstrate that favorable and unfavorable cytogenetic abnormalities are closely associated with distinct HOX expression patterns. When extended to a group of patients with either normal cytogenetics or those with chromosomal alterations of intermediate or unclear signifi-

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cance, the HOX expression patterns were similarly well demarcated and appear to be predictive of outcome, especially for the subset of patients with low levels of expression. Also, the levels of gene expression were clearly not random as there were highly significant correlations among various clustered HOX genes, PBX and MEIS family members.

Materials and methods

Homeobox amplification using degenerate primers

Two blocks of completely conserved amino acids, ELEKEF and KIWFQN, within the homeodomain were chosen for degenerate primers. These were ELEKEF: 5'-GCT CTA GA(A/G)(C/T)T(A/C/G/T) GA(A/G) AA(A/G) GA(A/G) TT and KIWFQN: 5'-GGA ATT C(A/G)T T(C/T)T G(A/G)A ACC A(A/G/T)A T(C/T)T T. PCR conditions consisted of 32–35 cycles of amplification (94°C, 1 min; 40°C, 1 min; 72°C, 1 min) with 4.5 mM MgCl₂ and 2 μM primers. A band of ~120 bp was gel isolated and cloned into a T vector. Individual clones were sequenced and analyzed using BLAST.

Real-time quantitative RT-PCR

Quantitative RT-PCR assays were performed using the ABI 5700 real-time system with SYBR-green fluorescence as previously described including the use of normalization to G3PDH.¹⁸ Briefly, RNAs were prepared from approximately 1–5 × 10⁶ frozen bone marrow cells and extensively treated with DNase using RNeasy columns and protocols recommended by the manufacturer (Qiagen, Valencia, CA, USA). First-strand cDNA was prepared using 1–2 μg of total RNA, SuperScript II (Invitrogen Life Technologies, Carlsbad, CA, USA) and conditions recommended by the manufacturer. For the quantitative RT-PCR, 20 μl reactions were utilized which included 0.5 μl cDNA, 1.6 mM Mg⁺⁺, 200 μM dNTPs, 0.1 μM primers and 0.1 μl AmpliTaq Gold (Applied Biosystems, Wellesley, MA, USA). A 'master mix' containing all components, except the cDNA template, was aliquoted to each reaction tube. The cDNA template was then added utilizing a small volume pipet to increase accuracy. Following an initial 95°C × 10 min to activate the DNA polymerase, 35 cycles of a two-stage PCR were used consisting of 95°C × 15 s and 60°C × 1 min. With this system, we initially began by performing the reactions in triplicate. However, the reproducibility is such that duplicate reactions are entirely sufficient. In the unusual case that duplicates differ by one PCR cycle, reactions are repeated. The PCR products were verified by gel analysis, were shown to consist of only a single band and in most cases were confirmed by DNA sequencing. The absence of contamination was verified by 'no template' controls and 'no reverse-transcriptase' controls were used to confirm the absence of DNA contamination.

The PCR primers were designed to work under a standard set of conditions and to produce products less than 200 bp. Primers were designed using PrimerExpress 1.0 (Applied Biosystems). The actual human gene sequences were obtained from GenBank. Primer sequences were as follows: HOXA1 for 5'-ACC CCT CGG ACC ATA GGA TTA C; HOXA1rev 5'-AAG GCG CAC TGA AGT TCT GTG; HOXA3 for 5'-TGC TTT GTG TTT TGT CGA GAC TC; HOXA3rev 5'-CAA CCC TAC CCC TGC CAA C; HOXA4 for 5'-GCT CTG TTT GTC TGA GCG

