The significance of *PTEN* and *AKT* aberrations in pediatric T-cell acute lymphoblastic leukemia

Linda Zuurbier,[1] Emanuel F. Petricoin 3rd,[2,3] Maartje J. Vuerhard,[1] Valerie Calvert,[2] Clarissa Kooi,[1] Jessica G.C.A.M. Buijs-Gladdines,[1] Willem K. Smits,[1] Edwin Sonneveld, Anjo J.P. Veerman,[4,5] Willem A. Kamps,[4,6] Martin Horstmann,[7,8] Rob Pieters,[1] and Jules P.P. Meijerink[1]

[1]Department of Pediatric Oncology/Hematology, Erasmus MC Rotterdam-Sophia Children's Hospital, Rotterdam, the Netherlands; [2]Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, VA, USA; [3]NCI-FDA Clinical Proteomics Program, Food and Drug Administration, Bethesda, MD, USA; [4]Dutch Childhood Oncology Group (DCOG), the Hague, the Netherlands; [5]Department of Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, the Netherlands; [6]Department of Pediatric Oncology, University of Groningen-Beatrix Children's Hospital, Groningen, the Netherlands; [7]German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL), Hamburg, Germany; and [8]Research Institute Children's Cancer Center Hamburg, Clinic of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

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Online Supplementary Design an Methods

Cell culture, γ-secretase inhibitor treatment and cell cycle analysis

T-ALL cell lines (DSMZ, Braunschweig, Germany) were cultured in RMPI-1640 supplemented with 10-20% fetal calf serum (Integro, Zaandam, the Netherlands), 100 IU/mL penicillin, 100 μg/mL streptomycin and 0.125 μg/mL fungizone (Invitrogen, Life Technologies, Breda, the Netherlands) at 37°C under 5% CO_2. T-ALL cell lines (JURKAT, CEM, LOUCY, SKW3, ALL SIL, HPBALL, PF382, HSB2, PEER, MOLT3, MOLT16, P12 ICHIKAWA, KARPAS45, RPMI8402, BE13, TALL1, SUPT1, KE37 and DND41) were grown under 1 μM Compound E (Enzo Life sciences (Alexis), Lausen, Switzerland) or 0.002% DMSO for four days, and $1x10^6$ cells were harvested. Cells were fixed with 70% cold ethanol and stained with propidium iodide (Invitrogen), after trypsin (Gibco BRL, Life Technologies, Breda, the Netherlands) and RNase A (Sigma, Zwijndrecht, the Netherlands) treatment. DNA content was measured and analyzed by flow cytometry (FACSCalibur, Becton Dickson, San Jose, CA, USA).

Genomic DNA and RNA extraction

Genomic DNA and RNA were isolated from at least $5x10^6$ leukemic cells using the Trizol reagent (Invitrogen) according to the manufacturer's instructions with minor modifications.[1] Copy-DNA synthesis of 1 μg of total RNA was performed as previously described before.[1] DNA was stored at 40°C, whereas RNA and cDNA were stored at -80°C.

Detection of mutations and splice variants

The phosphatase domain and C2-domain of *PTEN* (exons 1-9), the pleckstrin homology (PH) domain of *AKT1* (exon 4), the SH2-domain of *PIK3CA* (p85, exons 12 and 13) and the accessory domain of *PIK3RI* (p110, exon 10) were amplified and sequenced. Primers used are described in the *Online Supplementary Table S1*. PCR reactions were performed on 50 ng of DNA, 300 nM of primers, 200 μM of dNTPs, 4 mM $MgCl_2$, 1.25 U of ampli*Taq* gold (Applied Biosystems, Foster City, CA, USA) in 1 x PCR buffer II (Applied Biosystems) in a volume of 50 μL. After denaturation at 94°C for 5 min, PCR was performed for 40 cycles at 94°C for 15 min and 60°C for 1 min. Due to the GC-rich content, PCR of *PTEN* exon 1 was followed by a second asymmetric PCR for 10 cycles, using the Forward or Reverse primer. PCR products were purified with the Millipore Vacuum Manifold filter system (Millipore, Billerica, MA, USA) and sequenced (BigDye Terminator v3.1 Cycle sequencing Kit, Applied Biosystems) on an ABI PRISM 3130 DNA Analyzer (Applied Biosystems). Amplicons of patients who demonstrated two mutations were cloned using the TOPO-TA cloning kit (Invitrogen) to determine whether mutations occurred in *cis* or *trans*.

To examine promoter mutations, one primer set was used to amplify the promoter area. PCR-reactions were carried out as described above in the presence of 2 mM $MgCl_2$ and 5% DMSO. Annealing temperature started at 63°C, and was lowered by 0.5°C each cycle till a final annealing temperature of 58°C was reached. To investigate alternative *PTEN*-splicing, two primer pairs were used to amplify the complete *PTEN* transcript, using PCR conditions as described above in the presence of 2 mM $MgCl_2$. *NOTCH1* mutations were identified as described in our previous study.[2]

Methylation specific PCR (MSP)

For methylation specific PCR (MSP), sodium bisulfite conversion was carried out using the EZ DNA methylation kit (Zymo research, Orange, CA, USA). Primers used are listed in the *Online Supplementary Table S1*. PCR was performed using 0.6 U Hotstar Taq plus DNA polymerase (Qiagen, Venlo, the Netherlands), 1 x PCR buffer, 200 μM dNTPs, 300 nM primers, 1 x Q-solution, 3.5 mM MgCl2 and 100 ng converted DNA in a total volume of 50 μL. *Taq* polymerase was activated at 95°C for 5 min, followed by 35 PCR cycles at 95°C for 30 s, 59°C for 30 s and 72°C for 1 min, and a final elongation step at 72°C for 10 min. *In vitro* methylated DNA with CpG methyltransferase Sss1 and co-substrate S-adenosylmethionine (SAM, New England Biolabs, Ipswich, MA, USA) served as positive control, untreated genomic DNA served as negative control.

Fluorescence in situ hybridization analysis (FISH) and RQ-PCR

Rearrangements of the *TLX1*, *TLX3*, *TAL1*, *LMO2* and *MLL* loci were determined with fluorescence *in situ* hybridization analysis (FISH) as pre-

viously described.[1,3,4] *SET-NUP214*, *CALM-AF10* or *SIL-TAL1* fusion products or expression levels of *TLX1* or *TLX3* were detected by an RQ-PCR strategy as described.[1,3,4] BAC clones and RQ-PCR primers/probes are summarized in the *Online Supplementary Table S2*. To identify *PTEN* deletions by FISH, bacterial artificial chromosomes (BAC) clones RP11-846G17 and/or RP11-124B18 were used. Probe RP11-265I15 covering the X-chromosomal *BEX1* gene was used as control. BACs were obtained from BAC/PAC Resource Center (Children's Hospital, Oakland, CA, USA).

Microarray-based comparative genome hybridization (array-CGH)

Array-CGH analysis was performed on the human genome CGH Microarray 44A (n=33), 105K (n=2), and 400 K (n=78) (Agilent Technologies, Santa-Clara, CA, USA), which consists of 60-mer oligonucleotide probes that span both coding and non-coding sequences. The procedure was carried out as previously described.[3]

Western blot procedure

Western blot was performed as previously described.[2] Antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA) for PTEN (Cat#9552), phosphorylated (S380) PTEN (Cat#9551), phosphorylated (Thr308 and S473) AKT (Cat#9275 and 9271), phosphorylated (S2448 and S2481) mTOR (Cat#2971 and 2974), phosphorylated (Thr389) p70 S6 kinase (Cat#9205), phosphorylated (S65 and T70) 4E-BP1 (Cat#9451 and 9455), phosphorylated (Y1571) TSC2 (Cat#3614), phosphorylated (S256) FOXO1 (Cat#9461), intracellular NOTCH1 (ICN) Val1744 (Cat#2421), cMYC (Cat#9402) and MUSASHI1/2 (Cat#2154). To detect phosphorylated (S246) PRAS40, Cat#44-1100 from Invitrogen/Biosource was used. Total protein load was determined by staining for actin (Sigma, Cat#2547).

