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# *PHF6* mutations in T-cell acute lymphoblastic leukemia

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**AUTHOR CONTRIBUTIONS:** P.V.V. performed array CGH and mutation analysis of *PHF6* and wrote the manuscript. T.P. performed exon capture and next-generation sequencing of T-ALL samples and wrote the manuscript. H.K. analyzed next-generation sequencing data. J.V.d.M. performed additional array-CGH analysis and *PHF6* mutation screening in T-ALL and BCP-ALL samples. T.T., N.V.R. and A.W.L. performed experiments. M.C. and C.C.-C. performed and analyzed histological and immunohistochemical staining. J.P collaborated on *PHF6* mutation screening in BCP-ALL samples. C.J.H. and C.S. collaborated on additional screening for genomic *PHF6* deletions in T-ALL. Y.B., B.D.M and B.C. collaborated on the *PHF6* mutation screening. R.P. M.P., S.S. and J.S. collaborated on the multi-center array-CGH study. S.G.-G. and M.L.T. performed the isolation of T-cell progenitor cells for expression analysis of *PHF6*. X.Y. performed survival analysis of ECOG T-ALL patients. J.G. provided critical reagents and discussion. E.S. provided samples and correlative clinical data from DCOG. E.P., J.M.R. and P.H.W. provided samples and correlative clinical data from ECOG. J.M. and L.Z. collaborated on the multi-center array-CGH study and *PHF6* mutation analysis, provided molecular data on the characterization of T-ALL and performed survival analysis of *PHF6* mutations in the DCOG series. R.R. designed and directed the analysis of next generation sequencing results. F.S. and B.P. designed the studies and directed research. A.F. designed the studies, directed research and wrote the manuscript.

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## Abstract

Tumor suppressor genes on the X chromosome may skew the gender distribution of specific types of cancer[1,2]. T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological malignancy with an increased incidence in males[3]. In this study, we report the identification of inactivating mutations and deletions in the X-linked plant homeodomain finger 6 (*PHF6*) gene in 16% of pediatric and 38% of adult primary T-ALL samples. Notably, *PHF6* mutations are almost exclusively found in T-ALL samples from male subjects. Mutational loss of *PHF6* is significantly associated with leukemias driven by aberrant expression of the homeobox transcription factor oncogenes *TLX1* and *TLX3*. Overall, these results identify *PHF6* as a new X-linked tumor suppressor in T-ALL and point to a strong genetic interaction between *PHF6* loss and aberrant expression of TLX transcription factors in the pathogenesis of this disease.

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy in which multiple genetic defects collaborate in the transformation of T-cell progenitors[4,5]. Notably, T-ALL has a 3-fold higher incidence in males[3], while other immature hematologic tumors such as precursor B-lineage ALL (BCP-B-ALL) are equally frequent in males and females[3].

To identify a possible X-linked tumor suppressor in T-ALL, we performed an X chromosome targeted mutation analysis in tumor DNA samples from 12 male T-ALL cases. For each sample, we performed in-solution DNA capture of 7,674 regions encompassing 3,045,708 nucleotides corresponding to 5,215 X chromosome exons using the Agilent Sure Select oligonucleotide capture system[6]. DNA samples enriched for X chromosome exons were then analyzed by next generation sequencing using the SOLiD 3 platform. This analysis identified 66 candidate novel non-synonymous single nucleotide variants and 7 positions with high confidence calls for containing complex variants such as insertions or deletions (Fig. 1a). Dideoxynucleotide DNA sequencing of PCR products encompassing affected exons confirmed the presence of 61/66 (92%) of these single nucleotide variants and 4/7 (57%) of the more complex variants, including 2 insertions and 2 deletions (Supplementary Tables 1 and 2). Sequence analysis of paired DNA samples obtained at the time of clinical remission showed that most of these variants corresponded to previously unreported germline polymorphisms. However, and most notably, we also identified three somatically acquired changes corresponding to two non-synonymous single nucleotide substitutions (c.902A>G, p.T300A and c.990A>G, p.H330R) and a frameshift-creating insertion of 5 nucleotides (c.124_125insAGGCA, p.H43fs) in the *PHF6* (plant homeodomain finger 6) gene (Fig. 1a).