CC; HOXA4 rev 5'-AAT TGG AGG ATC GCA TCT TGG; HOXA5 for 5'-AGA TCT ACC CCT GGA TGC GC; HOXA5rev 5'-CCT TCT CCA GCT CCA GGG TC; HOXA7 for 5'-ATC ACT CTA CCT CGT AAA ACC GAC AC; HOXA7rev 5'-ACA TAA TAC GAA GAA CTC ATA ATT TTG ACC; HOXA9 for 5'-GAG TGG AGC GCG CAT GAA G; HOXA9rev 5'-GGT GAC TGT CCC ACG CTT GAC; HOXA10 for 5'-GAG AGC AGC AAA GCC TCG C; HoxA10rev 5'-CCA GTG TCT GGT GCT TCG TG; HOXB3 for 5'-CCG TGT AGA GAT GGC CAA CC; HOXB3rev 5'-CAA TCC CTT GGC CTT CTG G; HOXB6 for 5'-AGG ACA AGA GCG TGT TCG GC; HOXB6rev 5'-GGC CCA AAG GAG GAA CTG TTG; HOXB7 for 5'-CCA GCC TCA AGT TCG GTT TTC; HOXB7rev 5'-CCC GAA CCC GCT CCA TAG; HOXB9 for 5'-AAA AAG CGC TGT CCC TAC ACC; HoxB9rev 5'-ACA CCA AAT ACC AGA CGC TGG; HOXC8 for 5'-CAG AAC TCG TCT CCC AGC CTC; HoxC8rev 5'-GAC TTC AAT CCG GCG CTT TC; HOXC9 for 5'-GCC TGC TGC CTC AGC ACA G; HOXC9rev 5'-GAA CCC TCC CAA ATC GCA AG; MEIS1 for 5'-ATG TGA CAA TTT CTG CCA CCG; Meis1rev 5'-CCT GAA CGA GTA GAT GCC GTG; MEIS2 for 5'-GAT GAA AGA GAC GGC AGC TCC; Meis2rev 5'-GGG TTG AGG TTG CAT CAT CG; PBX1 for 5'-CCC ATC TCA GCA ACC CTT ACC; PBX1rev 5'-CCA TGG GCT GAC ACA TTG G; PBX2 for 5'-CGA ACA CTC GGA CTA TCG CAG; PBX2rev 5'-GCT CCC TCA GCA GGT TCA TG; PBX3 for 5'-GAG CTG GCC AAG AAA TGC AG; PBX3rev 5'-GGG CGA ATT GGT CTG GTT G; PRH for 5'-CTC CAA CGA CCA GAC CAT CG; PRHrev 5'-CCT GTC TCT CGC TGA GCT GC.

Cytogenetic and fluorescent *in situ* hybridization analyses

Chromosome analysis was performed at diagnosis using *in vitro* blood and bone marrow cell cultures (24 and 48 h). R-banding techniques were employed and the karyotype was assigned according to ISCN (1995).⁴⁴ FISH studies using a MLL-specific DNA probe (Appligene Oncor, Ventana Medical Systems, Tucson, AZ, USA) were performed in three cases with 11q23 rearrangement. In every case, FISH revealed a translocation of the MLL gene.

Results

Degenerate RT-PCR screening

We began our analysis of expressed HOX genes using degenerate RT-PCR amplifications in a subset of patient samples and cell lines. This was done primarily to identify HOX genes expressed at high levels relative to other HOX genes and genes which appeared to vary substantially among samples in order to ensure that these loci were included in subsequent real-time quantitative assays. Low-stringency degenerate RT-PCR amplifications, followed by subcloning and DNA sequence analysis, were successfully performed with 10 patient samples and four cell lines. These results are shown in Table 1 for HOXA and HOXB loci as well as the proline-rich homeodomain gene, PRH. We found that most patient samples expressed high relative levels of HOXA9 and HOXA10, although some cases predominantly expressed HOXA5 (patient 17) or HOXB3 (patient 14). Conversely, no HOXA1 or HOXB1 clones were identified suggesting that these genes were underexpressed. The degenerate RT-PCR amplifications used primers corresponding to two blocks of

Table 1 Degenerate RT-PCR: HOX loci as percent of total clones isolated

	No.	A1	A2	A3	A4	A5	A6	A7	A9	A10	B1	B3	B4	B5	B6	B7	B8	B9	PRH
<i>Patient</i>																			
01	33			3	21	3	6	6	27	18		15							
08	28			11	7	14	7	11	43	7									
09	34			3		3	6	3	15	24		15		3	24	3			3
10	38					8	5		34	13		37		3					
13	24			4		8	4		29	54									
14	29					21		3	17			48			3				7
16	27			11	4	4	4	4	26	15		26			4				
17	29			7	10	62			10	3									
19	38			13		35	10	1	25	13									
20	28			3		29			35	10				19		3			
<i>Cell lines</i>																			
HEL	28									36		4			4				50
LAMA84	44								11	7					2				77
RS4;11	29			3				3	72	21									
MV4;11	31								81	10			3						
<i>Genomic DNA</i>	153	11	2	3	7	3	1	5	8	5	12	1	1	1	5	2	1	5	0

Expressed HOX loci were amplified using degenerate primers and analyzed as described in Materials and methods. The total number of clones analyzed from each patient sample or cell line is indicated. For each of the HOX loci, the numbers refer to the percent of total clones derived from that locus. Spaces without numbers indicate that no corresponding clones were identified. Genomic DNA was used as a control for the ability of the degenerate primers to amplify particular loci. Only the relevant control HOX loci are shown here; the complete set of clones has been previously described.¹⁸

highly conserved amino acids, ELEKEF and KIWFQN, within the homeodomain. Since this region in the clustered HOX loci is uninterrupted by an intron, amplification of genomic DNA can be used as a control to assess amplification bias. We previously found that over 30 loci could be amplified from genomic DNA using these primers and conditions.¹⁸ Values for selected loci are shown in Table 1. Thus, the over-representation and under-representation of the HOX genes noted above is not due to PCR amplification biases.

