An early thymic precursor phenotype predicts outcome exclusively in HOXA-overexpressing adult T-cell acute lymphoblastic leukemia: a Group for Research in Adult Acute Lymphoblastic Leukemia study

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ABSTRACT

Gene expression studies have consistently identified a HOXA-overexpressing cluster of T-cell acute lymphoblastic leukemias, but it is unclear whether these constitute a homogeneous clinical entity, and the biological consequences of HOXA overexpression have not been systematically examined. We characterized the biology and outcome of 55 HOXA-positive cases among 209 patients with adult T-cell acute lymphoblastic leukemia uniformly treated during the Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL)-2003 and -2005 studies. HOXA-positive patients had markedly higher rates of an early thymic precursor-like immunophenotype (40.8% *versus* 14.5%, *P*=0.0004), chemoresistance (59.3% *versus* 40.8%, *P*=0.026) and positivity for minimal residual disease (48.5% *versus* 23.5%, *P*=0.01) than the HOXA-negative group. These differences were due to particularly high frequencies of chemoresistant early thymic precursor-like acute lymphoblastic leukemia in HOXA-positive cases harboring fusion oncoproteins that transactivate *HOXA*. Strikingly, the presence of an early thymic precursor-like immunophenotype was associated with marked outcome differences within the HOXA-positive group (5-year overall survival 31.2% in HOXA-positive early thymic precursor *versus* 66.7% in HOXA-positive non-early thymic precursor, *P*=0.03), but not in HOXA-negative cases (5-year overall survival 74.2% in HOXA-negative early thymic precursor *versus* 57.2% in HOXA-negative non-early thymic precursor, *P*=0.44). Multivariate analysis further revealed that HOXA positivity independently affected event-free survival (*P*=0.053) and relapse risk (*P*=0.039) of chemoresistant T-cell acute lymphoblastic leukemia. These results show that the underlying mechanism of *HOXA* deregulation dictates the clinico-biological phenotype, and that the negative prognosis of early thymic precursor acute lymphoblastic leukemia is exclusive to HOXA-positive patients, suggesting that early treatment intensification is currently suboptimal for therapeutic rescue of HOXA-positive chemoresistant adult early thymic precursor acute lymphoblastic leukemia. *Trial Registration: The GRAALL-2003 and -2005 studies were registered at http://www.clinicaltrials.gov as #NCT00222027 and #NCT00327678, respectively.*

Introduction

Modern management of acute leukemia is predicated upon the identification of biologically distinct subgroups whose prognosis might benefit from timely alterations in treatment intensity.[1] T-cell acute lymphoblastic leukemia (T-ALL) is associated with a wide range of acquired genetic abnormalities that contribute to developmental arrest and abnormal proliferation of malignant lymphoid progenitors.[2,3] Despite the diversity of observed mutations and deletions, transcriptional microarray studies have consistently shown that T-ALL can be classified by five recurrent patterns of gene expression, namely the Immature/LYL1, TAL1, TLX1, TLX3 and HOXA clusters.[4-6] The last subgroup is characterized by aberrant activation of the *HOXA* gene locus on chromosome 7. Homeobox (HOX) factors normally regulate the transcription of genes that are critical for development and proliferation[7,8] In murine models, Hoxa overexpression induces a hematopoietic differentiation block and leukemic transformation of normal progenitor cells,[9-11] suggesting that HOXA overexpression may directly affect the biology of human T-ALL.

HOXA-positive (HOXA^{Pos}) T-ALL is associated with a number of recurrent chromosomal translocations. Juxtaposition with *TCRB* regulatory elements *via* translocation (7;7)(p15;q34) or inversion(7)(p15q34) directly activates *HOXA* by a *cis*-like mechanism;[12,13] however, the majority of *HOXA* locus deregulation has been described to occur in *trans*. Fusion proteins that arise from rearrangements involving the Mixed Lineage Leukemia gene (*MLL*)[4], *MLLT10* (formerly *AF10*)[14-17] and the *SET-NUP214* translocation[18] have been shown to recruit DOT1 Ligand (DOT1L), which stimulates *HOXA* expression through aberrant methylation of Lys79 of Histone H3.[19,20] DOT1L is additionally known to methylate a range of target genes that are also likely to contribute to the leukemic phenotype,[21] and it is therefore probable that the molecular mechanisms of leukemogenesis within the HOXA^{Pos} subgroup are heterogeneous.

In support of this, *HOXA* dysregulation does not necessarily predict inclusion in the HOXA gene expression cluster, as a proportion of these cases segregate preferentially with the Immature/ LYL1 subgroup.[5,6] This immature cluster shows a high level of enrichment of transcripts that are associated with early thymic precursor (ETP)-ALL,[22] a subgroup of T-ALL that exhibit a stem cell/immature myeloid-like immunophenotype, resistance to treatment and poor outcome.[15,23-26] Genomic analysis of ETP-ALL has revealed high rates of mutations in factors involved in cytokine receptor and RAS signaling, hematopoiesis and epigenetic modification,[15] but the precise molecular basis of these patients' adverse prognosis remains unclear.

We analyzed the biological and clinical characteristics of a cohort of HOXA^{Pos} adult T-ALL patients who were treated as part of the Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL)-2003 and -2005 studies. Notably, we found that the underlying mechanism of *HOXA* deregulation is highly predictive of phenotypic immaturity and early treatment resistance. Survival analyses revealed that the HOXA^{Pos} group did not have an inferior overall outcome, and that poor prognosis was restricted to a subset of patients who had an ETP-like immunophenotype, chemoresistance and activation of the *HOXA* locus in *trans*. Strikingly, these parameters did not predict survival in the HOXA^{Neg} group, indicating that the adverse prognosis of ETP-ALL in adults is exclusive to HOXA^{Pos} patients.