In a complementary approach, we analyzed X chromosome array comparative genome hybridization (array-CGH) data from 246 primary T-ALL samples (179 male and 67 female)

in a multi-centre setting. These analyses revealed the presence of recurrent deletions in chromosomal band Xq26 in 8 out of 246 (~3%) T-ALL samples (Table 1). For 3 del(X)(q26) positive T-ALL cases, we performed array-CGH analysis against the corresponding remission material, which showed that these Xq26 deletions are somatically acquired leukemia-associated genetic events (Table 1). Re-analysis of all 8 del(X)(q26) positive T-ALL cases on a custom high-resolution chromosome X oligonucleotide array (Fig. 1b,c) narrowed down the common minimally deleted region to an area of 80 kb containing the *PHF6* gene. Consistently, quantitative PCR analysis confirmed loss of the *PHF6* locus in the del(X)(q26) positive cases (Fig. 1d). The convergent findings of our X chromosome exon mutation analysis and analysis of copy number alterations by array-CGH thus identified the *PHF6* gene as a new tumor suppressor mutated and deleted in T-ALL.

*PHF6* encodes a plant homeodomain (PHD) factor containing four nuclear localization signals and two imperfect PHD zinc finger domains[7] with a proposed role in the control of gene expression[7]. Notably, inactivating mutations in *PHF6* cause the Börjeson-Forssman-Lehmann syndrome (BFLS; MIM#301900), a relatively uncommon type of X-linked familial syndromic mental retardation which has not been associated with increased incidence of T-ALL[7-9]. Quantitative RT-PCR analysis demonstrated ubiquitous expression of *PHF6* transcripts in human tissues, with the highest levels of expression in thymus, ovary and thyroid and moderate levels of expression in spleen, testes and adipose tissue (Supplementary Fig. 1). Consistent with these results, PHF6 was readily detected by immunohistochemistry in mouse thymus (Supplementary Fig. 1). Finally, quantitative RT-PCR analysis of human thymocyte populations at different stages of development showed variable levels of *PHF6* expression, with marked upregulation of *PHF6* transcripts in CD4/CD8 double positive cells (Supplementary Fig. 1).

Mutation analysis of *PHF6* in an extended panel of pediatric and adult T-ALL primary samples identified truncating or missense mutations in *PHF6* in 38% (16/42) of adult and ~16% (14/89) of pediatric T-ALL samples (Fig. 2a and Table 1). In all available cases (7/30), analysis of matched buccal and/or bone marrow remission genomic DNA confirmed the somatic origin of *PHF6* mutations (4/21 frameshift mutations and 3/9 missense mutations) (Fig. 2b and Table 1). Finally, no mutations in *PHF6* were identified in DNA samples from BCP-B-ALLs ($n$ = 62), suggesting that mutational loss of *PHF6* in lymphoid tumors could be restricted to T-ALL.

Nonsense and frame-shift mutations accounted for 70% (21/30) of all *PHF6* mutations identified in our series and were evenly distributed throughout the gene. Missense mutations accounted for the remaining 30% (9/30) of *PHF6* lesions and recurrently involved codon C215 and the second zinc finger domain of the protein (Fig. 2a). DNA sequence analysis of *PHF6* in a panel of 15 well characterized T-ALL cell lines (Supplementary Table 3) showed the presence of truncating mutations in the *PHF6* gene in the DND41, HPB-ALL and T-ALL1 cell lines. Western blot analysis and immunohistochemical staining of PHF6 demonstrated robust expression and nuclear localization of PHF6 in *PHF6*-wild-type tumors and complete loss of PHF6 protein in T-ALL cell lines harboring mutations in *PHF6* (Fig. 2c,d).