Interestingly, each of the 10 patient samples that generated sufficient degenerate RT-PCR products for subcloning and sequence analysis was associated with poor prognostic cytogenetic features or was subsequently found to have high overall levels of HOX gene expression (see below). Identical experiments using samples from AMLs having favorable cytogenetics resulted in absent PCR products or weak products that had a high background of non-HOX loci after DNA sequencing (not shown). These included samples with the t(8;21) and t(15;17) translocations as well as the inversion 16. The same phenomenon occurred in cell lines. For example, degenerate RT-PCR products were readily obtained from leukemic cell lines such as HEL (erythroid/multipotent AML), LAMA84 (CML blast-crisis) and RS4;11 or MV4;11 (ALL/bi-phenotypic) and subclone isolates from these cell lines showed a predominance of HOXA9 or HOXB9 (Table 1). In contrast, weak or no degenerate RT-PCR products were obtained from the AML cell lines Kasumi-1 and NB4 which contain the t(8;21) and t(15;17) translocations, respectively (not shown). These results suggested that genes from the HOXA group, particularly HOXA3–HOXA10, as well as HOXB3, HOXB6 and, less frequently, HOXB9 are among the most abundantly expressed HOX genes in poor prognostic leukemias. These results also indicated that AMLs with favorable cytogenetic features are associated with lower levels of overall HOX gene expression.

Quantitative real-time RT-PCR assays

Initially, we utilized samples from 17 AML patients for which complete cytogenetic analyses were available (Table 2A, non-consecutive patient numbers 1–21). Subsequently, to verify and expand our findings we analyzed an additional 17 patients (non-consecutive numbers 23–39). The cytogenetic features, age, initial WBC counts/blast percentages and outcome are shown in Table 2A along with the quantitative HOX results (discussed below).

Quantitative real-time RT-PCR assays were established for 19 homeodomain containing genes which included 13 clustered HOX loci, five PBX and MEIS family members and PRH/HEX, a proline-rich homeotic gene which correlates inversely with differentiation in hematopoietic cells.^{22–24} The raw data were obtained in terms of Ct values which refers to the PCR cycle number during exponential amplification at which the product (measured in real-time by SYBR green fluorescence) crosses an arbitrary threshold. To adjust for variations in the amount of RNA, the Ct values for each gene were normalized against the Ct values for the housekeeping gene, G3PDH (ie $\Delta Ct = Ct_{\text{specific gene}} - Ct_{\text{G3PDH}}$). While the resulting ΔCt values are experimentally convenient, they are not readily intuitive (ie they reflect exponential amplification and higher ΔCt s represent lower expression). Instead, the results for a subset of the most informative HOX loci are shown in Table 2A in terms of their relative expression compared to G3PDH, ie expression = $(1/2^{\Delta Ct}) \times 1000$. In this format, a value of 100 is equivalent to 10% of the G3PDH expression level. If more than 34 PCR cycles were required to identify a product, the values are listed as ND (not detected). One technical note is that the identification of late-appearing RT-PCR products resulting from low-abundance transcripts is affected by the amount of input RNA/cDNA. Thus, some products listed as