Methods

The GRAALL-2003 and GRAALL-2005 studies

The GRAALL-2003 study was a phase II trial that enrolled 77 adults with T-ALL between November 2003 and November 2005.[27] The GRAALL-2005 study was the subsequent phase III trial that included randomized evaluation of hyper-fractionated cyclophosphamide in induction and late intensification. Two-hundred and sixty-one adults with T-ALL were enrolled between May 2006 and September 2011.

Informed consent was obtained from all patients at trial entry. Studies were conducted in accordance with the Declaration of Helsinki and approved by local and multicenter research ethical committees. The complete study protocols are detailed in the *Online Supplementary File* 'GRAALL_2003_2005 protocol'. At a point date on March 1st 2013, the median follow-up was 2.9 years (5.5 and 2.7 years for GRAALL-2003 and GRAALL-2005, respectively).

The sole criteria for inclusion in the current project were a diagnosis of T-ALL and availability of diagnostic material for *HOXA9* measurement. Survival outcomes of the 209 patients (42 GRAALL-2003 and 167 GRAALL-2005) who fulfilled these criteria did not differ from those of the remaining 129 T-ALL patients of the study cohorts. A full comparison of the clinical features of each group is shown in *Online Supplementary Table S1*.

Statistical analysis

The considered cut-off level for dichotomizing white blood cell count was 100x10^9/L. The considered cut-off ratio for dichotomizing HOXA status was defined as the lowest HOXA ratio associated with a genetic abnormality known to activate *HOXA*. Categorical data are presented as percentages and compared using Fisher exact tests. Continuous data are presented as medians and inter-quartile ranges and compared using Mann-Whitney tests. Censored data (i.e. overall survival, event-free survival and disease-free survival) were analyzed using Cox models. Competing risk events (i.e. cumulative incidence of relapse) were analyzed using Fine & Gray models. Overall survival and event-free survival were calculated from the date of pre-phase initiation. Events considered for event-free survival were induction failure, first hematologic relapse and death from any cause in first complete remission. The cumulative incidence of relapse and disease-free survival were calculated from the date of achieving complete remission. The chosen adjustment covariates were defined based on their clinical relevance, in order to minimize the risk of over-adjustment. The adjustment covariates were white blood count, stem cell transplantation, risk classifier, ETP status and chemosensitivity status. Stem cell transplantation was analyzed as a time-dependent covariate using the Mantel-Byar approach. Interactions were assessed by introducing interaction terms in the multivariate models. Specific hazards of relapse and hazard ratios are given with 95% confidence intervals. All tests were two-sided with a significance level of 0.05, except for interactions for which a significance level of 0.1 was considered. Statistical analyses were performed using Stata/mp 13.1 (Stata Corporation, College Station, TX, USA).

Additional details are provided in the *Online Supplementary Methods*.

Results

Definition of HOXA-positive adult T-cell acute lymphoblastic leukemia

In order to characterize the spectrum of *HOXA* deregulation in adult T-ALL, we measured the levels of *HOXA9* in the T-ALL cohort of the GRAALL-2003 and -2005 studies. Diagnostic material was available for 209 of 328 patients. *HOXA9* levels were normalized to a reference gene and expressed as a 'HOXA ratio' (see *Online Supplementary Methods*). This ratio varied greatly among samples, ranging from 0 to 66.2 (Figure 1A). Most patients had low HOXA ratios, and the median was 0.06.

As HOXA^{Pos} T-ALL comprises cases that express leukemic fusion proteins which have been shown to upregulate *HOXA* transcription directly, we defined the cut-off for positivity as the lowest HOXA ratio associated with a genetic abnormality known to activate the *HOXA* locus. This threshold of 0.66, as defined by the lowest ratio in a *PICALM-MLLT10* T-ALL, classified 55/209 cases as HOXA^{Pos}. Of note, 52 of these cases corresponded to the highest quartile of HOXA ratio in the entire study cohort.

Thirteen HOXA^{Pos} cases had sufficient diagnostic material available for evaluation of the global pattern of *HOX* locus transcription. As expected, the entire *HOXA* gene cluster was deregulated in 100% of these samples (Figure 1B). We then tested eight HOXA^{Neg} samples from the third quartile of expression, and found that 0% exhibited activation of *HOXA* gene transcription. We additionally quantified the levels of *HOXA5* by quantitative real-time polymerase chain reaction in 180 patients, and found that this measurement was strongly correlated with *HOXA9* (*Online Supplementary Figure S1*). We therefore conclude that global deregulation of the *HOXA* locus in adult T-ALL could be predicted by a HOXA ratio of 0.66, corresponding to about 25% of patients overall.

Molecular mechanism of HOXA *deregulation in adult T-cell acute lymphoblastic leukemia*

The 55 HOXA^{Pos} patients were extensively investigated for anomalies known to cause HOXA overexpression in T-ALL: translocations involving *MLL*, *PICALM-MLLT10*, *SET-NUP214* and *TCRB-HOXA* ('primary screen' in Figure 2A). Comprehensive assessment was completed in 52/55 cases. The three remaining cases (including one from the third quartile of HOXA ratio) did not undergo *TCRB-HOXA* testing due to a lack of sample availability. One of these three cases also lacked sufficient material for *MLL* fluorescence *in situ* hybridization, although the absence of any chromosome 11q abnormality by karyotyping made the possibility of *MLL* translocation unlikely.

