SUPPLEMENTARY APPENDIX

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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 doi:10.3324/haematol.2015.141218

Received: December 18, 2015.

Accepted: February 26, 2016.

Pre-published: March 4, 2016.

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Supplementary Methods

Quantification of *HOXA9* by qRT-PCR: RNA was extracted using an RNeasy MicroKit (Qiagen) and retrotranscribed using Superscript III reverse transcriptase (Life Technologies). The change-in-threshold (−ΔΔCT) method was used to quantify transcript levels following PCR amplification. *HOXA9* levels were calculated relative to the reference gene *ABL*, and *HOXA9*/ *ABL* was expressed as a HOXA ratio, whereby a ratio of 1 indicated equivalent expression of the two genes. Primer and Taqman probe sequences are shown in Supplementary Table S2.

Fluorescence *in situ* hybridization (FISH): The following Break Apart probes were used: *TCRB*: 5': RP11-1084E4 and RP11-615P18 (rhodamine-dUTP). 3': RP11-114L10 and CTD-2552B9 (FITC-dUTP). *HOXA*: 5': RP11-1036C18 (rhodamine-dUTP). 3': RP11-1132K14 (FITC-dUTP). *MLL*: 5': RP11-59N1 and RP11-112I9 (rhodamine-dUTP). 3': RP11-278O8 and RP11-30E1 (FITC-dUTP). *MLLT10*: RP11-469D16 (rhodamine-dUTP) and RP11-140P12 (FITC-dUTP).

RNA-sequencing: Poly(A)-enriched RNA-sequencing was performed using strand-specific and paired-end sequencing on Life Technologies SOLiD HQ5500XL. Poly(A)+ RNA was enriched from 3µg of total RNA using the MACS mRNA isolation kit (Miltenyi Biotec). Quantity and quality of mRNA was verified using RNA Pico chips on a 2100 Bioanalyzer (Agilent) and 200 ng of mRNA was used for mRNA-seq library preparation according to SOLiD Poly(A) RNA-Seq Kit (Life Technologies). The resulting library was sequenced on an AB SOLiD 5500xl using paired-end 75-35 bp sequencing chemistry following the manufacturer's instructions. High quality reads were matched to individual samples based on the barcode tag and using default parameters. Basecalls were performed using 5500 Series Genetic Analyzers Instrument Control Software v1.2 (Life Technologies). Mapping, coverage and fusion discovery were performed using default parameters of Lifescope™ (Life Technologies) after mapping of sequence reads to version hg19 of the human genome.

RT-PCR testing for leukemic fusion transcripts: Primer sequences are shown in Supplementary Table S3.

Taqman Low-density array (TLDA): Custom 48-well TLDA cards containing primers and probes for amplification of *HOX* genes were designed in collaboration with and ordered from Life Technologies. PCR was performed according to the manufacturer's instructions following retrotranscription of 200ng of RNA extracted from leukemic blasts at diagnosis. Expression of *HOX* genes was normalized to the *GAPDH* reference gene.

Supplementary Table S1: Characteristics of the HOXA study patients and the remainder of the T-ALL cohort in the GRAALL-2003 and GRAALL-2005 studies.

	Non-included cohort	**HOXA cohort**	***P-value***
Total, no.	129	209	
Clinical subsets analyzed			
Median age, y [Q1-Q3]	33·55 (25·32-45·10)	30·58 (23·78-40·47)	0·1754
WBC > 100 x 10^9/l, no. (%)	20 (15·50%)	54 (25·84%)	0·0256
CNS involvement, no. (%)	8 (6·20%)	27 (13·17%)	0·0429
CR, no. (%)	121 (93·80%)	196 (93·78%)	0·9945
CS, no. (%)	94 (73·44%)	113 (54·07%)	0·0004
CHS, no. (%)	75 (60·48%)	112 (54·37%)	0·2776
SCT, no. (%)	32 (26·45%)	77 (39·49%)	0·0178
Relapse rate, no. (%)	36 (30·00%)	60 (30·61%)	0·9086
DFS at 5 years [95% CI]	49·73% [40·08% - 60·29%]	46·65% [39·2% - 54·76%]	0·4018
OS at 5 years [95% CI]	47·42% [38·07 – 57·79%]	40·12% [33·04 – 48·10%]	0·1652