Table 2A Relative expression of selected HOX loci (x1000) vs G3PDH

Pt. No.	Age	FAB	WBC/blasts	Chromosome feature	Ct G3PDH	A3	A4	A5	A7	A9	A10	B3	B6	B9	MEIS1	MEIS2	PBX2	PBX3	Outcome
<i>AML – favorable cytogenetics</i>																			
05	50	M3	40K/95%	t(15;17)	16.6	0.2	0.2	0.1	0.1	ND	0.02	0.3	ND	0.2	ND	0.01	7	0.04	CR 40 mo.
21	19	M2	9K/75%	t(6;21)	17.2	3	0.2	ND	0.004	ND	0.9	0.9	0.01	2	ND	63	2	0.02	CR 18 mo.
18	70	M4	86K/81%	inv(16)	17.7	0.05	0.2	0.1	0.1	0.6	6	3	0.1	ND	5	1	5	0.4	CR 18 mo., relapsed
<i>AML – unfavorable cytogenetics</i>																			
01	62	M5	78K/80%	t(4;11)(q21;q23)[30] MLL+	20.9	0.9	10	0.6	0.7	2	6	4	0.3	ND	0.7	ND	0.7	0.5	CR 6 mo., relapsed
19	15	M1	22K/	t(10;19;11) MLL+	18.7	9	30	25	20	16	37	ND	0.03	ND	0.9	1	0.6	ND	CR, BMT, CR at 2 yr.
10	36	M0	83K/50%	inv(1)(p21;q24)-7	16.3	3	4	7	2	4	8	15	4	0.1	0.3	0.3	1	0.1	CR, BMT, died 12 mo.
16	62	M1	48K/92%	t(7)p10	19.1	4	18	12	2	7	24	21	7	ND	0.6	ND	2	0.5	CR 7 mo.
32	62	M1	48K/92%	46,XX[5]/46,XX,i(7)(p10)[31]	15.2	32	17	36	0.2	37	7	2	11	0.01	2	0.03	35	4	died 8 mo.
24	75	M1	53K/62%	46,XX[23]/45,XX,-7[7]	15.2	0.9	0.6	0.8	0.02	12	2	1	0.4	0.1	31	0.1	25	7	NR
36	65	M1	160K/90%	46,XX[28]/46,XX,del(7)(q21q31)[7]	15.8	30	15	98	1	107	6	39	40	94	30	1	17	26	died 1 mo.
25	61	M2	18K/82%	del(5)(q13q33),del(9)(q13q31)	15.7	3	2	3	0.1	43	9	16	17	0.1	27	0.1	38	4	NR
06	70	M4	23K/55%	inv(3)(p22;q26)	21.2	ND	2	ND	0.1	3	15	5	ND	0.4	ND	ND	ND	ND	NR, died
15	60	M5	128 K/2%	complex karyotype	17.6	0.02	0.1	0.03	0.01	0.01	0.2	0.2	ND	ND	0.01	2	1.0	0.1	CR, BMT, CR 15 mo.
31	62	M5	46K/4%	complex karyotype	14.3	0.1	2	0.2	0.001	0.2	0.1	0.6	0.01	0.001	0.03	5	25	2	CR 15 mo.
37	78	M5	n.a.	complex karyotype	15.1	0.1	0.2	0.04	0.002	1	0.8	0.2	0.02	0.004	0.1	24	18	1	DOA

Table 2A Continued

Pt. No.	Age	FAB	WBC/blasts	Chromosome feature	Ct G3PDH	A3	A4	A5	A7	A9	A10	B3	B6	B9	MEIS1	MEIS2	PBX2	PBX3	Outcome
AML – other chromosomal features:																			
HOX gene overexpression																			
07	20	M1	210K/99%	del(11)(q24)	22.3	189	134	180	12	44	8	102	29	0.2	129	ND	3	25	CR, BMT, died 4 mo.
08	40	M5a	2K/2%	t(9;11)+8 MLL+	18.6	58	39	26	5	19	30	ND	0.05	ND	34	0.1	7	13	CR, BMT, died 16 mo.
09	60	M5b	114K/69%	46,XX	17.3	17	6	7	3	5	40	17	32	2	2	1	6	0.6	died, 1 mo.
13	69	M4	131K/	46,XY	16.4	19	7	14	6	10	37	0.2	0.1	0.1	0.4	0.03	5	2	died, 1 mo.
14	77	M1	263K/90%	46,XX	17.5	31	50	78	8	27	43	117	40	128	45	0.1	23	21	not treated
17	74	M1	306K/98%	46,XX	17.2	63	33	55	20	17	36	ND	0.01	ND	31	0.5	17	9	not treated
20	52	M4	160K/87%	46,XX	17.6	14	35	25	4	18	50	33	20	0.1	10	ND	2	4	CR 24 mo.
23	51	M5	72K/6%	46,XX	15.6	54	44	77	1	80	9	32	46	25	40	0.02	17	25	CR 15 mo.
26	54	M1	3K/33%	analysis failed	15.6	67	31	108	0.6	134	16	31	10	12	76	98	28	20	Cr 46 mo.
28	69	M4	162/19%	46,XY[32]	15.2	80	78	65	2	199	22	0.2	0.2	0.2	21	0.2	25	29	died 1 mo.
29	65	M4	47K/45%	46,XY[32]	15.5	29	22	38	0.4	53	7	75	40	14	59	3	40	36	died 5 mo.
30	77	M1	263K/90%	46,XY[32]	14.9	74	93	201	1	113	13	167	72	184	51	0.1	47	47	DOA
33	35	M4	58K/51%	46,XY[26]	15.0	0.03	0.3	0.01	0.01	4	1	4	1	0.002	8	0.3	43	2	CR 25 mo.
34	74	M1	306K/98%	46,XX[35]	17.9	1	0.9	0.9	0.02	2	0.3	1	0.2	0.02	6	0.04	42	4	palliative Rx, died 6 mo.
35	72	M5	11K/98%	46,XX[15]/46,XX,add(7)(p22)[17]	15.8	0.05	0.1	0.1	0.01	46	15	0.02	0.1	0.01	67	1	7	0.6	CR 4 mo.
38	72	M2	9K/10%	46,XY[30]	15.6	30	18	26	0.7	63	3	13	34	44	4	0.3	20	17	CR 7 mo.
39	64	M1	94K/98%	46,XX[19]	15.6	70	41	116	3	219	16	8	105	200	84	1	26	44	CR 13 mo.
HOX gene underexpression																			
03	31	M2	126K/	46,XY[79]	18.3	0.7	0.3	0.1	0.3	ND	0.1	0.4	ND	0.4	0.1	0.8	20	0.05	CR 12 mo.
27	73	M1	21K/84%	46,XX[29]/47,XY,+8(1)	15.9	0.3	0.2	0.2	0.01	0.9	0.2	1	0.3	0.04	44	0.2	68	2	CR 8 mo.