This initial screen identified an explicatory translocation in 33 HOXA^{Pos} patients (Figure 2A,B and Table 1). These

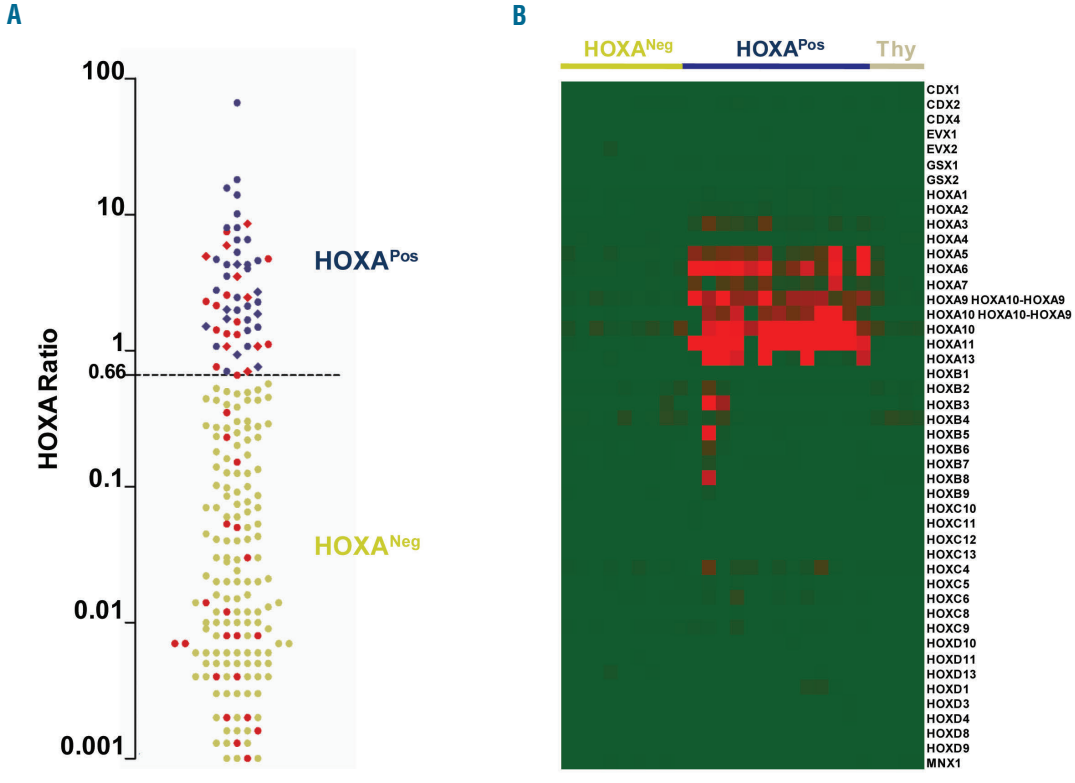

Figure 1. Definition of HOXA^{Pos} adult T-ALL. (A) HOXA ratios (*HOXA9/ABL*) were calculated for 209 T-ALL patients treated as part of the GRAALL-2003 and -2005 studies. Each point represents an individual measurement. The threshold of HOXA positivity (0.66) was defined by the lowest HOXA ratio associated with a known *HOXA*-deregulating abnormality (*PICALM-MLLT10*). For HOXA^{Pos} cases for which the etiology of *HOXA* locus activation remained undefined, the measurement point is indicated by a diamond. Cases with an ETP-like phenotype are shown in red. (B) Taqman low density array (TLDA) analysis of *HOX* gene expression in HOXA^{Neg} (n = 8) and HOXA^{Pos} (n = 13) patients, compared with normal thymus (Thy) (n = 3) controls. HOXA^{Pos} cases exhibit specific activation of the *HOXA* locus, while HOXA^{Neg} samples have uniformly low expression of all *HOX* genes.

comprised 11 *TCRB-HOXA*, eight *PICALM-MLLT10*, nine *SET-NUP214* and six *MLL* rearrangements. One patient had co-existing translocations of both *PICALM-MLLT10* and *TCRB-HOXA*. Two further patients were found to have the *NUP98-RAP1GDS1* translocation, while the presence of a visible t(10;12) (p12;q14) translocation during conventional karyotyping led us to investigate and confirm a *NAP1L1-MLLT10* translocation in another case (Figure 2C,D). For five patients from whom material was available, we also performed RNA-sequencing in an effort to identify cryptic *HOXA*-activating fusions. This revealed two additional *MLLT10* translocations, one involving the *XPO1* locus and the other involving the *DDX3X* locus (Figure 2E). We additionally performed fluorescence *in situ* hybridization screening for *MLLT10* in ten patients, identifying one further *MLLT10* rearrangement with an unknown partner.

After completion of these investigations, the etiology of *HOXA* activation remained undefined in 16/55 HOXA^{Pos} patients. Where possible (12/16 cases), we performed polymerase chain reaction testing for *NUP98-RAP1GDS1*, *XPO1-MLLT10*, *DDX3X-MLLT10* and *NAP1L1-MLLT10*, but all evaluated cases were negative for each translocation.

Clinico-biological characterization of HOXA-positive adult T-cell acute lymphoblastic leukemia

Initial comparison of the clinico-biological characteristics of HOXA^{Pos} and HOXA^{Neg} T-ALL revealed substantial heterogeneity within the HOXA^{Pos} group, whereby the patients with *cis*-activated *TCRB-HOXA* differed markedly from those with *trans*-activated *MLL*, *MLLT10*, *SET-NUP214* or *NUP98-RAP1GDS1*. For the ensuing analyses, the HOXA^{Pos} patients were therefore grouped according to the underlying mechanism of locus deregulation (Table 1). A comprehensive breakdown of the characteristics of all HOXA^{Pos} genetic subgroups is shown in *Online Supplementary Table S4*.