no: number; Q: Quartile WBC: white blood cell count; CNS: central nervous system; CR: complete remission; Cs: cortico-sensitive; CHs: chemo-sensitive; SCT: stem-cell transplantation; DFS: Disease-Free Survival; OS: Overall Survival.

Supplementary Table S2: Sequences of primers and probes used for quantification of *HOXA9*.

Gene	Forward Primer	Reverse Primer	Taqman Probe
HOXA9	GAAAACAATGCTGAGAATGAGAGC	CGCGCATGAAGCCAGTT	ACAAGCCCCCCATCGATCCCA
ABL	TGGAGATAACACTCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	CCATTTTTGGTTTGGGCTTCACACCATT

Supplementary Table S3: Sequences of primers used for PCR testing for fusion transcripts associated with HOXA positivity.

Fusion Transcript	Forward Primer	Reverse Primer
*PICALM-MLLT10**	GCAATCTTGGCATCGGAAAT	GCGCTTCAATGATCCAGATATAGAG **and** CCGTTTGCTCTTTTTCAGCTT
SET-NUP214	TTCCCGATATGGATGATG	CTTTGGGCAAGGATTTG
NUP98-RAP1GDS1	CTTACTACATTTGGAAGCAGC	CAGACAATCCAAGCATCCTTC
XPO1-MLLT10	GTTTCCCAGCATTCCTTGC	CAGTCCGGCAAACTGAGCG
DDX3X-MLLT10	TGCTGGCCTAGACCTGAACT	AGAGCGCTCCTACTTGTTGC
NAP1L1-MLLT10	CCCCTCCTGAAGTTCCTGAGAGTGGA	GCACCAGTGGCTGCTTTGCTTTCTC

*A multiplex PCR reaction was used in the detection of *PICALM-MLLT10*, therefore the sequences of two separate reverse primers are shown.