PHD finger-containing proteins have been implicated in numerous cellular functions, including transcriptional regulation and in some instances as specialized reader modules that recognize the methylation status of histone lysine residues[10]. In addition, PHF6 has been reported to be phosphorylated during mitosis[11] and by the ATM and ATR kinases upon DNA damage[12], which suggests a dynamic regulation of PHF6 during cell cycle and DNA repair. Consistent with this notion, shRNA knockdown of PHF6 resulted in increased levels of phosphorylated H2AX (gamma-H2AX), a posttranslational modification associated with the presence of DNA double strand breaks[13] (Fig. 2e).

Sex determination in humans is controlled by differential representation of the X and Y chromosomes, with presence of an XY pair in the male genome and two copies of chromosome X in females. The presence of numerous genes in the non-autosomal region of the X chromosome could result in a genetic imbalance between male and female cells, which is compensated by random chromosomal inactivation of one copy of chromosome X in female cells[14]. However, allelic expression analysis has shown that some genes can escape X chromosome inactivation in certain tissues[1,2,15]. To test the possibility that *PHF6* could escape X-inactivation in T-ALL cells, we performed allelic expression analysis of a silent SNP (rs17317724) located in the 3′ untranslated region of *PHF6* in lymphoblasts from 3 informative female T-ALL cases. In each of these samples, *PHF6* was monoallelilically expressed, suggesting that biallelic expression of *PHF6* is not commonly found in T-ALL (Fig. 3a). Most notably, we found that *PHF6* mutations are almost exclusively found in male T-ALL cases. *PHF6* mutations were present in 29/92 (32%) males and only in 1/39 (~2.5%) females ($P < 0.001$; Fig. 3b and Supplementary Table 4). Moreover, all 8 *PHF6* deletions identified by array-CGH analysis were found in male T-ALL cases, and each of the three cell lines with mutations in *PHF6* were derived from male T-ALL cases.

Immunohistochemical analysis of PHF6 expression in wild-type primary T-ALL samples showed positive PHF6 immunostaining ($n = 5$; 3 males and 2 females), while cases with *PHF6* truncating mutations ($n = 4$) (Fig. 3c) or a point mutation in C215 (p.C215F) were negative for PHF6 protein expression (Fig. 3c). In contrast, primary T-ALL cells harboring a *PHF6* point mutation in the PHD2 domain (p.T300A) were positive for PHF6 protein expression (Fig. 3c). Overall, these results suggest that truncating mutations and point mutations in C215 impair PHF6 expression, while amino acid substitutions in the PHD2 domain of PHF6 may selectively impair the tumor suppressor function of this protein.

Leukemic transformation of immature thymocytes is the result of a multistep process involving numerous genetic abnormalities, which can be associated with different clinical features, including age and prognosis. Notably, *PHF6* mutations were significantly more prevalent in adult (16/42; 38%) than in pediatric T-ALLs (14/89; 16%) ($P = 0.005$; Fig. 4a). Detailed genetic information was available for T-ALL cases treated in DCOG clinical trials ($n = 65$) (Supplementary Table 5). In this cohort, *PHF6* mutations were significantly associated with the aberrant expression of *TLX1* and *TLX3* ($P < 0.005$; Fig. 4b and Supplementary Table 5), two related oncogenes activated by chromosomal translocations in T-ALL[16-18]. No significant associations were observed between *PHF6* mutations and mutations in *NOTCH1*, *FBXW7* or *PTEN* in either pediatric ($n = 65$) or adult ($n = 34$) T-ALL cohorts (Supplementary Tables 5 and 6). Overall survival in *PHF6* wild-type pediatric

T-ALL cases treated on DCOG protocols[19] was 65% (33/51) vs. 71% (10/14) for *PHF6*-mutated cases (log-rank $P = 0.71$) (Fig. 4c). Overall survival in *PHF6* wild-type adult T-ALL leukemias treated in the ECOG2993 clinical trial was 36% (7/12) vs. 58% (8/22) for *PHF6*-mutated samples (log-rank $P = 0.24$) (Fig. 4d).