Reverse-phase protein microarray analysis (RPMA)

Reverse-phase protein microarray construction and analysis was performed essentially as previously described.[2,5,6]

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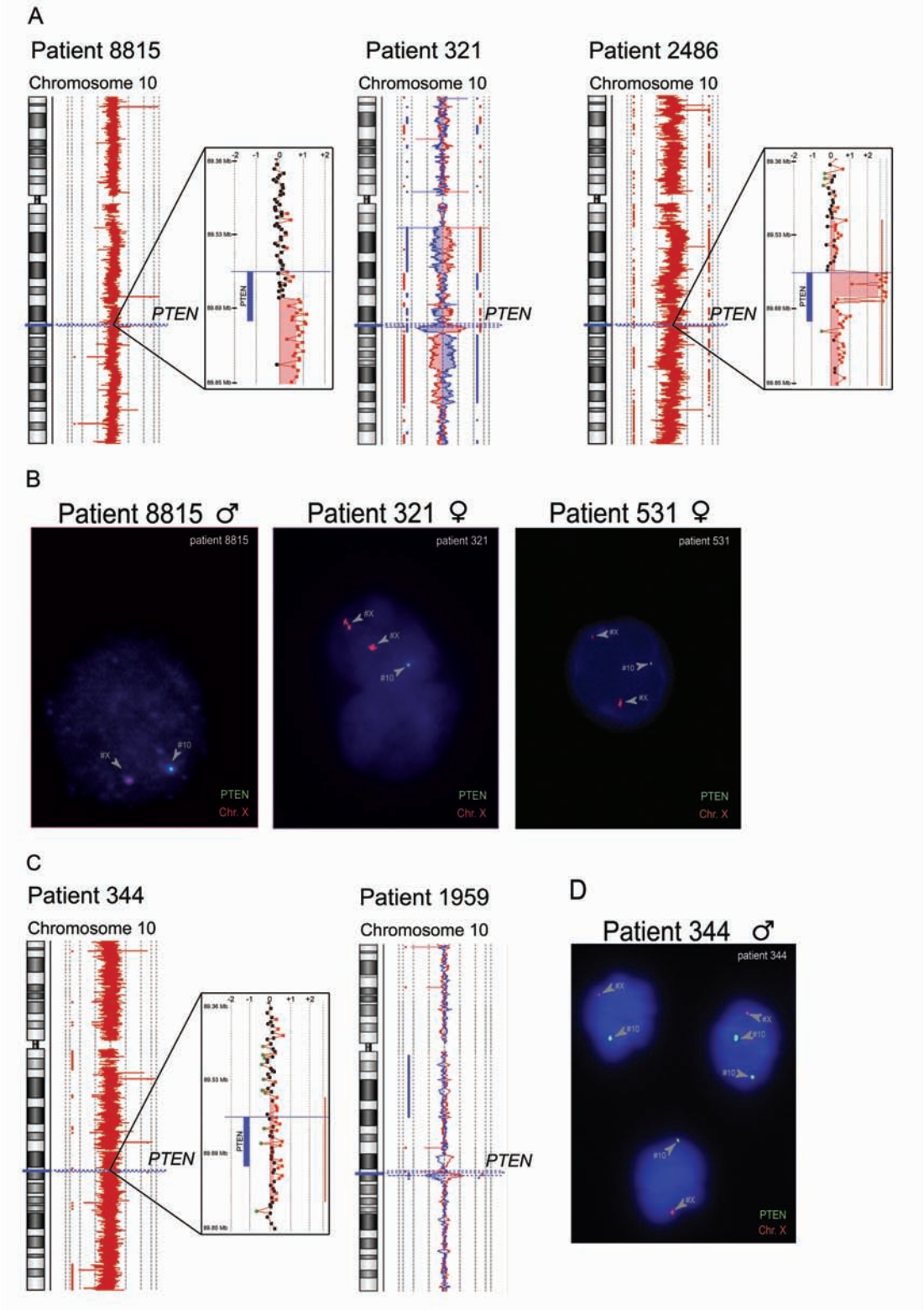
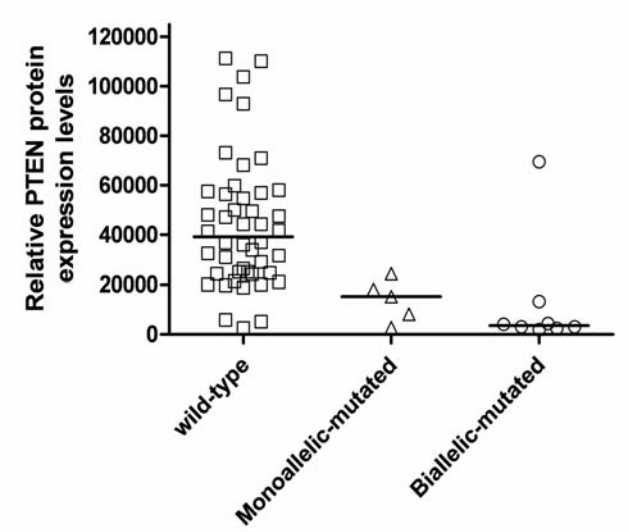
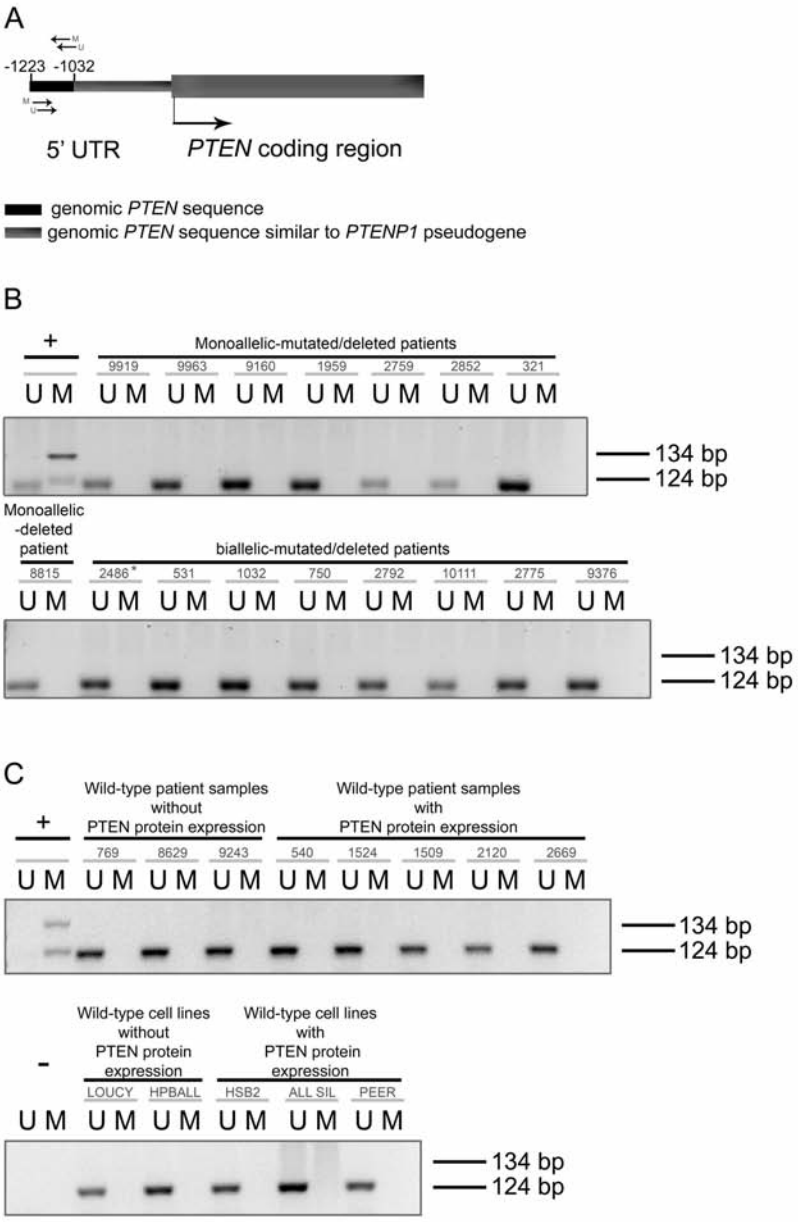