	HOXA^{Neg}	**HOXA^{Pos}**								**Total**	**p-value (χ^2)† Neg v Pos**	**p-value (χ^2)† Trans v cis**
Mechanism of *HOXA* activation	**NA**	**All HOXA^{Pos}**	**Trans**				**All Trans**	**Cis**	**Unknown**	**NA**		
Genetic classification	**NA**		**SET-NUP214**	**MLLT10**	**MLL**	**NUP98-RAP1GDS1**		**TCRβ-HOXA**	**NC**	**NA**		
n (%)	154 (74)	55 (100)	9 (17)	12 (22)	6 (11)	2 (4)	29/55 (53)	11 (20)	16 (29)	209		
***TCR* molecular status**												
Immature (IM0. Imd. Img)	22/133 (17)	29/51 (57)	7/9 (78)	7/11 (64)	4/6 (67)	1/1 (100)	19/27 (70)	1/10 (10)	9/15 (60)	51/184 (28)	**<0.0001*** **	**0.002** **
αβ lineage	96/133 (72)	14/51 (27)	1/9 (11)	2/11 (18)	0/6 (0)	0/1 (0)	3/27 (11)	7/10 (70)	4/15 (27)	110/184 (60)	**<0.0001*** **	**0.0011** **
γδ lineage	15/133 (11)	8/51 (16)	1/9 (11)	2/11 (18)	2/6 (33)	0/1 (0)	5/27 (19)	2/10 (20)	2/15 (13)	23/184 (13)	0.46	1
ETP immunophenotype	20/138 (15)	20/49 (41)	2/8 (25)	8/11 (73)	0/5 (0)	1/1 (100)	11/25 (44)	1/9 (11)	8/15 (53)	40/187 (21)	**0.0004*** **	0.11
EGIL												
1-2	33/144 23)	32/53 (60)	8/9 (89)	8/11 (73)	3/6 (50)	1/1 (100)	20/27 (74)	3/11 (27)	9/16 (56)	65/197 (33)	**<0.0001*** **	**0.01***
3	91/144 (63)	15/53 (28)	0/9 (0)	1/11 (9)	2/6 (33)	0/1 (0)	3/27 (11)	6/11 (55)	6/16 (38)	106/197 (54)	**<0.0001*** **	**0.009** **
4	20/144 (14)	6/53 (11)	1/9 (13)	2/11 (18)	1/6 (17)	0/1 (0)	4/27 (15)	2/11 (18)	1/16 (6)	26/197 (13)	0.81	1
***NOTCH1/FBXW7*^{mutated}**	111/154 (72)	36/55 (65)	4/9 (44)	5/12 (42)	2/6 (33)	2/2 (100)	13/29 (45)	11/11 (100)	13/16 (81)	147/209 (70)	0.39	**0.001** **
High risk classifier*	59/143 (41)	25/51 (49)	5/8 (63)	7/11 (64)	4/6 (67)	2/2 (100)	18/27 (67)	1/9 (11)	6/16 (38)	84/194 (43)	0.41	**0.006** **
Clinical subsets analyzed												
Age median	30.4	31.9	30.2	32,5	26.5	38.1	30,2	35	33	30.5	0.48	0.68
Age>35	61/154 (40)	22/55 (40)	4/9 (44)	5/12 (42)	1/6 (17)	1/2 (50)	11/29 (38)	5/11 (46)	7/16 (44)	83/209 (40)	1	0,73
Sex ratio M/F	3.8	1.9	8	2	1	1	2,2	1.8	1,7	3.1	0.22	0,7
WBC median	37.7	33	30.9	46,4	17.5	39.9	33	56.1	17,6	37	0.3	0.8
WBC>100 x 10^9/L	46/154 (30)	9/55 (16)	2/9 (22)	3/11 (25)	1/6 (17)	0/2 (0)	6/29 (21)	0/11 (0)	3/16 (19)	55/209 (26)	0.052	0.16
CNS involvement	17/151 (11)	10/54 (19)	2/9 (22)	3/11 (27)	0/6 (0)	0/2 (0)	5/28 (18)	2/11 (18)	3/16 (19)	27/205 (13)	0.23	1
Corticosensitivity	92/154 (60)	20/55 (36)	1/9 (11)	2/12 (17)	1/6 (17)	1/2 (50)	5/29 (17)	8/11 (73)	8/16 (50)	112/209 (54)	**0.004** **	**0.0017** **
Chemosensitivity	90/152 (59)	22/54 (41)	0/9 (0)	3/12 (25)	2/6 (33)	1/2 (50)	6/29 (21)	10/11 (91)	7/15 (47)	112/206 (54)	**0.026***	**<0.0001*** **
Complete Remission	145/154 (94)	51/55 (93)	8/9 (89)	12/12 (100)	6/6 (100)	2/2 (100)	28/29 (97)	11/11 (100)	13/16 (81)	196/209 (94)	0.75	1
Relapse	46/145 (32)	13/51 (26)	3/8 (38)	4/12 (33)	1/6 (17)	0/2 (0)	8/28 (29)	1/11 (9)	4/13 (31)	59/196 (30)	0.48	0.4
Death	49/154 (32)	19/55 (35)	2/9 (22)	7/12 (58)	2/6 (33)	0/2 (0)	11/29 (38)	3/11 (27)	5/16 (31)	68/209 (33)	0.74	0.72
MRD ≥ 10^{-4}	19/81 (24)	16/33 (48)	5/7 (71)	5/8 (63)	2/3 (67)	1/1 (100)	13/19 (68)	0/7 (0)	3/8 (38)	35/114 (31)	**0.01***	**0.0052** **

Supplementary Table S4: Patient Characteristics (Corresponds to Table 1)

Patient numbers and percentages are indicated.