Overall, these results identify *PHF6* as a new X-linked tumor suppressor gene and strongly suggest a specific interaction between the oncogenic programs activated by aberrant expression of TLX transcription factors and the mutational loss of *PHF6* in the pathogenesis of T-ALL.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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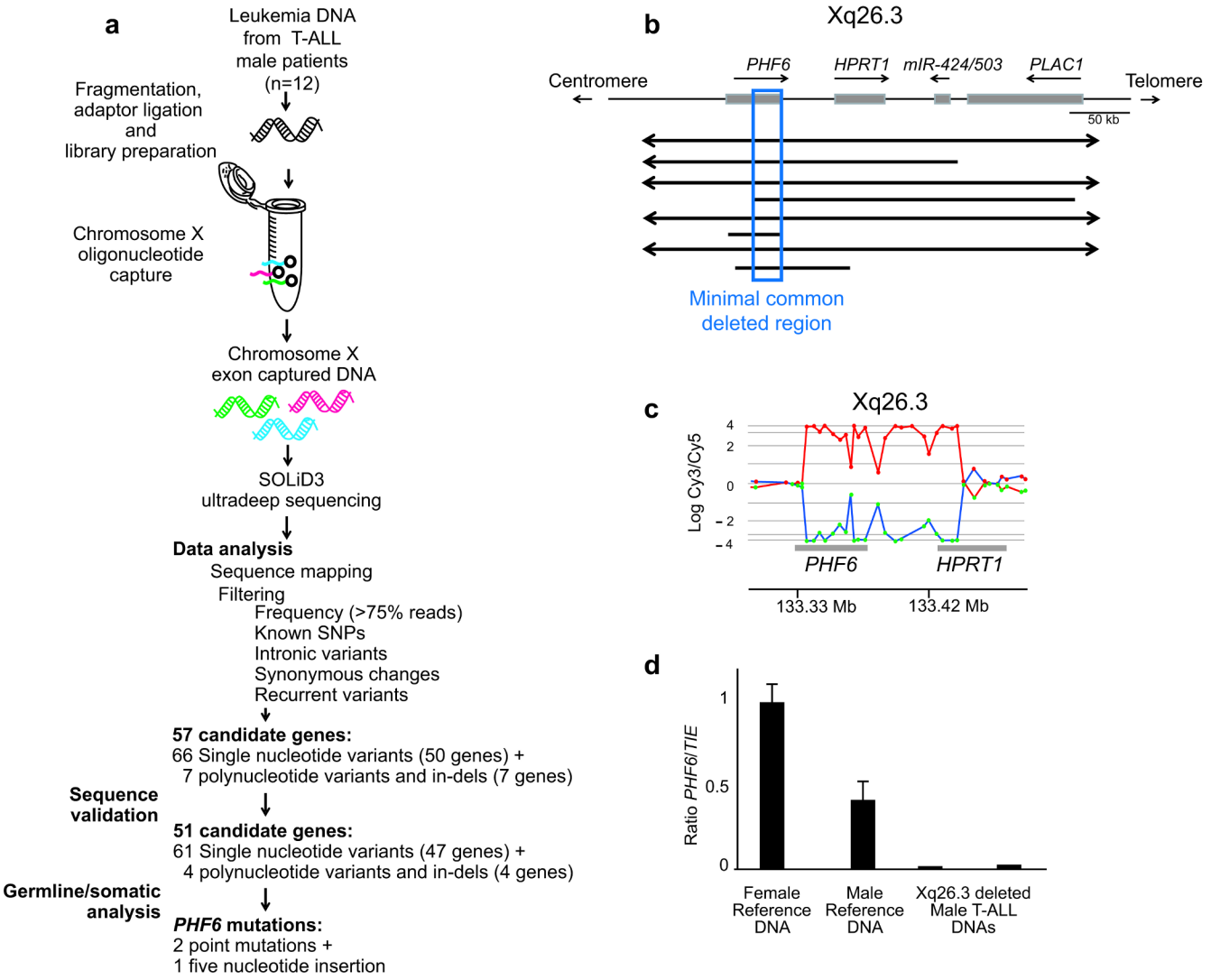