HOXA^{Pos} and HOXA^{Neg} cases did not differ significantly with regard to sex (male:female ratio 1.9 *versus* 3.8; *P*=0.22), median age (30.4 *versus* 31.9 years; *P*=0.4), white blood cell count (33.0 *versus* 37.7x10^9/L; *P*=0.3) or central nervous system involvement (18.5% *versus* 11.3%; *P*=0.23). There were similar rates of *NOTCH1/FBXW7* mutations (65.5% *versus* 72.1%; *P*=0.39). In addition, analysis using our recently described oncogenetic risk predictor[28] that includes classification by mutations in *NRAS*, *KRAS* and *PTEN* did not reveal any differences between the two groups (49% HOXA^{Pos} high risk *versus* 41.3% HOXA^{Neg} high risk; *P*=0.41).

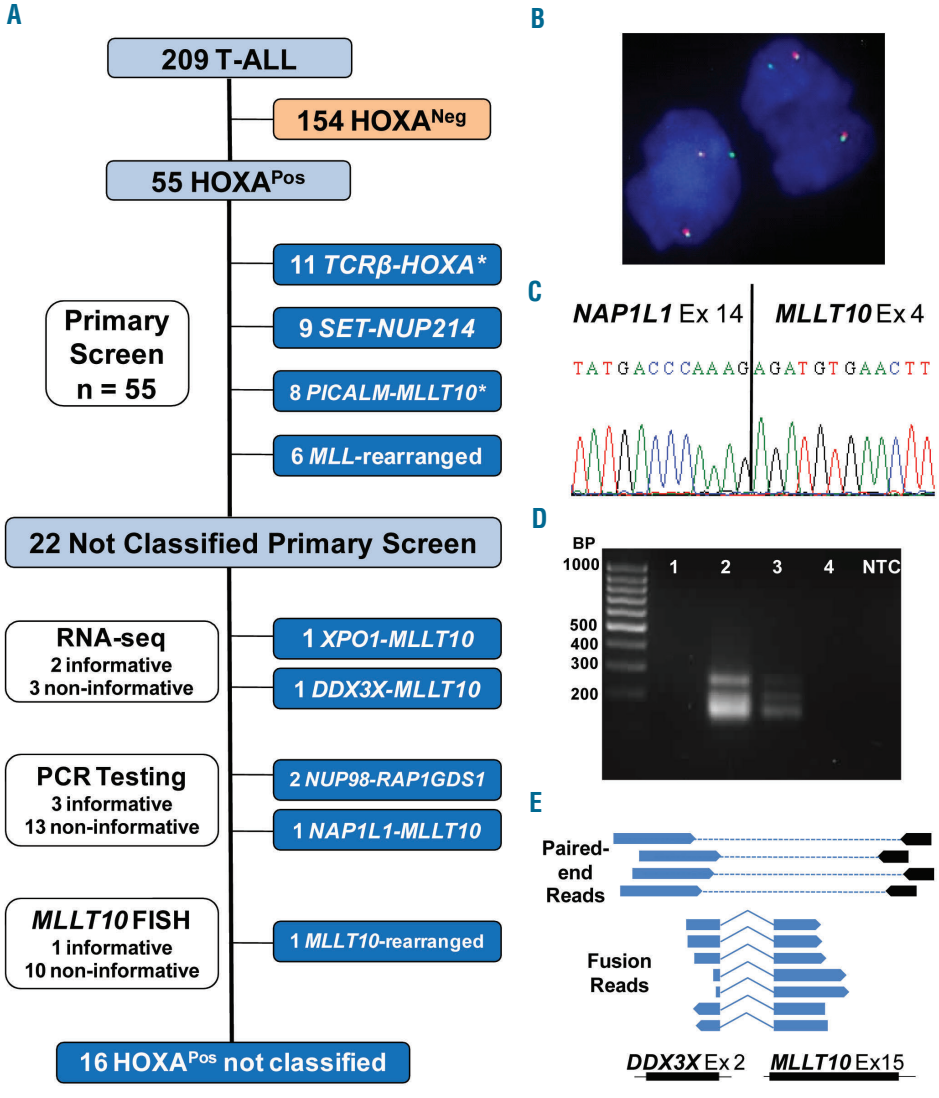

Figure 2. Molecular mechanism of *HOXA* deregulation in adult T-ALL. (A) Flowchart of investigation of the etiology of HOXA positivity. Numbers of patients in each diagnostic subgroup are shown. *One patient had co-existing diagnoses of both *TCRβ-HOXA* and *PICALM-MLLT10*. RNA-seq = RNA-sequencing. FISH = Fluorescence *in situ* hybridization. Representative results are depicted in panels (B) – (E). (B) Positive FISH for *TCRβ-HOXA*. (C) Direct (Sanger) sequencing confirms the presence of the *NAP1L1-MLLT10* translocation. Exon numbers are indicated. (D) Reverse transcriptase polymerase chain reaction amplification of *NUP98-RAP1GDS1* using fusion-specific primers. Patients 2 and 3 are positive for *NUP98-RAP1GDS1*, while patients 1 and 4 are negative. NTC = no template control. (E) Diagnosis of *DDX3X-MLLT10* by RNA-sequencing. A schematic representation of paired-end and fusion-spanning reads is shown. Plain lines indicate split reads spanning two exons, and dotted lines indicate two reads of the same fragment. Exon numbers are indicated.