TCR, T-cell receptor, EGIL, European Group for the immunological classification of leukaemia, ETP, Early Thymic Precursor, WCC, White Blood Cell Count; CNS, Central Nervous System

† t test (Mann-Whitney test) or Fisher's exact test were used where appropriate.

Supplementary Table S5: Multivariate models of the interaction of HOXA with other covariates.

OS	Haz. Ratio	[95% Conf.	Interval]	P>z
WBC>100	2·288	1·127	4·647	0·022
SCT	0·701	0·350	1·406	0·317
ETP	1·099	0·430	2·808	0·844
HOXA pos	1·300	0·682	2·477	0·425
risk classifier high	2·469	1·334	4·571	0·004
EGIL 3-4 vs 1-2	0·721	0·353	1·475	0·371
cs pos	1·092	0·576	2·071	0·787
chs pos	0·835	0·398	1·751	0·632

EFS	Haz. Ratio	[95% Conf.	Interval]	P>z
WBC>100	1·388	0·802	2·401	0·241
SCT	0·807	0·433	1·501	0·497
ETP	1·452	0·629	3·351	0·382
HOXA pos	1·806	0·884	3·687	0·105
risk classifier high	2·529	1·489	4·296	0·001
EGIL 3-4 vs 1-2	1·281	0·595	2·758	0·528
cs pos	0·898	0·501	1·609	0·716
chs pos | HOXA neg	1·111	0·541	2·283	0·774
chs pos | HOXA pos	0·277	0·071	1·074	0·063

DFS	Haz. Ratio	[95% Conf.	Interval]	P>z
WBC>100	1·396	0·757	2·575	0·285
SCT	1·056	0·530	2·107	0·876
ETP	1·692	0·663	4·319	0·271
HOXA pos	1·758	0·784	3·943	0·171
risk classifier high	2·922	1·643	5·195	0·000
EGIL 3-4 vs 1-2	1·504	0·647	3·493	0·343
cs pos	0·755	0·408	1·397	0·371
chs pos | HOXA neg	1·500	0·625	3·596	0·364
chs pos | HOXA pos	0·362	0·091	1·441	0·149

Relapse	SHR	[95% Conf.	Interval]	P>z
WBC>100	1·322	0·691	2·526	0·399
SCT	0·556	0·270	1·145	0·111
ETP	2·812	0·852	9·280	0·090
HOXA pos	2·049	0·836	5·020	0·117
risk classifier high	3·664	1·987	6·757	0·000
EGIL 3-4 vs 1-2	2·662	0·807	8·780	0·108
cs pos	0·904	0·481	1·701	0·755
chs pos | HOXA neg	1·461	0·619	3·446	0·387
chs pos | HOXA pos	0·119	0·013	1·085	0·059

Multivariate Cox models were performed for studying OS, EFS and DFS. Multivariate Fine & Gray models adapted for competing risk events were performed for studying CIR.

The effect of HOXA was adjusted on WCC, SCT, EGIL classification, Risk classifier, ETP status, Corticosensitivity (CS) and Chemosensitivity (CHS).

Interactions between Chemosensitivity and HOXA status have been highlighted for EFS ($p = 0.053$), CIR ($p = 0.039$) and DFS ($p = 0.071$).

Conditional effects of CHS depending on HOXA status are presented when the interaction is highlighted

(chs pos | HOXA neg:
effect of chemosensitivity when HOXA is negative)

(chs pos | HOXA pos:
effect of chemosensitivity when HOXA is positive).