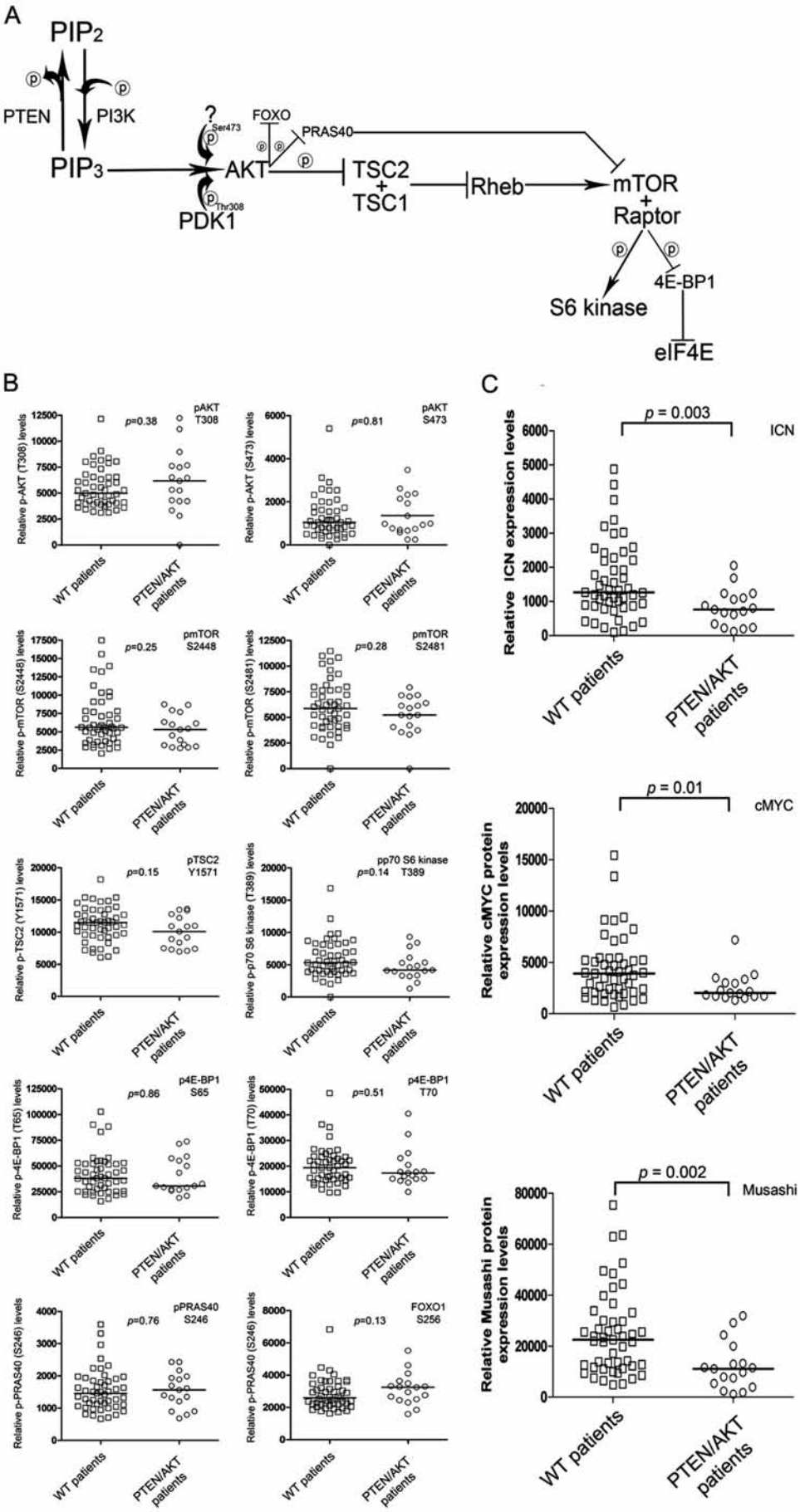
Online Supplementary Figure S1. *PTEN* deletions in T-ALL patients detected by array CGH and FISH analysis. (A) Array CGH and (B) FISH results of T-ALL patients with a clonal *PTEN* deletion. (C) Array CGH and (D) FISH results of patients with a subclonal *PTEN* deletion.

Online Supplementary Figure S2. PTEN protein expression levels in *PTEN* wild-type patients, *PTEN* monoallelic and biallelic-mutated T-ALL patients.

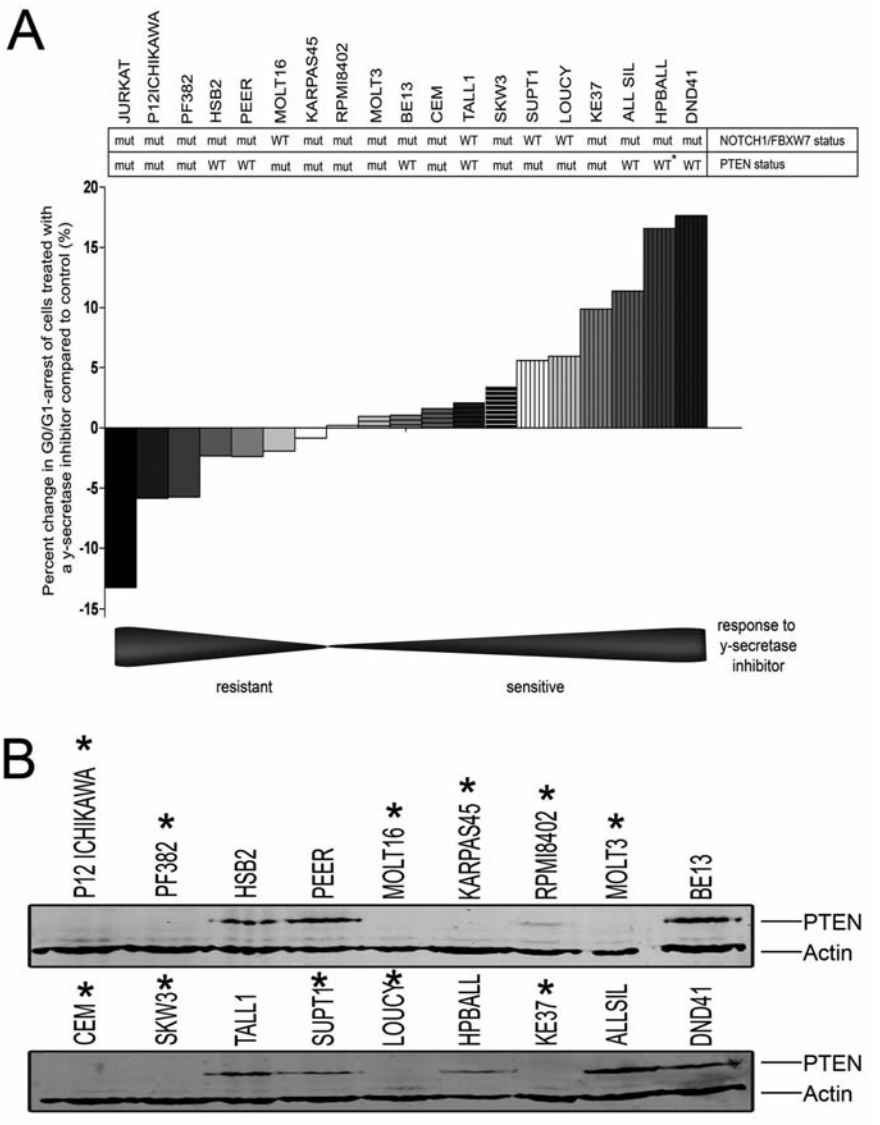




Online Supplementary Figure S3. Methylation-specific PCR of the *PTEN* promoter region in T-ALL patients. (A) Schematic overview of PTEN promoter area depicting overlapping sequences between the *PTEN* gene and the *PTENP1* pseudogene in gray and the unique *PTEN* sequence in black. Primers used for methylation-specific PCR are indicated by arrows where M indicates primers used for the amplification of methylated DNA and U indicates primers used for the amplification of unmethylated DNA. -1223 and -1032 indicate the numbers of base pairs before the *PTEN* start site. (B) PCR results of methylation-specific PCR in mono-allelic and bi-allelic mutated/deleted patients and (C) in *PTEN* wild-type patients and cell lines with and without PTEN expression. + indicates the the positive control. U shows the PCR result for unmethylated DNA (124bp) and M for methylated DNA (134bp).