Notably, HOXA^{Pos} leukemias were more likely to be both genotypically and phenotypically immature than their HOXA^{Neg} counterparts. Genotypic immaturity, as defined by lack of detectable rearrangement of the *TCRB* locus in the leukemic blasts,[29] was considerably more common in HOXA^{Pos} cases (56.9% *versus* 16.5%; *P*<0.0001). Furthermore, HOXA^{Pos} samples had significantly higher rates of an ETP-like immunophenotype,[25] as defined by low expression of CD5, lack of CD1a/CD8, and expression of at least one stem cell or myeloid antigen (CD34, CD13, CD33, CD117). Rates of ETP-like immunophenotype were 40.8% for HOXA^{Pos} patients, compared with 14.5% for HOXA^{Neg} cases (*P*=0.0004). Strikingly, this immaturity was not observed amongst the *TCRB-HOXA* subgroup, which presented a more mature cortical profile of developmental arrest. As the *TCRB-HOXA* rearrangement is the only subgroup of HOXA^{Pos} T-ALL that presents a *cis*-activation of the *HOXA* locus, this suggests that the stage of differentiation block of T-ALL blasts correlates with the mechanism of *HOXA* deregulation.

There were also major differences between the groups with regard to initial treatment response. The HOXA^{Pos} subgroup had significantly lower proportions of both early corticosteroid response (36.4% *versus* 59.7%; *P*=0.0045) and early bone marrow chemosensitivity (40.7% *versus* 59.2%; *P*=0.026) in comparison with the HOXA^{Neg} cases. These differences were not seen when the patients with *cis*-activated *TCRB-HOXA* were analyzed separately, as these had comparatively high rates of both corticosensitivity (72.7%) and chemosensitivity (90.9%). Assessment of minimal residual disease (MRD) response gave similar results, as HOXA^{Pos} cases were more likely than HOXA^{Neg} cases to have positive (>10^{-4}) MRD1 after induction (48.5% *versus* 23.5%; *P*=0.01). Again, these differences were confined to the *trans*-activated HOXA^{Pos} subgroup, as all *TCRB-HOXA* patients who were assessed were negative for MRD1. Taken together with the observed heterogeneity of developmental arrest between *cis*- and *trans*-activated cases, these results suggest that the underlying mode of *HOXA* activation affects the biological phenotype of HOXA^{Pos} T-ALL.

HOXA positivity is not directly linked to altered clinical outcome in adult T-cell acute lymphoblastic leukemia

In order to determine whether HOXA positivity correlates with prognosis in adult T-ALL, we performed global survival comparisons of HOXA^{Pos} and HOXA^{Neg} cases. There were very similar outcomes in the two cohorts for 5-year overall survival (55.0% for HOXA^{Pos} *versus* 58.1% for HOXA^{Neg}; *P*=0.91), event-free survival (45.9% *versus* 48.9%; *P*=0.95) and disease-free survival (50% *versus* 51.1%; *P*=0.92) (Figure 3A,B and *Online Supplementary Figure S2A*). Additional analysis according to HOXA ratio revealed no differences in survival between quartile groups (*Online Supplementary Figure S2B-D*), further indicating a lack of direct correlation between the degree of *HOXA* locus activation and patient outcome. The limited size of the HOXA^{Pos} subgroups precluded satisfactory analysis of the survival risks associated with individual translocations (*Online Supplementary Figures S3A-C*).

Table 1. Clinico-biological characteristics of HOXA^{Pos} and HOXA^{Neg} adult T-ALL.

	HOXA^{Neg}	HOXA^{Pos}				Total	*P* value† Neg *vs.* Pos	*P* value† Trans *vs.* cis
***HOXA* activation mechanism**	**NA**	**All HOXA^{Pos}**	***Trans***	***Cis***	**Unknown**	**NA**		
N. (%)	154 (74)	55 (100)	29 (53)	11 (20)	16 (29)	209		
***TCR* molecular status**								
Immature (IM0. Imd. Img)	17%	57%	70%	10%	60%	28%	**<0.0001*** **	**0.002** **
αβ lineage	72%	27%	11%	70%	27%	60%	**<0.0001*** **	**0.0011** **
γδ lineage	11%	16%	19%	20%	13%	13%	0.46	1
ETP immunophenotype	15%	41%	44%	11%	53%	21%	**0.0004*** **	0.11
EGIL 1-2	23%	60%	74%	27%	56%	33%	**<0.0001*** **	**0.001** *
EGIL 3	63%	28%	11%	55%	38%	54%	**<0.0001*** **	**0.009** *
EGIL 4	14%	11%	15%	18%	6%	13%	0.81	1
***NOTCH1/FBXW7*^{mutated}**	72%	65%	45%	100%	81%	70%	0.39	**0.001** **
High risk classifier*	41%	49%	67%	11%	38%	43%	0.41	**0.006** **
Clinical subsets analyzed								
Age median, years	30.4	31.9	30.2	35	31.2	30.5	0.48	0.68
Age >35 years	40%	40%	38%	46%	41%	40%	1	0,73
Sex ratio M/F	3.8	1.9	2.2	1.8	1.8	3.1	0.22	0.7
White blood cell count median, x10^9/L	37.7	33	33	56.1	22.2	37	0.3	0.8
White blood cell count >100x10^9/L	30%	16%	21%	0%	24%	26%	0.052	0.16
Central nervous system involvement	11%	19%	18%	18%	24%	13%	0.23	1
Corticosensitivity	60%	36%	17%	73%	47%	54%	**0.004** **	**0.0017** **
Chemosensitivity	59%	41%	21%	91%	44%	54%	**0.026***	**<0.0001*** **
Complete remission	94%	93%	97%	100%	82%	94%	0.75	1
Relapse	32%	26%	29%	9%	29%	30%	0.48	0.4
Death	32%	35%	38%	27%	35%	33%	0.74	0.72
MRD ≥ 10^{-4}	24%	48%	68%	0%	33%	31%	**0.01***	**0.0052** **