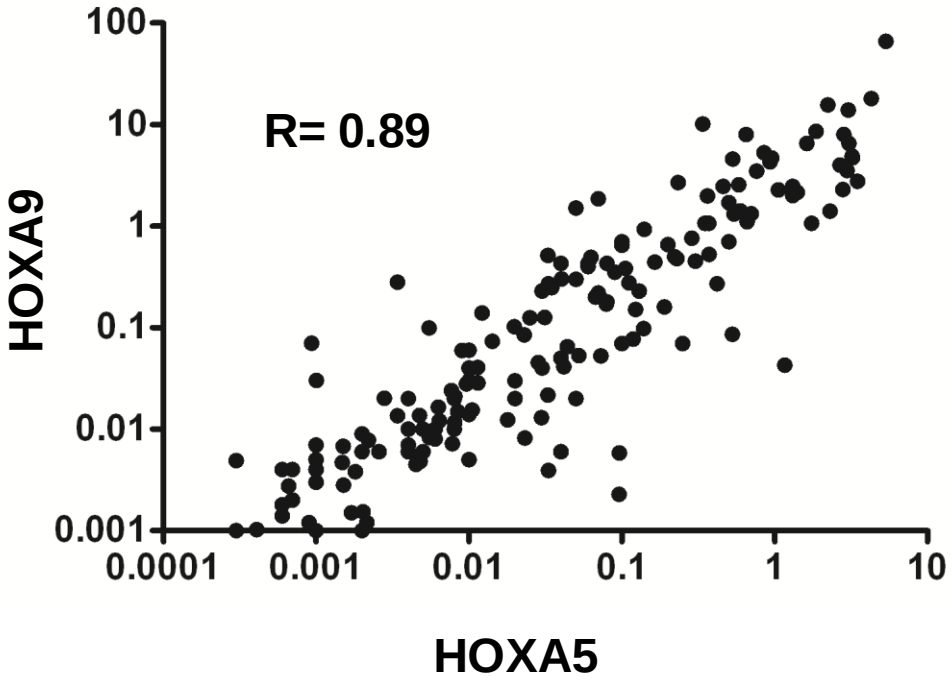
OS = Overall Survival.
EFS = Event-Free Survival.
DFS = Disease-Free Survival.
WBC = White blood cell count.
SCT = Stem Cell Transplant.
ETP = Early Thymic Precursor-like phenotype.
EGIL = European Group for the immunological classification of leukaemia.

Supplementary Table S6: Summary of the genetics of HOXA^{Neg} and HOXA^{Pos} T-ALL.

	Total	**HOXA^{Neg}**	**HOXA^{Pos}**
All patients	209	154	55
SIL-TAL1	18	18 (154)	0 (55)
TLX1	44	42 (154)	2 (55)
TLX3	25	23 (154)	2 (55)
MLL	6	0 (80)	6 (54)
PICALM-MLLT10	8	0 (154)	8 (55)
TCRβ-HOXA	11	0 (50)	11 (52)
SET-NUP214	9	0 (154)	9 (55)
No abnormality detected	87	71	16