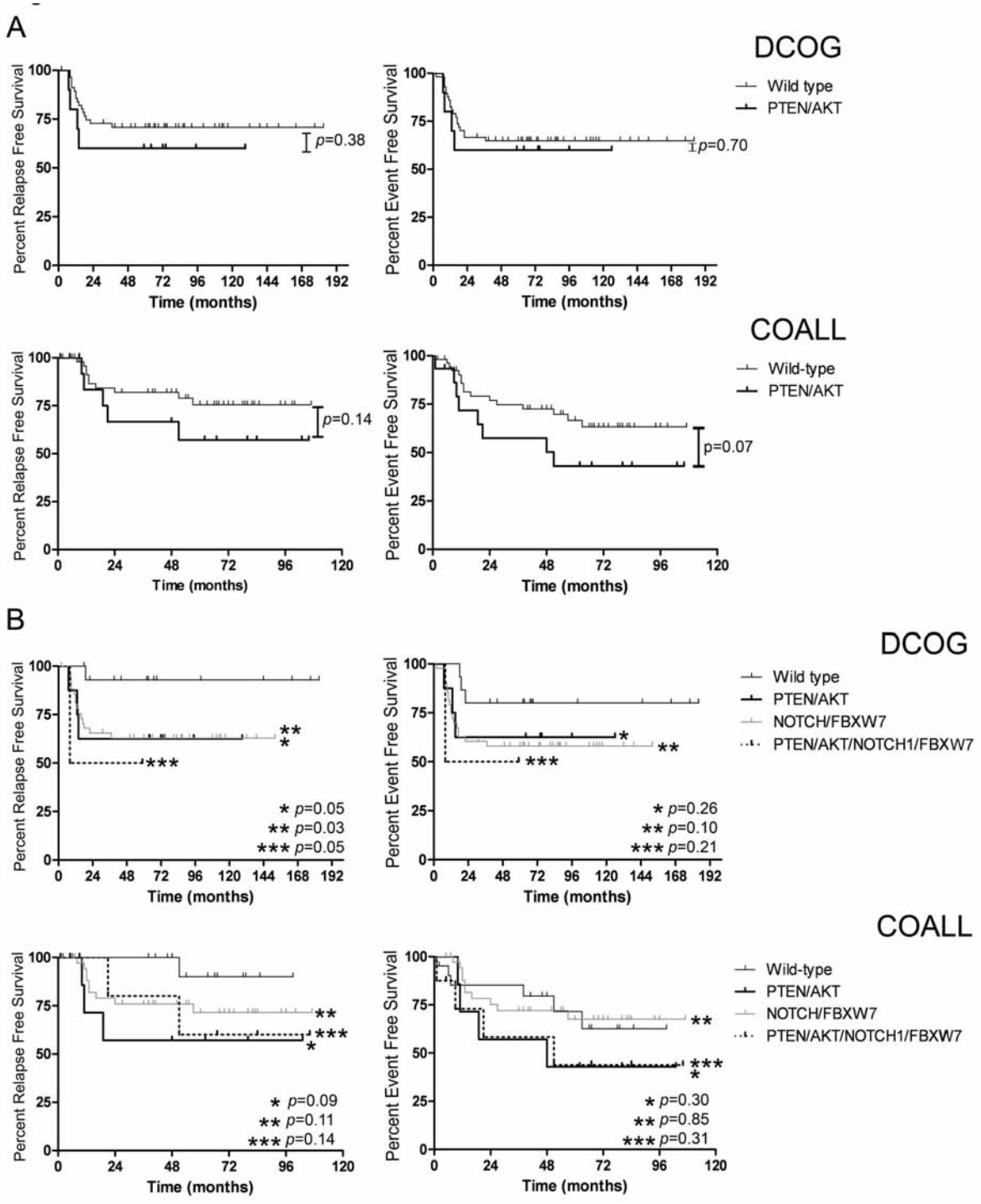


Online Supplementary Figure S4. Total and phosphorylated levels of PTEN/AKT and NOTCH pathway mediators in T-ALL. (A) Schematic overview of AKT and its potential downstream signaling partners. (B) The expression of phosphorylated AKT (T308 and S473) levels as well as the activation status of potential downstream signaling components in PTEN/AKT mutant *versus* PTEN/AKT non-mutated (wild-type) patients, analyzed by reverse-phase protein microarray. Potential downstream targets include mTOR (S2448 and S2481), p70 S6 kinase (T389), 4EBP1 (S65 and T70), TSC2 (Y1571), PRAS40 (S246), and FOXO1 (S256). (C) The expression of intracellular NOTCH1 (ICN), the NOTCH1 target molecule cMYC, and the indirect NOTCH1 activator MUSASHI1/2 (MSI1/2), in PTEN/AKT mutant and PTEN/AKT non-mutated T-ALL patient samples. The *P* value for each comparison is indicated.



Online Supplementary Figure S5. PTEN mutations are not necessarily related with resistance towards the γ-secretase inhibitor compound E. (A) Response of indicated T-ALL cell lines towards the γ-secretase inhibitor compound E, measured by G0/G1-arrest following 96 h of γ-secretase inhibitor treatment relative to DMSO-treated control cells. Cell lines that do not undergo G0/G1-arrest are indicated as resistant cell lines, whereas cell lines that do undergo G0/G1-arrest following incubation with compound E are indicated as sensitive. *Cell lines with reduced PTEN expression through mutations, deletions or aberrant splicing. PTEN and NOTCH1/FBXW7 mutational status. Genetic aberrations in the HPBALL cell line that result in low or loss of PTEN protein levels have not been identified. (B) Western blot analysis of PTEN protein levels. Cell lines have been ordered based on their compound E resistance with most resistant cell lines at the upper left corner to most sensitive cell lines in the lower right corner. β-actin is used as loading control.

Online Supplementary Figure S6. Survival curves of T-ALL patients in both cohorts separately. (A) Relapse free survival and event free survival of *PTEN/AKT* wild-type (gray line) and mutant patients (black line) in DCOG and COALL cohorts. (B) Relapse free survival and event free surival of *PTEN/AKT* and *NOTCH1/FBXW7* wild-type patients (dark gray line), *PTEN/AKT* mutant (black line), *NOTCH1/FBXW7* mutant (light gray line) and of patients with *PTEN/AKT* as well as *NOTCH1/FBXW7* mutations (dotted black line) in DCOG and COALL cohorts.



Online Supplmentary Table S1. Primers used for PCR amplification of PTEN, AKT and PI3K.

Exon	Forward primer sequence	Reverse primer sequence	Product (Bp)
PTEN exon 1	5'-AGCTTCTGCCATCTCTCTC-3'	5'-TTTCGCATCCGTCTACTC-3'	203
PTEN exon 2	5'-ACATTGACCACCTTTTATTACTC-3'	5'-GGTAAGCCAAAAAATGATTATAG-3'	368
PTEN exon 3	5'-ATGGTGGCTTTTTGTTTGT-3'	5'-GCTCTTGGACTTCTTGACTTA-3'	229
PTEN exon 4	5'-TCAGGCAATGTTTGTTAGTATT-3'	5'-ATCGGGTTTAAGTTATACAACATA-3'	175
PTEN exon 5	5'-TTGTATGCAACATTTCTAAAGTT-3'	5'-ATCTGTTTTCCAATAAATTCTCA-3'	393
PTEN exon 6	5'-ACGACCCAGTTACCATAGC-3'	5'-TAGCCCAATGAGTTGAACA-3'	405
PTEN exon 7	5'-AATCGTTTTTGACAGTTTGAC-3'	5'-TCACCAATGCCAGAGTAAG-5'	378
PTEN exon 8	5'-GATTGCCTTATAATAGTCTTTGTG-3'	5'-TTTTTGACGCTGTGTACATT-3'	594
PTEN exon 9	5'-GCCTCTTAAAGATCATGTTTG-3'	5'-GGTCCATTTTCAGTTTATTCA-3'	399
PTEN cDNA PCR I	5'-TCCATCCTGCAGAAGAAG-3'	5'-CAGATGATTCTTTAACAGGTAGC-3'	639
PTEN cDNA PCR II	5'-AGAGGCGCTATGTGTATTATTAT-3'	5'-GTCCATTTTCAGTTTATTCAAG-3'	764
PTEN USP	5'-TTTTGAGGTGGTTTGGGTTTTTGGT-3'	5'-ACACAATCACATCCCAACACCA-3'	124
PTEN MSP	5'-TTTTTTTTCGGTTTTTCGAGGC-3'	5'-CAATCGCGTCCCAACGCCG-3'	134
PTEN promoter	5'-CCTGCATTTCCCTCTACA-3'	5'-GCTGCACGGTTAGAAAAG-3'	801
AKT1 exon 4	5'-CAGGGCCGTTTCTGTC-3'	5'-CCCAGCCAGTGCTTGT-3'	434
PIK3RI (p110) exon 10	5'-GTTGGCTAACTTCAGCAGTTAC-3'	5'-TGTGCCAACTACCAATGTAGTA-3'	605
PIK3CA (p85) exon 12+13	5'-CTGGGAAACCATAGTGAAACT-3'	5'-ATGGCACTGAGTTTATACATTTTC-3'	573

Online Supplementary Table S2. RQ-PCR primer and probes and FISH BAC-clones.