HOXA^{Pos} cases are grouped according to the underlying mechanism of HOXA *locus activation:* trans- *(*MLL, MLLT10, SET-NUP214, NUP98-RAP1GDS1*),* cis- *(*TCR -HOXA*) or unknown. TCR: T-cell receptor; EGIL: European Group for the Immunological classification of Leukaemia; ETP: early thymic precursor. *High risk classifier incorporating the effects of* RAS *and* PTEN *mutations*[25]. †*t test (Mann-Whitney test) or Fisher exact test were used where appropriate. Statistically significant differences are highlighted in bold.*

Overall, these results suggest that despite the high associated rates of early treatment resistance, HOXA positivity does not influence patient outcomes directly.

An early thymic precursor-like immunophenotype is associated with an inferior prognosis in HOXA-positive, but not HOXA-negative adult T-cell acute lymphoblastic leukemia

Patients with HOXA^{Pos} or HOXA^{Neg} T-ALL had markedly different profiles of both genotypic and phenotypic maturity (Table 1). In particular, cases with *trans*-activation of the *HOXA* locus had very high rates of an ETP-like immunophenotype. This led us to speculate that HOXA^{Pos} ETP-ALL may constitute a distinct subgroup of adult T-ALL, and that HOXA overexpression might modulate the biology of ETP-ALL.

We initially performed univariate survival analyses after division of the HOXA^{Pos} and HOXA^{Neg} patients into ETP and non-ETP cohorts. We found that the presence of an ETP-like immunophenotype correlated with marked differences in outcome within the HOXA^{Pos} group for overall survival (31.2% in HOXA^{Pos} ETP *versus* 66.7% in HOXA^{Pos} non-ETP; P=0.03), event-free survival (25% *versus* 52.8%; P=0.02), disease-free survival (28.6% *versus* 53.6%; P=0.02) and cumulative incidence of relapse (53.7% *versus* 25.4%; P=0.0095) at 5 years (Figure 4). In contrast, these survival differences were not seen in HOXA^{Neg} patients, among whom ETP and non-ETP cases had similar 5-year overall survival (74.2% in HOXA^{Neg} ETP *versus* 57.2% in HOXA^{Neg} non-ETP; P=0.44), event-free survival (60.8% *versus* 50.7%; P=0.72), disease-free survival (64.7% *versus* 52.2%; P=0.9) and cumulative incidence of relapse (29.2% *versus* 39.2%; P=0.57) (Figure 4).

Multivariate analysis revealed that the statistical interaction between HOXA positivity and ETP-like phenotype did not reach independent significance when other prognostic factors were included in the model (*Online Supplementary Table S5*). As an ETP-like phenotype was usually associated with a profile of additional biological characteristics that might also influence patient outcome (Table 1 and *Online Supplementary Table S4*), we used multivariate statistical models to determine which clinical covariates were specifically modulated by the presence of HOXA. These analyses revealed a significant interaction between HOXA positivity and chemosensitivity, whereby HOXA positivity conferred significant decreases in both the event-free survival and cumulative incidence of relapse of chemoresistant patients (P=0.053 and P=0.039, respectively). Strikingly, this effect was independent of white blood cell count, stem cell transplantation, EGIL classification, and our recently reported risk classifier that integrates the prognostic effects of mutations of *NOTCH1*, *FBXW7*, *RAS* and *PTEN*[25] (*Online Supplementary Table S5*). Taken together, these analyses indicate that the prognostic value of an ETP-like chemoresistant phenotype in adult T-ALL is specific to the HOXA^{Pos} cohort.

Discussion

We have characterized the clinico-biological consequences of HOXA positivity in a large cohort of adult T-ALL patients uniformly treated as part of the GRAALL-2003 and -2005 studies. We found that *HOXA9* transcript levels robustly predicted global *HOXA* locus activation, thereby justifying this measurement as a proxy for definition of HOXA positivity. T-ALL cases exhibited a wide and continuous range of HOXA ratios, making rigid categorization of HOXA^{Pos} T-ALL difficult. In order to arrive at a practical cut-off, we chose the lowest ratio associated with a known *HOXA*-activating translocation. The legitimacy of this approach was supported by the finding that the *HOXA* locus was globally activated exclusively in HOXA^{Pos} patients, while diagnostic screening revealed no evidence of *HOXA*-activating translocations in patients with borderline ratios. We nevertheless cannot exclude the possibility that some cases classified as HOXA^{Neg} may have lesser degrees of *HOXA* activation which might affect disease biology. In addition, HOXA positivity was unexplained in 16 patients, despite extensive investigation. These cases had similar clinico-biological profiles to those of the *trans*-activated HOXA^{Pos} cohort, suggesting the presence of similar mechanisms of *HOXA* deregulation which remain to be discovered.