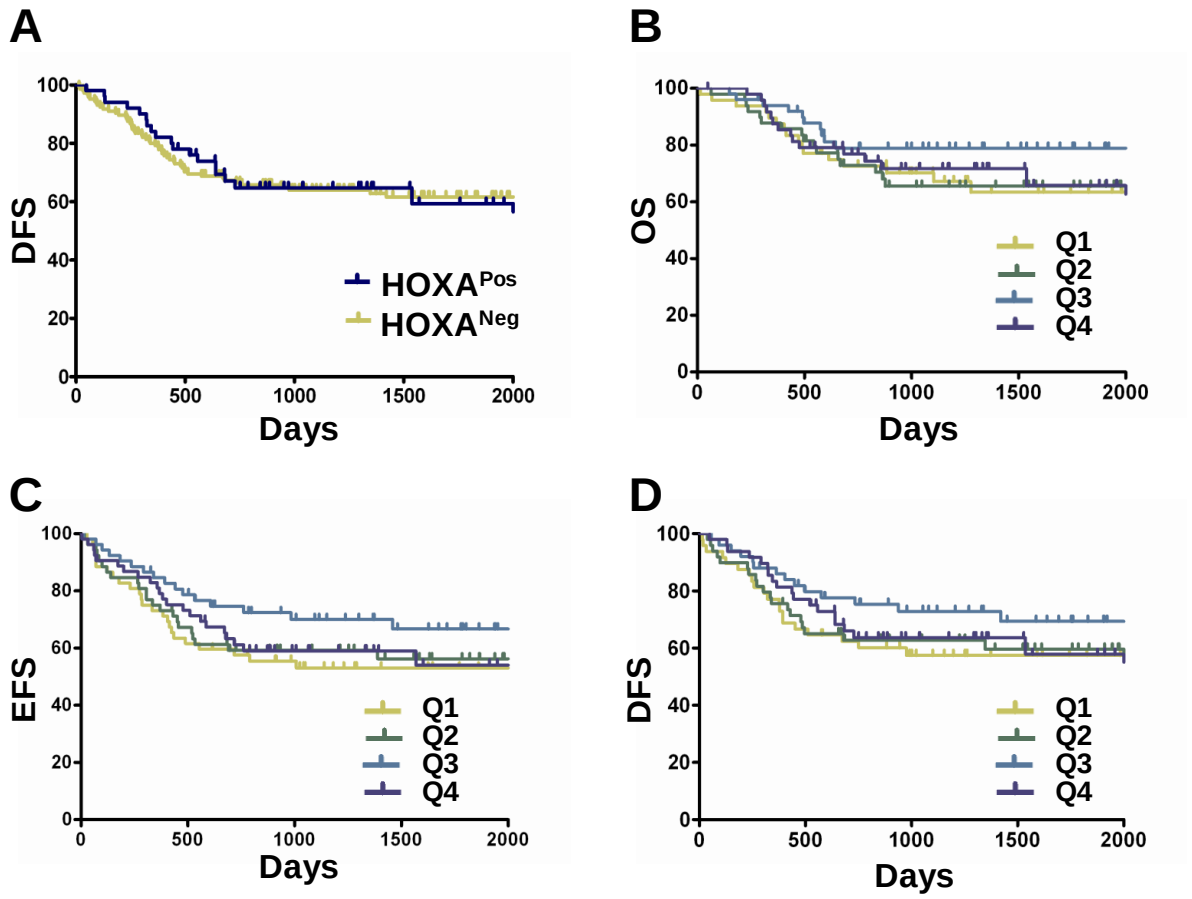
Diagnostic T-ALL samples were tested for the genetic abnormalities as shown. Figures in brackets indicate the number of patients tested in each case.

Supplementary Figure S1. Correlation of HOXA5 and HOXA9 ratios.