Delta Ct values were converted to 'relative expression' as described in the text. A value of 1000 is equal to the expression of G3PDH and a value of 100 is equivalent to 10% of the G3PDH level, etc. The Ct values for G3PDH are shown as an indication of the amount of template (RNA/cDNA) used. Patient characteristics are indicated including age, FAB classification, WBC count at presentation and the percent of blasts. Only the most salient karyotypic features are shown.

ND, not detected after 34 cycles; DOA, died on admission; CR, complete response; NR, no response; BMT, bone marrow transplant.

Table 2B Cut-off values based on ROC curves (see text)

A3	A4	A5	A7	A9	A10	B3	B6	B9	Meis1	Meis2	PBX2	PBX3
0.8	0.44	0.42	0.02	1.69	1.33	1.19	0.037	0.24	0.65	0.29	31.8	3

'ND' might have been detectable with the use of more template. Therefore, the Ct values for G3PDH reflecting template input are indicated in Table 2A. Other loci which were tested but not shown include HOXA1, HOXB7, HOXC8, HOXC9 and PBX1 which were either absent or expressed at low levels in most samples. In contrast, PRH was abundantly expressed (not shown) but did not appear to correlate with outcome (not shown).

We first compared the HOX expression patterns for patient samples having favorable and unfavorable chromosomal alterations (Table 2A). There were three patients with favorable cytogenetics (patients 5, 18 and 21). These included the acute promyelocytic leukemia t(15;17) translocation, as well as the t(8;21) and inv(16) rearrangements affecting the core binding factor alpha and beta components, respectively. Twelve patients had poor prognostic cytogenetic features which consisted of MLL gene rearrangements excluding the t(9;11) translocation, deletions of chromosome 5 or 7, rearrangements involving 3q and complex karyotypes defined as four or more abnormalities. As can be seen, patients with favorable cytogenetics have considerably lower levels of HOX expression than do patients with unfavorable cytogenetic features based on chromosomal deletions or rearrangements. This was especially evident for the HOXA loci, HOXA3–A10. However, several samples with unfavorable cytogenetics also had substantially higher levels of HOXB3, HOXB6 and the TALE (three amino acid loop extension) family members, MEIS1, PBX2 and PBX3. In contrast, each of the three patients with a complex karyotype had low HOX expression.

There were 19 additional patients with either normal cytogenetics ($n = 14$) or alterations of unclear significance. These included additional 7p material (patient 35), trisomy 8 in a single metaphase (patient 27), a deletion of 11q24 (patient 07) and one patient (No. 08) with a t(9;11) translocation which has been reported to convey a less adverse prognosis than other MLL gene rearrangements.²¹ In addition, one patient (No. 26) had no cytogenetic results. Of these 19 patients, two (Nos 03 and 27) had low overall levels of HOX expression. Two other patients (Nos 33 and 34) had low overall HOX levels except for HOXA9 and PBX2.

In our initial studies, we observed that HOXB9 was overexpressed in three of five leukemic cell lines which also generally overexpressed other HOX genes (eg HEL, LAMA84 and K562). However, far fewer patient samples had elevated levels of HOXB9. Interestingly, the four highest levels of HOXB9 were all obtained from samples having an AML-M1 FAB subtype (patients 39, 30, 14 and 36) although, conversely, not all AML-M1 phenotypes had elevated HOXB9 expression. These same cases also tended to have the highest levels of expression for several other of the HOXA and HOXB genes as well as for MEIS1 (Table 2A). Using the Wilcoxon sum-rank test, we compared the quantitative HOX expression results in FAB subtype M1 vs non-M1 subtypes in all 34 patients. Indeed, statistically significant higher levels of expression were observed for HoxA3 ($P = 0.0168$), HoxA4 ($P = 0.0391$), HoxA5 ($P = 0.0041$), MEIS1 ($P = 0.0105$) and PBX3 ($P = 0.0376$) in AML-M1. However, the correlation between

HOXB9 and the M1 phenotype was not statistically significant as several of the patients with AML-M1 lacked elevated expression. We also noted that several patient samples with normal cytogenetics had higher levels of multiple HOX genes than cases with poor prognostic cytogenetics. We have not examined this in more detail but these findings were observed from two sets of samples (non-consecutive numbers 1–21 and 23–39) prepared and studied at different times indicating that this is a reproducible observation.