Although *HOXA* overexpression has been linked to adverse prognosis in acute myeloid leukemia,[30-33] the clini-

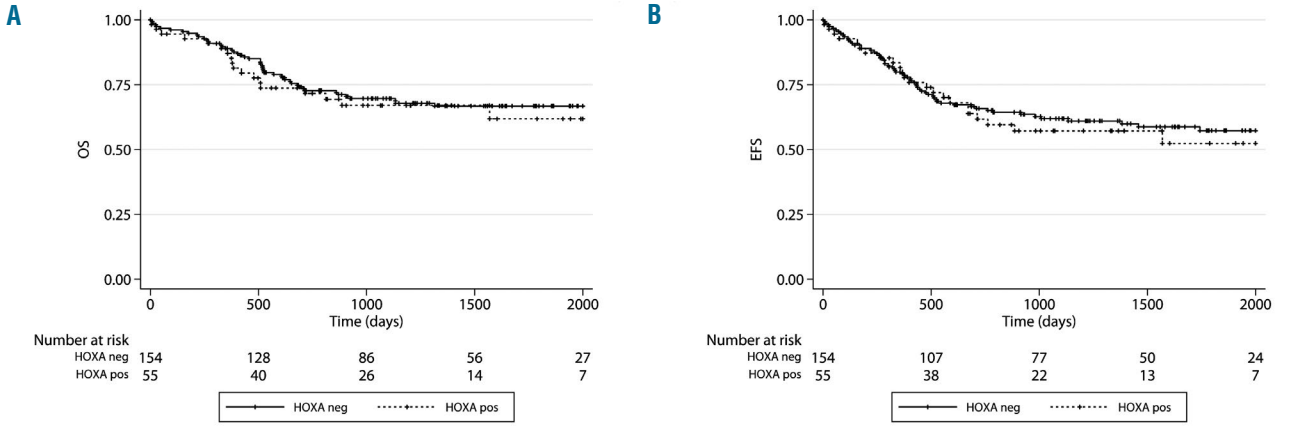

Figure 3. HOXA^{Pos} and HOXA^{Neg} adult T-ALL patients have similar survival outcomes. (A) Overall survival (OS) and (B) event-free survival (EFS) of HOXA^{Pos} and HOXA^{Neg} patients are shown. Five-year OS was estimated to be 55% (95% CI, 38.46% - 68.79%) in the HOXA^{Pos} group, as compared with 58.1% (95% CI, 48.50% - 66.49%) in HOXA^{Neg} cases. The corresponding figures for 5-year EFS were 45.9% (95% CI, 30.58% - 59.91%) for HOXA^{Pos} and 48.9% (95% CI, 39.73% - 57.53%) for HOXA^{Neg}.

cal impact of HOXA positivity in T-ALL has not previously been examined. We found that survival did not differ between the HOXA^{Pos} and HOXA^{Neg} patients when the groups were compared as a whole, but that significant disparities in outcome within the HOXA^{Pos} cohort were intimately linked to clinico-biological phenotype. Notably, cases that harbored an activation of the *HOXA* locus in *trans* had a high rate of an ETP-like immunophenotype that was typically associated with early treatment resistance and inferior survival. In keeping with these findings, HOXA positivity has recently been reported to be associated with an ETP-like gene expression profile and induction failure in pediatric T-ALL.[34] Of note, the incidence of HOXA overexpression in that study was 25%, which is very similar to what we observed in this adult T-ALL cohort, and in excess of previous estimates of HOXA positivity based purely on transcriptomic clustering.

Immunophenotypic classification of ETP-ALL in adults is complicated by a higher frequency of cases with overlapping patterns of antigen expression than that which is seen in children.[35] This variability in the estimation of the incidence of ETP-ALL has in turn hindered identification of prognostic factors that may help predict the outcome of the disease.[36] We found that the negative outcome of adults with ETP-ALL in this study was exclusive to the HOXA^{Pos} cohort, despite similarly high rates of chemoresistance in HOXA^{Pos} and HOXA^{Neg} ETP-ALL. Our results therefore identify HOXA positivity as a novel prognostic variable in adults with ETP-ALL, and the results of multivariate statistical analysis suggest that HOXA overexpression is directly correlated with the outcome of chemoresistant ETP-ALL. These results are also consistent with previous reports of poor outcome in HOXA^{Pos} PICALM-MLLT10 T-ALL, which seems to be confined to cases with an immature immunophenotype.[37,38]

The negative prognosis that was originally described in pediatric ETP-ALL appears to have improved with the implementation of targeted treatment intensification