Gene	Aberration	RQ-PCR primer/probe	FISH type	BAC clones	Refs
TAL1	del(1)(p32)	FW 5'-CGC TCC TAC CCT GCA AAC A-3'	Fusion	Dako	Gabert *et al*., Leukemia 2003[7]
	or t(1;14)(p32;q11)	RV 5'-CCG AGG AAG AGG ATG CAC A-3'			
	or t(1;7)(p32;q34)	5'-(FAM)-ACC TCA GCT CCG CGG AAG TTG C-(TAMRA)-3'			
LMO2	del(11)(p12p13		Split	RP11-646J21 (telomeric)	Van Vlierberghe *et al*., Blood 2006[1]
				RP11-98C11 (telomeric)	
				RP11-603J2 (telomeric)	
				RP11-36H11 (centromeric)	
				RP11-769M16 (centromeric)	
				RP11-465C16 (centromeric)	
LMO2	t(11;14)(p13;q11)		Fusion	RP11-646J21	Van Vlierberghe *et al*., Blood 2006[1]
	or t(7;11)(q34;p13)			RP11-98C11	
CALM-AF10	t(10;11)(p13;q14)	FW 5'-TTA ACT GGG GGA TCT AAC TG-3'	Split	RP11-29E15 (centromeric *CALM*)	Van Grotel *et al*., Haematologica 2006[8]
		5' transcript RV 5'-GCT GCT TTG CTT TCT CTT C-3'		RP11-12D16 (telomeric *CALM*)	
		3' transcript RV 5'-CCC TCT GAC CCT CTA GCT TC-3'	Fusion	RP11-12D16 (telomeric *CALM*)	Van Grotel *et al*., Haematologica 2006[8]
		5'-(FAM)-CTT GGA ATG CGG CAA CAA TG-(TAMRA)-3'		RP11-399C16 (centromeric *AF10*)	
TLX1	t(10;14)(q24;q11)	FW 5'- CTC ACT GGC CTC ACC TT-3'	Split	Dako	
	or t(7;10)(q34;q24)	RV 5'-CTG TGC CAG GCT CTT CT-3'			
		5'-(FAM)-CCT TCA CAC GCC TGC AGA TC-(TAMRA)-3'			
TLX3	t(5;14)(q35;q32) #	FW 5'-TCT GCG AGC TGG AAA A-3'	Split	Dako	
		RV 5'-GAT GGA GTC GTT GAG GC-3'			
		5'-(FAM)-CCA AAA CCG GAG GAC CAA GT-(TAMRA)-3'			
MLL	11q23 rearrangements		Split	Dako	
SET-NUP214	del(9)(q34)	FW 5'-TTC CCG ATA TGG ATG ATG-3'			Van Vlierberghe *et al*., Blood 2008[3]
		RV 5'-CTT TGG GCA AGG ATT TG-3'			
GAPDH		FW 5'-GTC GGA GTC AAC GGA TT-3'			Stam *et al*., Blood 2003[8]
		RV 5'-AAG CTT CCC GTT CTC AG-3'			
		5'-(FAM)-TCA ACT ACA TGG TTT ACA TGT TCC AA-(TAMRA)-3'			

Primer and probe combinations to identify the SIL-TAL1 *deletion, CALM-AF10 5'or 3' fusion transcripts, TLX1 transcripts, TLX3 transcripts, or the* SET-NUP214 *gene fusion by RQ-PCR analysis. For the detection of mRNA transcripts, GAPDH is used as normalization control. BAC clones for the various FISH analyses to identify TAL1 rearrangements (including the* SIL-TAL1 *deletion) the* LMO2 *deletion or translocation variants, the* CALM-AF10 *translocation,* TLX1 *translocations, the* TLX3 *translocation and #other* TLX3 *translocation or MLL rearrangements.*

Online Supplementary Table S3. *PTEN* and *AKT* aberrations in pediatric T-ALL.

Patient	PTEN mutation		Affected	PTEN	PTEN	PTEN	AKT	NOTCH/FBXW7
	Allele A	Allele B	exon(s)	deletion	splicing	protein	mutation	mutation
335	R129G	T231fsX24	ex5 & ex7	WT/WT	ND	+	WT	WT
344	F144fsX37	R232fsX23	ex5 & ex7	**subclonal**	ND	absent	ND	PEST
531*	P246fsX11	-	ex7	**del/WT**	ND	absent	WT	WT
750	D235fsX9	P245fsX12	ex7	ND	ND	absent	WT	PEST
1032	R232fsX13	Q244fsX8	ex7	WT/WT	ND	absent	WT	WT
1959	R129fsX4/P245fsX3	WT/-	ex5 & ex7	**subclonal**	ND	ND	WT	WT
2759	R232*	WT	ex7	WT/WT	WT	ND	WT	WT
2775	R233fsX10	P245fsX9	ex7	WT/WT	ND	ND	WT	HD
2792	R232fsX10	P243fsX18	ex7	WT/WT	ND	ND	WT	WT
9160	C249fsX10	WT	ex7	WT/WT	WT	absent	WT	WT
9376	R232fsX10	P245fsX14	ex7	WT/WT	ND	absent	WT	FBXW7
9577	L180fsX2	I305fsX7	ex6 & ex8	WT/WT	ND	absent	WT	HD
9919	T231fsX14	WT	ex7	WT/WT	WT	absent	WT	HD/FBXW7
9963	T276A	WT	ex8	WT/WT	WT	absent	WT	WT
10111	C104fsX2	K236fsX5	ex5 & ex7	WT/WT	ND	absent	WT	WT
2852	P245fsX3	WT	ex7	WT/WT	**altered/WT**	ND	WT	WT
321	WT	-	-	**del/WT**	WT	absent	WT	PEST
2486	-	-	-	**del/del**	ND	absent	WT	WT
8815	WT	-	-	**del/WT**	**altered**	absent	WT	WT
9243	WT	WT	-	WT/WT	**altered**	absent	WT	WT
769	WT	WT	-	WT/WT	WT	absent	WT	FBXW7
8629	WT	WT	-	WT/WT	WT	absent	WT	HD
2781	WT	WT	-	WT/WT	ND	ND	**E17K**	WT
2787	WT	WT	-	ND	ND	ND	**E17K**	WT
3028	WT	WT	-	ND	ND	ND	**E17K**	FBXW7

Homozygous mutations are marked by an asterisk; ND: not done; WT: wild-type; Del: deletion. NOTCH1*-activating mutations are indicated as heterodimerization domain (HD) or proline, glutamic acid, serine, and threonine rich domain (*PEST*) mutations or mutations that occur in the E3-ubiquitin ligase* FBXW7 *gen.*

Online Supplementary Table S4. PTEN genetics of T-ALL cell lines.

Patient	PTEN mutation		Affected	PTEN	PTEN	PTEN	AKT	NOTCH/FBXW7	PTEN
	Allele A	Allele B	exon(s)	splicing	deletion	protein	mutation	mutation	reference
JURKAT	P245fsX9	WT	ex7	ND	ND	ND	ND	JM/FBXW7	new
MOLT16	P245fsX12	P245fsX12	ex7	ND	ND	absent	ND	WT	new
MOLT3	D267fsX?	WT	ex8	ND	ND	absent	ND	HD/PEST	18
PF382	V84fsX?	WT	ex4	ND	ND	absent	ND	HD/PEST	23
CEM	Y28fsX?	WT	ex2	ND	ND	absent	ND	HD/FBXW7	18,23
P12ichikawa	W274X	WT	ex8	ND	ND	absent	ND	FBXW7	18,23
KARPAS45	R334*	WT	ex7	ND	ND	absent	ND	HD/FBXW7	18
RPMI8402	K236fsX?	R159S	ex7	ND	ND	low present	ND	FBXW7	18,23
SUPT1	R172C	WT	ex6	ND	**del/WT/WT/WT**	low present	ND	WT	new
LOUCY	WT	-	-	**altered**	**del/WT**	absent	ND	WT	18+confirmed
SKW3	ND	-	-	ND	**del/WT**	absent	ND	PEST	ND
KE37	WT	WT	-	no transcript	**del/WT/del/WT**	absent	ND	PEST	18
HPBALL	WT	WT	-	WT	WT/WT	**low present**	ND	HD/PEST	18,23
HSB2	WT	WT	-	no transcript	WT/WT	+	ND	HD/FBXW7	new
PEER	WT	WT	-	ND	ND	+	ND	HD/FBXW7	new
ALLSIL	WT	WT	-	ND	ND	+	ND	HD/PEST	23
DND41	WT	WT	-	ND	ND	+	ND	HD/PEST	18,23
TALL1	WT	WT	-	ND	ND	+	ND	WT	18+confirmed
BE13	WT	WT	-	ND	ND	+	ND	HD/FBXW7	18+confirmed

Online Supplementary Table S5. Patients' characteristics and mutation overview for 146 pediatric T-ALL patients.