Finally, there was good correlation between the degenerate RT-PCR data and the specific quantitative analyses. For example, the majority of degenerate RT-PCR products from patient 1 were derived from HOXA4, A9, A10 and B3. High expression of these genes was confirmed by specific RT-PCR assays. Similarly, by degenerate RT-PCR, HOXB3 transcripts comprised 48% of the total clones isolated from patient 14. By real-time quantitative RT-PCR, this same sample was found to express HOXB3 at levels which were approximately 11.7% of G3PDH expression. Conversely, no HOXA1 subclones were identified by degenerate RT-PCR and the subsequent quantitative assays confirmed it was generally expressed at low levels (not shown). Although specific PCR primers can accurately assess the relative expression of individual HOX loci among samples, individual primer pairs may amplify their targets with different efficiencies making a direct comparison between genes less precise. These degenerate RT-PCR amplifications provided a useful complementary approach and a degree of confirmation for the real-time assays.

Analysis of patient outcome

Our study utilized patient samples that had been cytogenetically characterized for the purpose of analyzing quantitative HOX expression in different types of AML. While we were able to obtain some degree of clinical follow-up, the number of samples and the degree of follow-up were too limited for an optimal analysis of response and survival correlations. Of the 34 patient samples, 26 were evaluable for outcome and 25 of these were available for initial response to therapy. Patients were excluded if they died within 1 month of admission (patients 9, 13, 28, 30, 36, 37), were not treated (patients 14, 17), or treated only with palliative intent (patient 34) as indicated in Table 2A. For each of the genes, we determined cut-off values using ROC curves^{25,26} to optimize sensitivity and specificity. These cut-off values are indicated in Table 2B.

With this data set and Fisher's exact test, no statistically significant differences were observed in the complete response (CR) rate for high vs low expression of HOXA9 or any of the other HOX genes. However, the overall CR rate was high in this group of patients. While 7/7 patients with low HOXA9 expression (<1.69, Table 2B) achieved a CR, so did 15/18 patients with higher levels of expression. Next, we used the Spearman correlation coefficients to examine the relationship between age and gene expression. None of the genes was significantly correlated with age. In addition, the Wilcoxon sum-rank test was applied to compare the gene expression values

between patients below and above 60 years of age. No significant correlations were identified. Finally, we used the Cox regression model and log-rank test to look at event-free survival with an event defined as either no response to induction chemotherapy, relapse or death after 1 month. We found that HOXA7 values less than 0.02 were significantly correlated with an improved event-free survival ($P = 0.0474$). Similarly, low expression of other HOX genes was marginally significant for improved event-free survival, ie HoxA9 ($P = 0.0795$), HoxA4 ($P = 0.0744$) and HoxA5 ($P = 0.0925$) reflecting the limited number of patients studied.

HOX gene interactions

We used the quantitative data from the real-time RT-PCR measurements of all 34 AML samples to look for significant inter-HOX gene correlations. These results are shown in Table 3. There was a high correlation between each of the HOXA3–HOXA10 family members and each other. The same relationship existed for those members of the HOXB group that were tested. Similarly, expression levels of nearly all the HOXA and B family members examined were significantly correlated with MEIS1 and PBX3 expression, but generally not with MEIS2 and PBX2. In some cases, based on the specific loci studied, we were able to analyze the expression levels between paralogous genes. For instance, HOXA3 expression was significantly correlated with HOXB3 and, likewise, HOXA9 levels were correlated with HOXB9. However, HOXA3 was also correlated with the expression of HOXB6 and HOXB9 and similar relationships can be seen for other HOXA and B loci. In con-

trast, MEIS1 and MEIS2 levels were unrelated whereas PBX2 and PBX3 expression was significantly correlated. MEIS1 levels were best correlated with PBX3 although a significant relationship was also seen with PBX2. Thus, the picture that emerges from these quantitative data is one of multiple HOX gene underexpression or overexpression and also one in which MEIS1 and PBX3 are the most likely cooperating TALE-family members in adult AML.

Discussion

In this report, we have been able to take advantage of technological improvements in quantitative gene expression using real-time RT-PCR to study chromosomally defined cases of AML with clinical follow-up. Strikingly, we identified substantially different patterns of HOX expression between leukemias associated with favorable vs unfavorable cytogenetic features. Importantly, these differences appear to extend to cases lacking characteristic chromosomal alterations. While the number of patients was too small to show a difference in initial complete response rates, we could demonstrate an improved event-free survival for low expression of HOXA7 and there was marginal significance for other HOXA genes. This is not surprising given the very high correlation among the expression levels of various HOXA and B loci (Table 3).