**Figure 1.**

Next generation sequencing and array CGH analysis of the X chromosome identifies *PHF6* mutations in human T-ALL. (**a**) Overview of mutation screening approach of the human X chromosome exome in a panel of tumor DNA samples from 12 male T-ALL cases using oligonucleotide sequence capture and next generation sequencing with SOLiD3. After filtering and confirmation of high throughput sequencing data, analysis of corresponding remission DNA samples led to the identification of three somatically acquired changes in the *PHF6* gene. (**b**) Schematic overview of the recurrent genomic deletions involving chromosomal band Xq26.3 in 8 human T-ALL samples. Specific genes located in Xq26.3 are shown. (**c**) Detailed view of a representative oligo array-CGH plot of leukemia DNA/control DNA ratios (blue tracing) versus the dye-swap experiment (red tracing) in a patient harboring an Xq26.3 deletion. (**d**) DNA quantitative PCR analysis of *PHF6* copy number dose in female and male reference genomic DNAs and 2 primary samples from male T-ALL cases harboring Xq26.3 deletions.

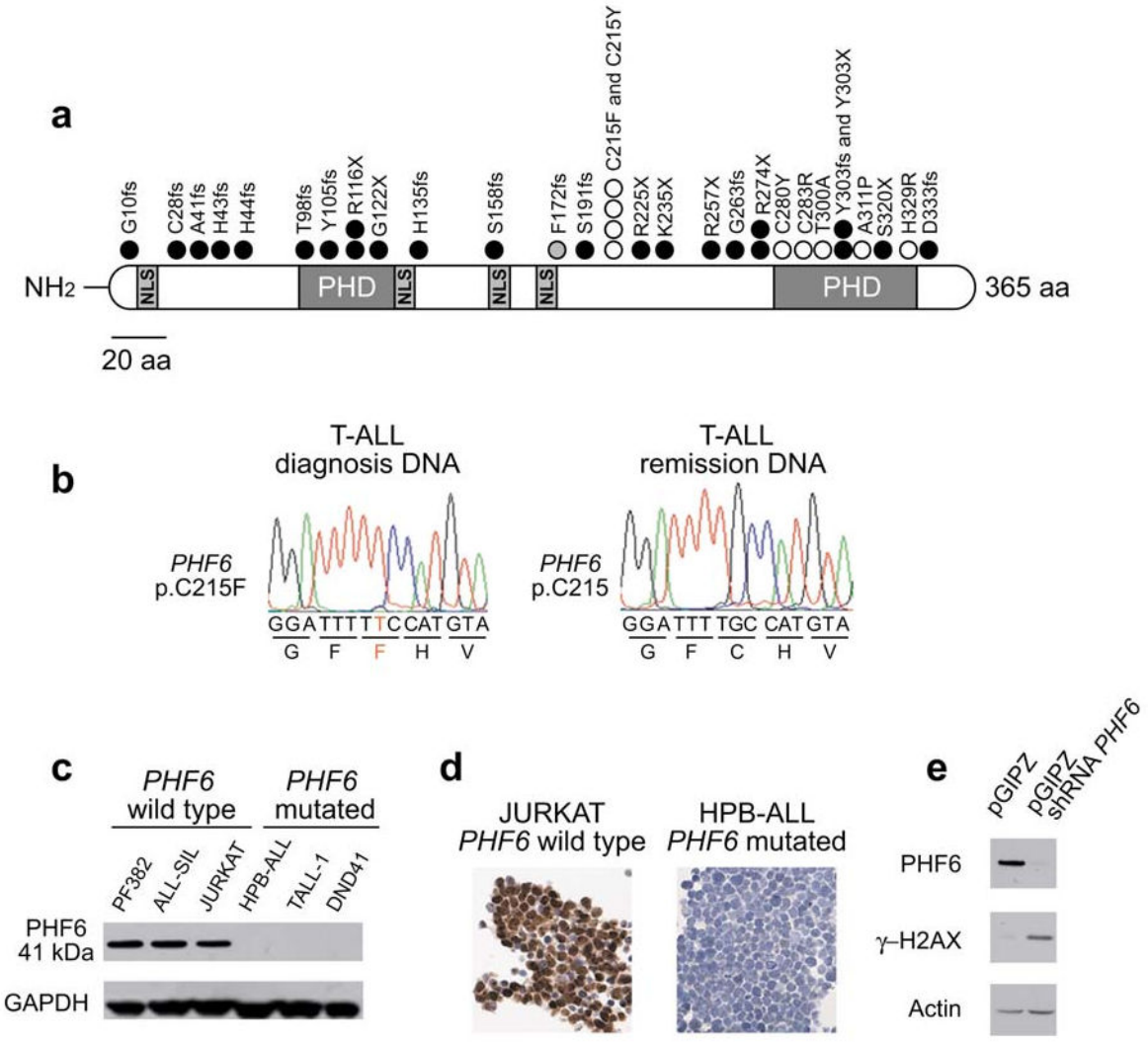


**Figure 2.**

*PHF6* mutations and expression in T-ALL lymphoblasts. (**a**) Structure of the PHF6 protein including four nuclear localization signals and two imperfect PHD zinc finger domains. Overview of all *PHF6* mutations identified in primary T-ALL samples and T-ALL cell lines. Filled circles represent nonsense and frameshift mutations, whereas missense mutations are depicted as open circles. Circles filled in gray indicate mutations identified in female T-ALL cases. (**b**) Representative DNA sequencing chromatograms of paired diagnosis and remission genomic T-ALL DNA samples showing a somatic mutation in exon 7 of *PHF6*. (**c**) Western blot analysis of T-ALL cell lines revealed complete loss of PHF6 protein expression in the *PHF6* mutated T-ALL cell lines. (**d**) PHF6 immunostaining in the Jurkat and HPB-ALL, wild-type and mutant T-ALL cell lines, respectively. (**e**) Western blot analysis of PHF6 and gamma-H2AX expression in HEK293T cells upon PHF6 shRNA knockdown. Actin levels are shown as loading control.