Table

Patients	Stratum	Gender	Age	WBC	GEP	Unsupervized cluster	TAL	LMO	TLX3	HOXA	MEF2C	TLX1	NKX2-1/NKX2-2	unknown	NOTCH1/FBXW7	PTEN/AKT/low PTEN protein	NOTCH1/FBXW7/PTEN/AKT status	del(9)(p21)	WT1	PHF6
1	1	1	7,5	130	1	1	1	0	0	0	0	0	0	0	1	0	HD/PEST	1	0	0
2	1	2	8	132	0	9999	1	0	0	0	0	0	0	0	0	1	PTEN	9999	0	0
3	1	1	3,3	590	1	1	1	0	0	0	0	0	0	0	0	1	PTEN	9999	0	0
4	1	1	13,4	41	1	1	1	0	0	0	0	0	0	0	1	0	HD	1	0	0
5	1	1	11,9	110	1	1	1	0	0	0	0	0	0	0	0	0	WT	1	0	0
6	1	1	4,3	205	1	1	1	0	0	0	0	0	0	0	9999	9999	9999	9999	0	9999
7	1	1	10,4	112	1	1	1	0	0	0	0	0	0	0	0	0	WT	1	0	0
8	1	2	9,8	28	0	9999	1	0	0	0	0	0	0	0	1	0	PEST	1	0	0
9	1	2	13	95	1	1	1	0	0	0	0	0	0	0	1	0	HD	1	0	0
10	1	1	6,4	188	0	9999	1	0	0	0	0	0	0	0	1	0	HD/PEST/FBXW7	9999	0	1
11	1	1	14,9	300	1	1	1	0	0	0	0	0	0	0	1	0	HD	9999	0	0
12	1	1	11,3	192	1	1	1	0	0	0	0	0	0	0	1	0	PEST	1	0	0
13	1	1	9,3	310	1	1	1	0	0	0	0	0	0	0	1	0	HD/FBXW7	9999	0	1
14	1	1	15,4	200	1	1	1	0	0	0	0	0	0	0	1	0	HD/FBXW7	9999	0	0
15	1	1	10,8	347	0	9999	0	0	0	1	0	0	0	0	1	0	HD/PEST	0	0	1
16	1	1	7,9	124	1	2	0	0	0	1	0	0	0	0	1	0	HD/PEST	1	0	0
17	1	1	10,1	14	1	4	0	0	0	1	0	0	0	0	0	0	WT	0	0	0
18	1	1	5,5	185	1	2	0	0	1	0	0	0	0	0	0	0	WT	1	0	1
19	1	1	3,2	417	1	2	0	0	1	0	0	0	0	0	1	0	HD	1	1	1
20	1	1	5,9	276	1	2	0	0	1	0	0	0	0	0	1	0	FBXW7	1	0	0
21	1	2	12,3	34	0	9999	0	0	1	0	0	0	0	0	1	0	HD/PEST	9999	0	0
22	1	1	6,3	174	1	2	0	0	1	0	0	0	0	0	1	0	PEST	1	0	1
23	1	1	5,1	80	1	2	0	0	1	0	0	0	0	0	1	0	HD	1	0	0
24	1	1	5,3	32	0	9999	0	0	1	0	0	0	0	0	1	0	HD/FBXW7	9999	1	0
25	1	2	8,8	89	1	2	0	0	1	0	0	0	0	0	1	0	HD/FBXW7	0	0	0
26	1	1	5,1	45	0	9999	0	0	1	0	0	0	0	0	1	0	HD/FBXW7	1	0	0
27	1	1	5	140	0	9999	0	0	1	0	0	0	0	0	1	0	HD	1	1	0
28	1	1	5,8	500	0	9999	0	0	1	0	0	0	0	0	1	0	HD	1	0	1
29	1	1	4,5	405	1	2	0	0	1	0	0	0	0	0	1	0	JM	9999	1	1
30	1	1	7,2	354	0	9999	0	0	1	0	0	0	0	0	0	0	WT	1	0	9999
31	1	1	6,2	69	0	9999	0	0	1	0	0	0	0	0	1	0	HD	9999	0	0
32	1	1	6,3	36	1	2	0	0	1	0	0	0	0	0	1	0	HD/FBXW7	0	0	0
33	1	2	7,8	90	1	2	0	0	1	0	0	0	0	0	9999	9999	9999	9999	0	9999
34	1	2	6,4	98	1	2	0	0	1	0	0	0	0	0	1	0	HD	1	1	0
35	1	1	10,3	149	0	9999	0	0	0	0	0	1	0	0	1	0	HD	1	0	0
36	1	1	11,2	54	1	3	0	0	0	0	0	1	0	0	1	0	HD	1	0	0
37	1	1	9,9	27	1	3	0	0	0	0	0	1	0	0	0	1	AKT1	1	1	1
38	1	1	3,5	57	1	3	0	0	0	0	0	1	0	0	0	1	PTEN	1	0	0
39	1	2	4,4	280	1	2	0	0	0	0	0	1	0	0	1	0	HD/PEST	1	0	1
40	1	1	7,8	159	1	2	0	0	0	0	0	1	0	0	9999	9999	9999	9999	0	1
41	1	2	2,2	153	0	9999	0	0	0	1	0	0	0	0	0	0	WT	1	0	0
42	1	2	12	41	0	9999	0	0	0	1	0	0	0	0	1	0	HD	1	0	0
43	1	1	9	95	1	1	0	1	0	0	0	0	0	0	1	0	PEST	1	0	0
44	1	1	1,8	250	0	9999	0	1	0	0	0	0	0	0	0	0	WT	0	0	0
45	1	2	9,3	900	1	1	0	1	0	0	0	0	0	0	1	0	JM	1	0	0
46	1	1	2,3	125	1	1	0	1	0	0	0	0	0	0	0	0	WT	1	0	0
47	1	2	16,7	86	1	1	0	0	0	0	0	0	0	1	0	0	WT	9999	0	0
48	1	1	13,8	129	0	9999	0	0	0	0	0	0	0	1	0	1	PTEN	9999	0	0
49	1	1	9,1	226	1	1	0	1	0	0	0	0	0	0	1	0	HD	9999	0	0
50	1	1	1,5	135	1	3	0	0	0	0	0	0	0	1	0	0	WT	1	0	0
51	1	1	15,7	129	1	3	0	0	0	0	0	0	1	0	1	0	HD	0	0	1
52	1	1	3,8	92	1	3	0	0	0	0	0	0	0	1	1	0	HD	1	0	0
53	1	1	15,9	136	1	1	0	0	0	0	0	0	0	1	1	1	HD/PTEN	1	0	0
54	1	2	2,8	58	0	9999	0	0	0	0	0	0	0	1	0	0	WT	9999	0	0
55	1	1	6,2	191	0	9999	0	1	0	0	0	0	0	0	1	0	FBXW7	1	0	0
56	1	1	3,9	435	1	4	0	0	0	0	0	0	0	1	0	0	WT	1	0	0
57	1	2	13,2	50	1	1	0	0	0	0	0	0	0	1	1	0	FBXW7	9999	0	0
58	1	1	1,1	530	0	9999	0	0	0	0	0	0	0	1	0	0	WT	9999	0	0
59	1	2	5,3	60	0	9999	0	0	0	0	0	0	0	1	1	0	HD	9999	1	0
60	1	2	8,4	15	1	4	0	0	0	0	0	1	0	0	1	0	PEST	1	0	0
61	1	2	3,8	158	1	1	1	0	0	0	0	0	0	0	1	1	FBXW7/No PTEN protein	9999	0	0
62	1	1	8,5	130	0	9999	0	0	0	0	0	0	0	1	1	0	HD	1	0	0
63	1	1	13,9	600	1	1	0	1	0	0	0	0	0	0	0	1	PTEN	1	0	0
64	1	1	13,7		1	1	1	0	0	0	0	0	0	0	0	0	WT	1	0	9999
65	1	2	1,9	387	0	9999	0	0	0	0	0	0	0	1	1	0	HD	1	0	9999
66	1	1	1,6	50	1	1	0	0	0	0	0	0	1	0	9999	9999	9999	1	1	9999
67	1	2	4,7	212	0	9999	0	1	0	0	0	0	0	0	1	0	PEST	9999	0	0
68	1	2	15,9	231	0	9999	0	0	0	0	0	0	0	1	1	0	HD	1	0	9999
69	1	2	4,3	5	0	9999	0	0	0	0	0	0	0	1	0	1	PTEN	0	0	0
70	1	1	6,2	167	1	1	0	1	0	0	0	0	0	0	0	1	AKT1	9999	0	0
71	1	1	2,8	649	0	9999	0	1	0	0	0	0	0	0	0	0	WT	1	0	9999
72	1	1	1,3	177	0	9999	0	0	0	0	0	0	0	1	1	0	HD/FBXW7	0	0	9999
73	2	1	2,2	63	1	3	1	0	0	0	0	0	0	0	1	1	PTEN/PEST	1	0	9999
74	2	1	4,3	252	1	1	1	0	0	0	0	0	0	0	0	0	WT	1	0	9999
75	2	1	5,8	31	1	1	1	0	0	0	0	0	0	0	0	0	WT	9999	0	9999
76	2	2	9,9	246	1	1	1	0	0	0	0	0	0	0	0	1	PTEN	1	0	9999
77	2	1	7,5	156	1	3	1	1	0	0	0	0	0	0	1	0	PEST	1	0	9999
78	2	1	4,6	57	1	1	1	0	0	0	0	0	0	0	0	0	WT	9999	0	9999
79	2	1	4,3	178	1	1	1	0	0	0	0	0	0	0	0	1	PTEN	1	0	9999
80	2	2	2,7	156	1	1	1	0	0	0	0	0	0	0	0	0	WT	1	0	9999
81	2	1	4,3	450	1	1	1	0	0	0	0	0	0	0	0	1	WT /No PTEN protein	9999	0	9999
82	2	1	7,3	118	1	1	1	0	0	0	0	0	0	0	0	1	PTEN	1	0	9999
83	2	1	4,9	30	1	3	1	0	0	0	0	0	0	0	1	1	HD/No PTEN protein	0	0	9999
84	2	1	9,9	61	1	1	1	0	0	0	0	0	0	0	1	0	PEST	1	0	9999
85	2	1	15,4	16	1	1	1	0	0	0	0	0	0	0	0	0	WT	1	0	9999