While these results must be confirmed in larger studies, they suggest that patients with low HOX expression have a favorable outcome. Since each of the good prognostic cytogenetics cases had low HOX expression and each of the poor prognostic group based on chromosomal deletions or rearrangements

Table 3 Spearman (S) correlation coefficients and *P* values

	A3	A4	A5	A7	A9	A10	B3	B6	B9	MEIS1	MEIS2	PBX2	PBX3
A3	1.0000	0.89177 <0.0001	0.93595 <0.0001	0.72605 <0.0001	0.77218 <0.0001	0.54996 0.0008	0.35367 0.0402	0.60875 0.0001	0.45581 0.0067	0.59396 0.0002	-0.1255 0.4796	0.2171 0.2175	0.75921 0.0001
A4		1.0000	0.90029 <0.0001	0.79825 <0.0001	0.74328 <0.0001	0.65413 <0.0001	0.45216 0.0073	0.60603 0.0001	0.36672 0.0329	0.54358 0.0009	-0.3059 0.0785	0.08533 0.6314	0.70057 <0.0001
A5			1.0000	0.72315 <0.0001	0.83809 <0.0001	0.54115 0.001	0.48392 0.0037	0.72178 <0.0001	0.45867 0.0064	0.69207 <0.0001	-0.1372 0.439	0.26333 0.1324	0.81977 <0.0001
A7				1.0000	0.49425 0.003	0.78545 <0.0001	0.22525 0.2003	0.38208 0.0258	0.16439 0.3529	0.33476 0.053	-0.328 0.0583	-0.2785 0.1108	0.3396 0.0494
A9					1.0000	0.55868 0.0006	0.42664 0.0119	0.73446 <0.0001	0.48736 0.0035	0.76011 <0.0001	-0.0521 0.7696	0.32354 0.062	0.81767 <0.0001
A10						1.0000	0.19021 0.2812	0.35529 0.0392	0.10158 0.5676	0.36845 0.032	-0.215 0.2221	-0.2452 0.1622	0.26553 0.1291
B3							1.0000	0.81314 <0.0001	0.61242 0.0001	0.39812 0.0197	-0.2313 0.1882	0.17407 0.3248	0.46052 0.0061
B6								1.0000	0.58492 0.0003	0.66169 <0.0001	-0.1463 0.4089	0.37357 0.0295	0.7284 <0.0001
B9									1.0000	0.33101 0.0559	0.05176 0.7713	0.30313 0.0814	0.49777 0.0027
MEIS1										1.0000	-0.0029 0.9869	0.49196 0.0031	0.79384 <0.0001
MEIS2											1.0000	0.17013 0.3361	-0.0401 0.8221
PBX2												1.0000	0.6021 0.0002

The Spearman coefficients (upper number) and corresponding *P* value (lower number) are shown for various combinations.

had high expression (Table 2), we anticipate that quantitative HOX analysis may be most useful prognostically for patients with normal karyotypes, complex karyotypes and other rearrangements of intermediate or unclear significance. While speculative, our initial results suggest that low HOX expression may be similar to good prognostic cytogenetics. In contrast, while patients with high HOX expression do less well overall, there are clear exceptions (eg patients 20 and 26). Our results are consistent with the report of Golub *et al*¹⁷ using gene expression microarrays, who noted that HOXA9 was the single best predictor of outcome in patients with AML. Our findings extend those observations and demonstrate that multiple genes of the HOXA and B class, especially HOXA3 to HOXA10, are jointly overexpressed in poor prognostic AMLs along with certain TALE family members. Previously, Magli *et al*²⁷ reported that blocks of HOX genes were coordinately expressed in AML cell lines, which supports our findings in direct patient samples. We also found that overexpression of several HOX genes was significantly more frequent in the FAB-M1 subtype although our series lacked cases with FAB-M6 and M7 subtypes.

A more basic question concerns the mechanism of HOX gene overexpression. Using normal CD34⁺ subsets and degenerative RT-PCR, Sauvageau *et al*²⁸ found that approximately 90% of isolated subclones were from the HOXA cluster and most were derived from HOXA4 to HOXA9 transcripts. Kawagoe *et al*,²⁹ as well as Lawrence *et al*,³⁰ found that HOX expression was normally downregulated in bone marrow cells as they progressed from CD34⁺ to CD34⁻ and that this downregulation was lost in AMLs. Therefore it appears, at least in part, that the spectrum of HOX gene expression we observed in AML may not be qualitatively different from corresponding normal cells. Rather the difference might be either quantitative in nature or involve deregulation such that HOX expression is constitutive. For instance in the mouse, constitutive expression of HOX genes including Hoxa7, a9, a10, b3 and b8 results in acute leukemia.¹¹⁻¹⁴ Similarly, recurrent chromosomal translocations in humans involving HOXA9,⁷ PBX1¹⁰ and the homeodomain containing gene, HOX11,⁸ result in deregulated expression and leukemia. Our results demonstrate that there are major differences between good and poor prognostic AMLs in terms of quantitative HOX gene expression. These quantitative alterations may be extremely important. For example, Greer *et al*⁴ demonstrated that paralogous HOX genes can be functionally equivalent and that it is the expression level of the paralogous group as a whole that is critical for correct development.