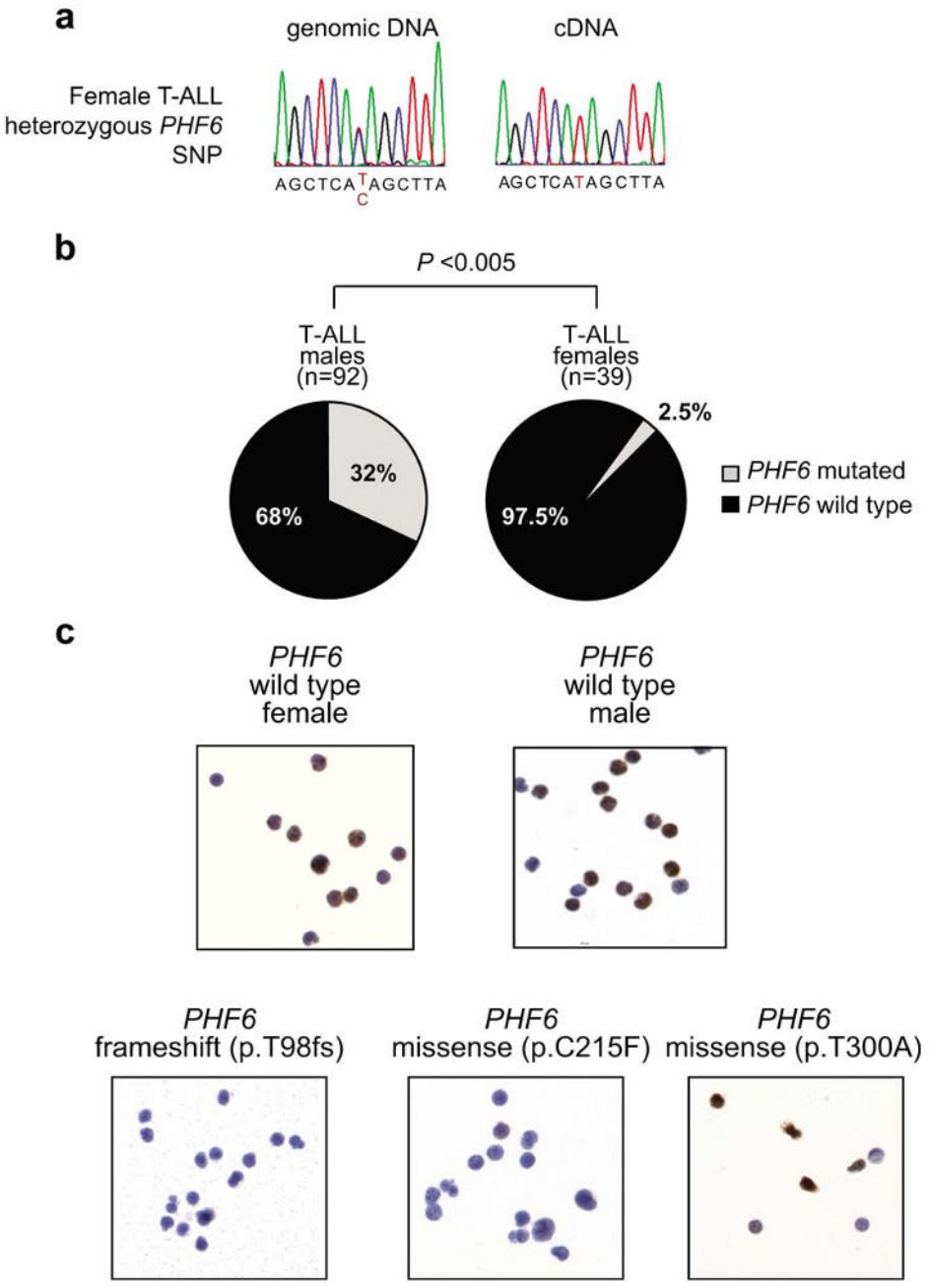


**Figure 3.**

*PHF6* expression in T-ALL lymphoblasts. (**a**) Sequence analysis of paired genomic DNA and cDNA samples shows monoallelic expression of *PHF6* SNP rs17317724 in lymphoblasts from a wild-type *PHF6* female T-ALL case. (**b**) Differential distribution of *PHF6* mutations in T-ALL samples from male and female cases. (**c**) Immunohistochemical analysis of PHF6 expression in wild type and mutant T-ALL lymphoblasts.