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86	2	1	9,7	4	1	2	0	0	1	0	0	0	0	0	1	0	HD/FBXW7	1	1	9999
87	2	1	6,8	77	1	2	0	0	1	0	0	0	0	0	1	0	HD	1	0	9999
88	2	2	11,7	81	1	2	0	0	1	0	0	0	0	0	1	0	PEST	1	1	9999
89	2	1	8,6	46	1	2	0	0	1	0	0	0	0	0	0	0	WT	1	0	9999
90	2	1	5,3	3	1	2	0	0	1	0	0	0	0	0	1	0	HD	1	0	9999
91	2	1	5	64	1	3	0	0	1	0	0	0	0	0	1	0	HD/FBXW7	9999	1	9999
92	2	1	14,9	30	1	3	0	0	1	0	0	0	0	0	1	1	FBXW7/AKT1	9999	1	9999
93	2	1	10,5	2	1	2	0	0	1	0	0	0	0	0	0	0	WT	1	0	9999
94	2	1	12	350	1	2	0	0	1	0	0	0	0	0	1	0	HD	9999	1	9999
95	2	1	7,5	120	1	2	0	0	1	0	0	0	0	0	1	0	HD	9999	0	9999
96	2	2	14,1	289	1	2	0	0	1	0	0	0	0	0	9999	0	9999	9999	0	9999
97	2	1	5,8	170	1	2	0	0	1	0	0	0	0	0	1	0	HD	1	1	9999
98	2	2	13,2	107	1	2	0	0	0	0	0	1	0	0	0	0	WT	1	0	9999
99	2	2	5,5	89	1	3	0	0	0	0	0	1	0	0	1	0	HD	1	0	9999
100	2	1	8	180	1	1	0	1	0	0	0	0	0	0	1	0	HD	1	0	9999
101	2	2	12,1	388	1	1	0	1	0	0	0	0	0	0	0	0	WT	1	0	9999
102	2	1	2,1	235	1	1	0	1	0	0	0	0	0	0	0	0	WT	1	0	9999
103	2	1	13,4	110	1	1	0	1	0	0	0	0	0	0	1	0	FBXW7	1	0	9999
104	2	2	14,9	109	1	1	0	1	0	0	0	0	0	0	1	0	HD/FBXW7	1	0	9999
105	2	1	2,6	185	0	9999	0	1	0	0	0	0	0	0	1	1	PTEN/PEST	1	0	9999
106	2	1	9,4	169	1	1	0	1	0	0	0	0	0	0	1	0	HD	1	0	9999
107	2	1	16,4	171	1	4	0	0	0	0	0	0	0	1	1	0	PEST	0	0	9999
108	2	2	12,4	94	1	4	0	0	0	0	0	0	0	1	1	1	PTEN/PEST	1	0	9999
109	2	2	6,4	207	1	4	0	0	0	0	0	1	0	0	1	0	HD/PEST	0	0	9999
110	2	2	10,8	248	1	4	0	0	0	0	0	1	0	0	1	0	PEST	0	0	9999
111	2	1	10,9	2	1	4	0	0	0	0	0	1	0	0	0	0	WT	0	0	9999
112	2	1	4,2	108	1	4	0	0	0	0	0	0	0	1	1	0	HD/PEST	0	0	9999
113	2	1	3,1	88	1	4	0	0	0	0	0	1	0	0	0	0	WT	0	0	9999
114	2	2	10,5	213	1	1	0	0	0	0	0	0	0	1	0	0	WT	1	0	9999
115	2	2	3,7	137	1	4	0	0	0	0	0	1	0	0	0	1	PTEN/HD	1	0	9999
116	2	1	16,1	9	1	4	0	0	0	0	0	0	0	1	1	0	PEST	0	0	9999
117	2	2	17,1	15	1	2	0	0	0	1	0	0	0	0	1	0	HD/PEST	1	0	9999
118	2	2	15,1	46	1	4	0	0	0	1	0	0	0	0	0	0	WT	0	0	9999
119	2	1	16,7	69	1	3	0	0	0	1	0	0	0	0	1	0	FBXW7	1	0	9999
120	2	1	17,8	57	1	2	0	0	0	1	0	0	0	0	1	0	JM	0	0	9999
121	2	1	13,2	6	1	4	0	0	0	1	0	0	0	0	1	0	PEST	0	0	9999
122	2	1	7,5	234	1	2	0	0	0	1	0	0	0	0	1	0	PEST	1	1	9999
123	2	2	10,5	142	1	2	0	0	0	1	0	0	0	0	1	0	HD/FBXW7	0	1	9999
124	2	2	15,4	213	1	2	0	0	0	1	0	0	0	0	1	0	HD/FBXW7	0	0	9999
125	2	1	7	152	1	1	0	0	0	0	0	0	0	1	0	0	WT	1	1	9999
126	2	1	7,8	119	1	1	0	0	0	0	0	0	0	1	1	0	JM/PEST	0	0	9999
127	2	2	10	18	1	1	0	0	0	0	0	0	0	1	0	0	WT	9999	0	9999
128	2	2	2,9	204	1	1	0	0	0	0	0	0	0	1	0	1	PTEN	1	0	9999
129	2	1	10,1	80	1	1	0	0	0	0	0	0	0	1	1	0	HD	1	0	9999
130	2	1	7,7	491	1	1	0	0	0	0	0	0	0	1	1	0	JM	1	0	9999
131	2	1	14,9	271	1	1	0	0	0	0	0	0	0	1	0	0	WT	1	0	9999
132	2	1	5,2	36	1	1	0	0	0	0	0	0	0	1	0	0	WT	1	0	9999
133	2	1	7,6	41	1	1	0	0	0	0	0	0	0	1	1	0	FBXW7	1	0	9999
134	2	1	2,2	89	1	1	0	0	0	0	0	0	0	1	0	1	PTEN	1	0	9999
135	2	1	2,9	183	1	1	0	1	0	0	0	0	0	0	1	1	PTEN/FBXW7	1	0	9999
136	2	1	9,4	287	1	1	0	0	0	0	0	0	0	1	0	1	PTEN	1	0	9999
137	2	1	4,6	57	1	3	0	0	0	0	0	0	1	0	1	0	HD	1	0	9999
138	2	1	3,8	76	1	3	0	0	0	0	0	0	0	1	1	0	HD/PEST	1	0	9999
139	2	2	11,1	214	1	1	0	0	0	0	0	0	0	1	0	0	WT	1	0	9999
140	2	2	5,4	15	1	4	0	0	0	0	0	0	0	1	0	0	WT	1	0	9999
141	2	1	6,9	30	1	3	0	0	0	0	0	0	1	0	1	0	HD	0	0	9999
142	2	2	6,3	67	1	3	0	0	0	0	0	0	1	0	1	1	PTEN/HD/FBXW7	0	0	9999
143	2	2	1,7	29	1	3	0	0	0	0	0	0	1	0	1	0	PEST	0	0	9999
144	2	2	12,8	192	1	3	0	0	0	0	0	0	1	0	0	0	WT	1	0	9999
145	2	1	7,5	295	1	2	0	0	1	0	0	0	0	0	1	0	HD	9999	0	9999
146	2	2	17,3	40	0	9999	0	0	0	0	0	0	0	1	1	0	FBXW7	1	0	9999

Legend for Table S5

Stratum, patients treated on DCOG (1) or COALL protocols (2); Gender, Male (1) or Female (2); Age in years; WBC, white blood cell counts (*10E9/liter); GEP, Gene expression data present (1) or absent (0); Unsupervized clusering, TAL/LMO (1), TLX (2), proliferative (3), Immature (4) (ref **Homminga et al., Cancer Cell. 2011, 19(4):484-497.**); Cytogenetics: TAI1, TAL1 or TAL2-rearranged (1); LMO, LMO1-, LMO2 or LMO3-rearranged (1); TLX3, TLX3-rearranged; HOXA, MLL-rearranged or CALM-AF10-positive or SET-NUP214-positive or Inv(7)(p15q34) (1); MEF2C, MEF2C-rearrangements or other rearrangements (involcing NKX2-5, SPI1, ETV6-NCOA2, RUNX1-AFF3) that activate MEF2C (1) (see ref **Homminga et al., Cancer Cell. 2011, 19(4):484-497**); TLX1, TLX1-rearranged (1), NKX2-1/NKX2-2, NKX2-1 or NKX2-2 rearranged (1); NOTCH1/FBXW7, cases having NOTCH1-activating mutations (1), PTEN/AKT/low PTEN protein, cases with PTEN or AKT aberrations or cases that lack PTEN protein expression due to unknown mechanisms (1); NOTCH1/FBXW7/PTEN/AKT status, mutations in NOTCH1, FBXW7, PTEN and/or AKT or deletion of PTEN or no PTEN protein detected; del(9)(p21), cases with deletion of CDKN2A and CDKN2B genes (1); WT1, WT1-mutated cases (1); PHF6, PHF6-mutated cases (1); Missing data in patients have been indicated by "9999"; patient lacking described rearrangements or matations are indicated by "0" .