One genetic mechanism associated with HOX gene overexpression involves rearrangements of MLL. Each of our patient samples which included t(4;11), t(9;11) and t(10;19;11) translocations, as well as two ALL/biphenotypic cell lines with t(4;11) translocations, had HOX gene overexpression. Rozovskaia *et al*¹⁶ recently reported that HOXA9 and MEIS1 were upregulated in ALL with the 4;11 translocation. In AML, Lawrence *et al*³¹ reported that both HOXA9 and MEIS1 were frequently co-expressed, which our results confirm. However, in our AML patient samples, only one of the three MLL rearrangements was associated with MEIS1 overexpression, whereas most of the patients with normal cytogenetics or other chromosomal features had MEIS1 overexpression (Table 2). While MLL rearrangements have been described in the absence of chromosomal rearrangements by standard metaphase analysis,³² the reported frequency (11%) is far too low to account for our observations. Interestingly, we identified the same dichotomy of high and low HOX

expression in lung cancer¹⁸ where MLL rearrangements have not been reported. Thus, it is possible that deregulation of other components in a common pathway leads to similar HOX expression phenotypes in different malignant diseases.

Most of the clustered HOX genes that have been directly implicated by retroviral activation in murine leukemia (eg HOXA7, A9, A10, B3 and B8) were identified as overexpressed in our degenerate RT-PCR survey and subsequently confirmed by real-time quantitative assays. An exception was HOXB8 which we did not observe. The effects of constitutively expressing these genes in mouse hematopoietic cells are profound. For example, HOXA9 infected bone marrow cells develop a typical appearing AML with a high percentage of immature cells and blasts.³³ HOXA9 also immortalizes and confers a proliferative advantage to primary murine myeloid cells.³⁴ Similarly, HOXA10 overexpression causes leukemia in mice¹² and, in human cord blood CD34⁺ progenitors, leads to increased myeloid proliferation and the appearance of blasts.³⁵ Expression of HOXB3 in mice results in a marked proliferation of myeloid cells with tissue infiltration and splenomegaly, although the cells retain some capacity to differentiate.³³ In addition to the above genes, we found that HOXA4 and HOXA5 were overexpressed. A paralogous gene in mouse, HOXB4, causes a 50-fold increase in transplantable murine stem cells³⁶ and overexpression of human HOXA5 causes a shift in the differentiation of CD34⁺ cells from erythroid to myeloid especially when more primitive CD34⁺ subsets were infected.³⁷

Similar results have been obtained with deregulation of TALE homeodomain genes which were initially found to be activated in murine leukemias via retroviral integrations.^{38,39} We observed that MEIS1 and PBX3 were frequently overexpressed, especially in conjunction with other HOX loci. PBX and HOX proteins form hetero-dimers which together recognize longer and more specific DNA target sequences,⁴⁰ and at least part of the HOXA9 effects on myeloid cells require its Pbx interaction domain.³⁴ Recently, PBX1 has been shown to bind transcriptional repressor elements (N-CoR) and co-precipitate the SIN3a repressor complex.⁴⁰ Thus, transcriptional repression by HOX-PBX heterodimers is likely to be an important mechanism in human leukemogenesis. In this regard, Ferrara *et al*⁴¹ demonstrated that 100% of AML blasts underwent *in vitro* differentiation in response to combined treatment with retinoic acid and trichostatin A, an inhibitor of histone deacetylase proteins which are contained in repressor complexes. MEIS proteins regulate the nuclear accumulation of PBX and together form a trimeric complex with HOX proteins.^{42,43} Thus, mechanistically the overexpression of HOX, PBX and MEIS family members very likely leads to a greater effects on HOX target genes.

In conclusion, the quantitative analysis of HOX gene expression has the potential to add new information to the clinical management of patients especially where characteristic chromosomal alterations are lacking. Moreover, it seems likely that the overexpression of multiple HOX genes contributes to the pathogenesis of poor prognostic AML. While there are many remaining scientific questions to be answered, the mechanism(s) of HOX overexpression and its molecular consequences are particularly relevant.

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