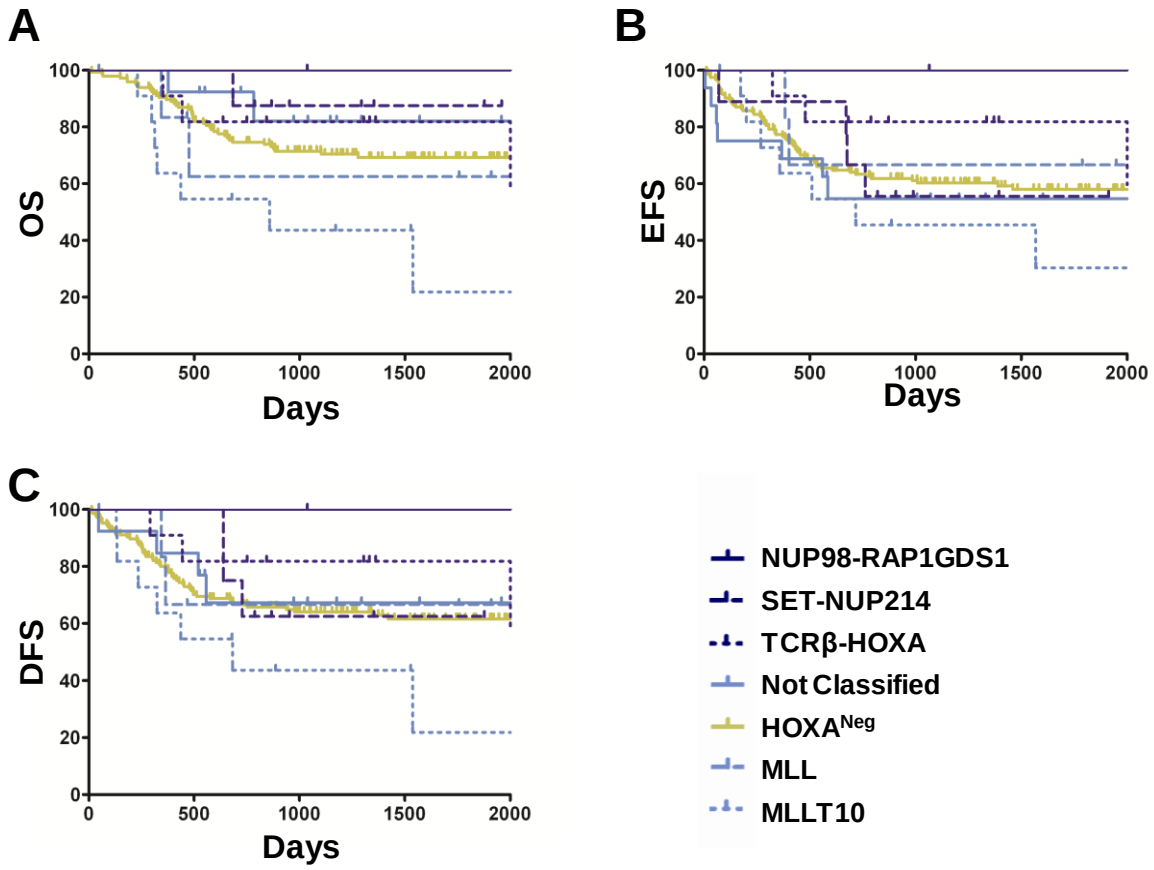
HOXA5 was measured by qRT-PCR and normalized to the expression of *ABL1*. Comparison with *HOXA9* levels (HOXA ratio) is shown. Each point represents *HOXA5* and *HOXA9* levels for an individual patient. The indicated R value corresponds to the calculated Spearman's correlation coefficient.

Supplementary Figure S2 (Corresponds to Figure 3: HOXA^{Pos} and HOXA^{Neg} adult T-ALL patients have similar survival outcomes)



(A) 5 year DFS was estimated at 50·0% (95% CI: 33·4% - 64·5 %) in HOXA^{Pos} patients and 51·1% (95% CI: 41·5% - 59·9%) in HOXA^{Neg} patients. Survival analysis revealed no differences in **(B)** OS, **(C)** EFS or **(D)** DFS when patients were separated by quartile of HOXA ratio.

Supplementary Figure S3. Survival analysis of HOXA^{Pos} oncogenetic subgroups.



Outcome analyses were performed according the underlying oncogenetic abnormality. Survival graphs for **(A)** OS, **(B)** EFS and **(C)** DFS are shown. There was an observed trend towards poorer prognosis in the MLLT10 subgroup, but limited patient numbers (n= 12) preclude definitive conclusions. 5 year OS for MLLT10 patients was estimated at 30% (95% CI: 7·11% - 57·79%). The corresponding figure for EFS was 23·8% (95% CI: 4·7% - 50·9%), and for DFS 23·8% (95% CI: 4·7% - 50·9%).

Supplementary Figure S4. Consort Diagram