Online Supplementary Table S6. Clinical and immunophenotypic data of *PTEN/AKT* and *NOTCH1/FBXW7*-mutated *versus* wild-type patients.

	PTEN/AKT+NOTCH/FBXW7 aberration		
	WT	Mut	
Total n. patients (n=141)	**36**	**105**	***P***
Male	24	73	0.75
Female	12	32	
Median age (range)	7.1 (1.1-16.7)	7.6 (1.3-17.8)	**0.05**
Median WBC (range)	125 (2.0-649.0)	124 (3.0-900.0)	0.61
Events (n=49)	**WT n(%)**	**Mut n(%)**	***P***
	9	**40**	
Relapse (%)	2 (22%)	33 (83%)	**0.002**†
Toxic death / 2nd malignancy (%)	4/3 (78%)	4/3 (17%)	**0.03**
Immunophenotypic (n=138)	**WT n(%)**	**Mut n(%)**	***P***
Pre-T/Pro-T +	9 (23%)	30 (77%)	0.61
Cortical T +	12 (21%)	46 (79%)	0.20
Mature T +	15 (37%)	26 (63%)	0.06

Significant P values are indicated in bold. All P values were calculated using Pearson's χ^2 test, unless indicated; WT: wild-type; Mut, mutant; P: P value; Median age indicated in years; ▫ Mann-Whitney-U test; WBC: white blood cell count; white blood cell counts are indicated as number of blasts (x10⁹/L); †Fisher's exact test.

Online Supplementary Table S7. Relapse free survival of patient groups based on clinical, (cyto)genetic or biological characteristics.

Clinical (n=146)	DCOG 5-year RFS (%±SD)	*P*	COALL 5-year RFS (%±SD)	*P*	Overall stratified analysis 5-year RFS (%±SD)	*P*
Male (n=100) *vs.* female (n=46)	84±8 *vs.* 66±7	0.19	91±6 *vs.* 62±9	0.03	88±5 *vs.* 65±5	**0.01**
Age <10 (n=96) *vs.* ≥10 year (n=50)	62±11 *vs.* 74±6	0.53	82±8 *vs.* 69±8	0.31	74±7 *vs.* 72±5	0.81
WBC <50 (n=31) *vs.* ≥50 *10^9/L (n=114)	69±6 *vs.* 80±13	0.4	70±7 *vs.* 84±10	0.13	70±5 *vs.* 83±8	0.09
Cytogenetics (n=146)						
TAL1 + (n=27) *vs. TAL1* - (n=119)	92±7 *vs.* 66±6	0.07	62±15 *vs.* 76±6	0.27	79±9 *vs.* 71±5	0.47
LMO2 + (n=14) *vs. LMO2* - (n=132)	57±19 *vs.* 73±6	0.43	75±22 *vs.* 74±6	0.73	64±15 *vs.* 74±4	0.70
TLX3 + (n=29) *vs. TLX3* - (n=117)	57±12 *vs.* 75±6	0.11	54±17 *vs.* 78±6	0.17	56±10 *vs.* 77±4	**0.04**
TLX1 + (n=8) *vs. TLX1* - (n=138)	80±18 *vs.* 70±6	0.54	73±6 *vs.* 100±0	0.46	86±13 *vs.* 72±4	0.37
HOXA + (n=13) *vs. HOXA* - (n=133)	40±23 *vs.* 74±6	0.15	83±15 *vs.* 73±6	0.62	64±15 *vs.* 74±4	0.53
□ *MEF2C*+ (n=6) *vs. MEF2C* - (n=140)	73±6 *vs.* 71±6	0.56	80±18 *vs.* 100±0	0.85	83±15 *vs.* 72±4	0.65
□ *NKX2-1*+ (n=7) *vs. NKX2-1* - (n=139)	100±0 *vs.* 70±6	0.40	100±0 *vs.* 71±7	0.20	100±0 *vs.* 71±4	0.13
†Unknowns (n=42) *vs.* knowns (n=104)	75±10 *vs.* 70±7	0.81	78±10 *vs.* 73±7	0.88	77±7 *vs.* 71±5	0.78
Gene expression clusters (n=117)‡						
TAL/LMO + (n=53) *vs.* TAL/LMO - (n=64)	73±10 *vs.* 67±10	0.70	69±10 *vs.* 79±7	0.36	71±7 *vs.* 75±6	0.70
TLX + (n=30) *vs.* TLX - (n=87)	60±14 *vs.* 74±8	0.25	65±13 *vs.* 79±6	0.36	63±9 *vs.* 77±5	0.15
Proliferative + (n=19) *vs.* Proliferative - (n=98)	83±15 *vs.* 68±8	0.40	89±11 *vs.* 73±7	0.34	87±9 *vs.* 71±5	0.20
Immature/(ETP)ALL + (n=15) *vs.* Immature/(ETP)ALL - (n=102)	50±35 *vs.* 70±7	0.97	91±9 *vs.* 72±7	0.23	85±10 *vs.* 71±5	0.32
Type B mutations						
PTEN/AKT mutant (n=25) *vs.* wild-type (n=117)	60±16 *vs.* 71±6	0.38	57±15 *vs.* 78±6	0.14	59±11 *vs.* 75±4	0.09
NOTCH1/FBXW7 mutant (n=90) *vs.* wild-type (n=51)	62±8 *vs.* 82±8	0.10	70±8 *vs.* 80±9	0.41	66±5 *vs.* 81±6	0.08
PTEN/AKT/NOTCH1/FBXW7 mutant (n=105) *vs.* wild-type (n=36)	62±7 *vs.* 93±7	0.03	68±7 *vs.* 90±10	0.07	65±5 *vs.* 92±6	**0.005**
PHF6 mutant (n=12) *vs.* wild-type (n=51)	73±13 *vs.* 67±7	0.69	ND		ND	
WT1 mutant (n=17) *vs.* wild-type (n=129)	63±17 *vs.* 72±6	0.47	49±23 *vs.* 77±6	0.21	59±13 *vs.* 75±4	0.17
Del *9p21* (n=88) *vs.* wild-type (n=25)	72±7 *vs.* 63±17	0.67	68±8 *vs.* 94±6	0.06	70±5 *vs.* 84±7	0.24

Significant log rank P *values for DCOG or COALL cohort analyses are indicated in bold; RFS: relapse free survival; SD: standard deviation;* P: P *value; WBC: white blood cell count;* □*Different genetic aberrations have been identified that all result in the activation of the* MEF2C *or* NKX2-1/NKX2-2 *oncogenes that define novel genetic T-ALL subtypes*[10]*;* †*All patients who have one of the above described cytogenetic aberrations (known)* versus *all patients without any of these above described aberrations (unknown);* ‡*113 out of 117 T-ALL patients included in the gene expression profiling study*[10] *had a known* PTEN *and* AKT *mutation status. T-ALL patients were assigned to the TAL/LMO group based on the presence of* TAL1 *or* LMO2 *rearrangements or by having a* TAL/LMO *expression signature.*[10]

Online Supplementary Table S8. *NOTCH1*-activating and *PTEN/AKT* mutations predict for poor outcome in pediatric T-ALL.

	N.	Univariate analyses using Cox's regression model Hazard ratio	95% CI	*P*
Male gender	146	0.3	0.122-0.820	0.02
TLX3	146	2.1	1.030-4.339	0.04
PTEN/AKT/NOTCH1/FBXW7	141	6.1	1.456-25.310	0.01

	N.	Multivariate analyses using Cox's regression model Hazard ratio	95% CI	*P*
Male gender	141	0.37	0.141-0.959	0.04
TLX3	141	1.7	0.843-3.629	0.13
PTEN/AKT/NOTCH1/FBXW7	141	5.6	1.341-23.437	0.02

Univariate and multivariate Cox's regression analyzes using relapse free survival for various parameters that were significantly associated with good or poor relapse free survival (see Online Supplementary Table S6).