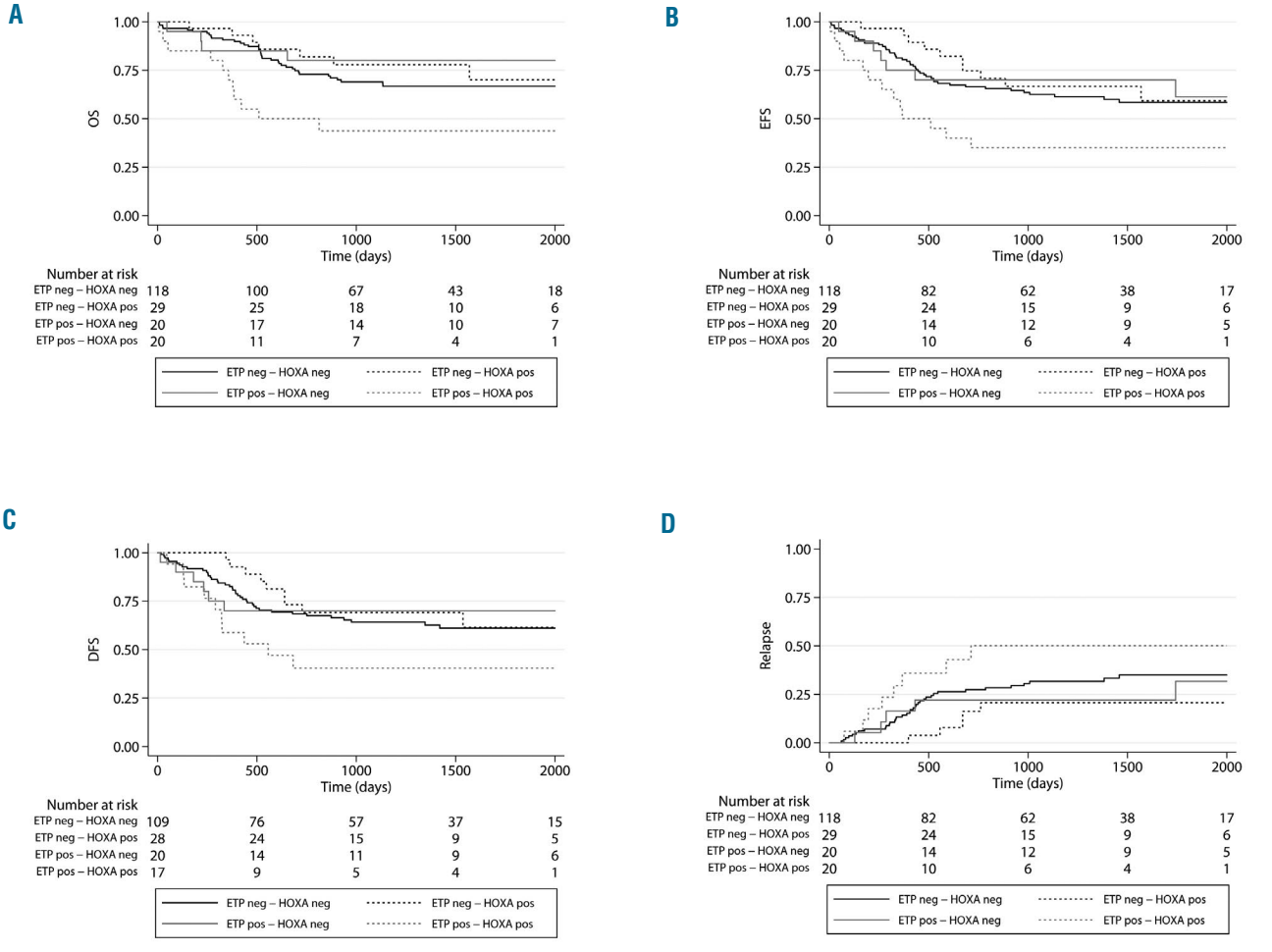

Figure 4. An ETP-like immunophenotype is associated with an inferior prognosis in HOXA^{Pos}, but not HOXA^{Neg} adult T-ALL. The results of survival analyses after separation of the HOXA^{Pos} and HOXA^{Neg} groups according to the presence or absence of an ETP-like immunophenotype are shown. The 5-year survival figures were as follows: (A) overall survival (OS) for HOXA^{Pos} ETP 31.3% (95% CI, 11.4% - 53.7%), HOXA^{Pos} non-ETP 66.7% (95% CI, 42.5% - 82.5%), HOXA^{Neg} ETP 74.2% (95% CI, 45% - 89.4%), HOXA^{Neg} non-ETP 57.2% (95% CI, 46.1% - 66.9%). (B) Event-free survival (EFS) for HOXA^{Pos} ETP 25% (95% CI, 10.2% - 43.1%), HOXA^{Pos} non-ETP 52.7% (95% CI, 35.5% - 67.4%), HOXA^{Neg} ETP 60.8% (95% CI, 39.4% - 76.6%), HOXA^{Neg} non-ETP 50.7% (95% CI, 41.1% - 59.5%). (C) Disease-free survival (DFS) for HOXA^{Pos} ETP 28.6% (95% CI, 11.7% - 48.2%), HOXA^{Pos} non-ETP 53.6% (95% CI, 35.9% - 68.5%), HOXA^{Neg} ETP 64.7% (95% CI, 43.1% - 79.8%), HOXA^{Neg} non-ETP 52.2% (95%CI, 42.1% - 61.3%). (D) Cumulative incidence of relapse (CIR) for HOXA^{Pos} ETP 53.7% (95% CI, 34.3% - 75.6%), HOXA^{Pos} non-ETP 25.4% (95% CI, 13.6% - 44.4%), HOXA^{Neg} ETP 29.2% (95% CI, 15.1% - 51.6%), HOXA^{Neg} non-ETP 39.2% (95% CI, 30.6% - 49.3%).

based on early MRD assessment.[25,26,39,40] It remains to be seen whether similar strategies will improve the outcome of HOXA^{Pos} adult ETP-ALL patients. The introduction of more intensive pediatric-based regimens has been central to recent improvements in the outcome of hitherto resistant adult ALL.[41-43] Patients included in the GRAALL-2003 and -2005 studies received enhanced induction and/or salvage therapy in the event of poor early treatment response; however, systematic early MRD monitoring was not performed for all patients. Nevertheless, our results suggest that this approach offered significant survival benefits for the HOXA^{Neg} cohort, as ETP-like and non-ETP-like cases ultimately had comparable outcomes. Conversely, we found that these treatment modifications were inadequate for therapeutic rescue of the majority of chemoresistant HOXA^{Pos} ETP-ALL cases, and that the outlook for these patients remains poor. We propose that the dramatically inferior prognosis of this group mandates consideration of alternative treatments in the context of future clinical trials. Previous data revealing the requirement for DOT1L activity in HOXA-overexpressing acute myeloid leukemia[21] suggest that pharmacological DOT1L inhibition might also have therapeutic benefit in T-ALL. In addition, recent evidence from *in vitro* and animal models suggests that combined inhibition of glycogen synthase kinase and poly(ADP-ribose) polymerase effectively suppresses growth of chemoresistant HOXA-overexpressing acute myeloid leukemia,[44] suggesting a similar potential avenue of investigation in HOXA^{Pos} T-ALL.

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