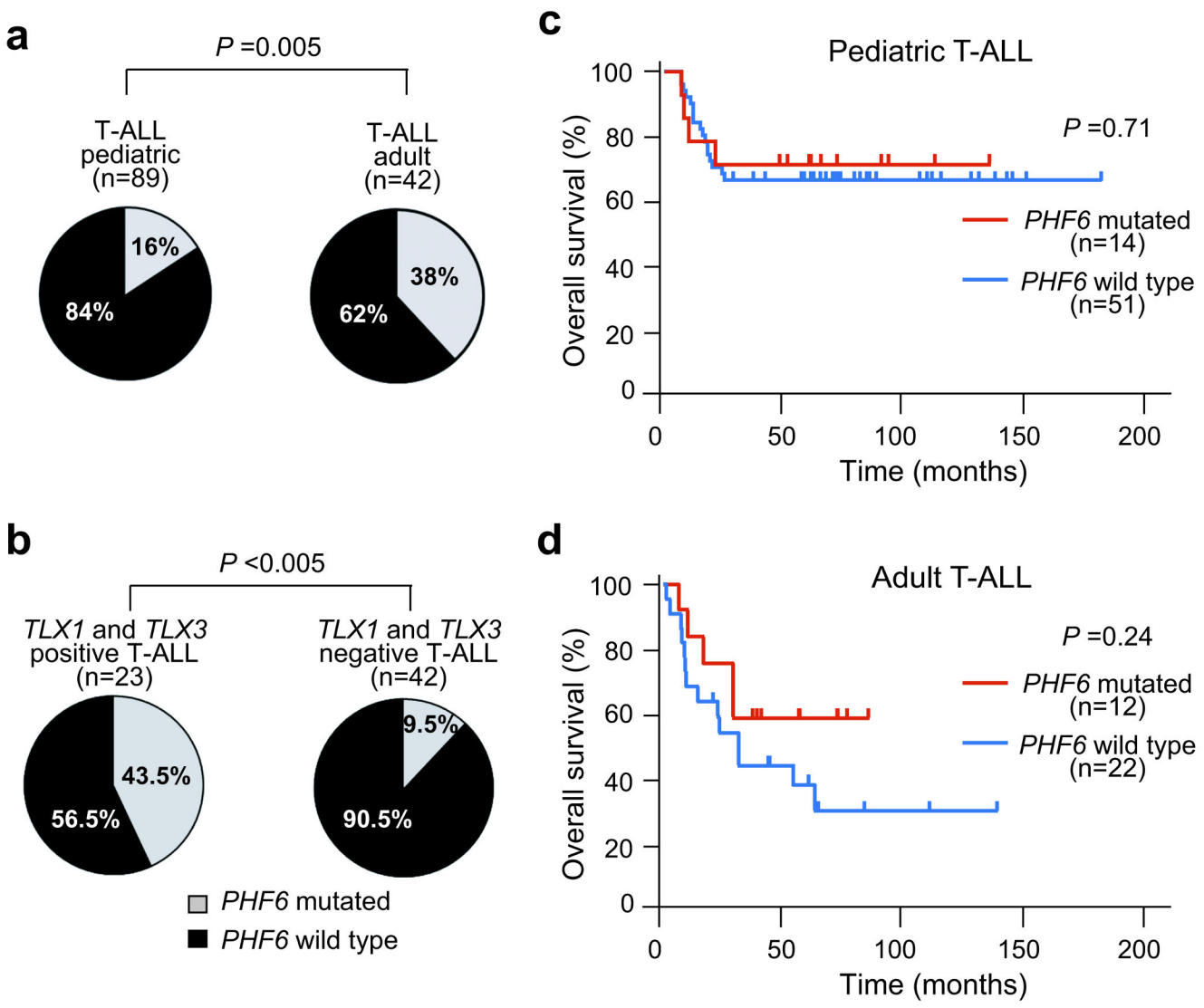


**Figure 4.**

Clinical and biological characteristics associated with *PHF6* mutations in T-ALL. (**a**) Frequencies of *PHF6* mutations in pediatric and adult T-ALL samples. (**b**) Differential distribution of *PHF6* mutations in *TLX1/TLX3* positive and negative T-ALL samples. (**c**) Kaplan-Meier curve of overall survival in pediatric T-ALL patients from DCOG trials ALL7, ALL8 and ALL9 with and without *PHF6* mutations. (**d**) Kaplan-Meier survival curve in adult T-ALL patients with and without mutations in *PHF6* treated in ECOG clinical trial ECOG2993.

**Table 1**

**Characteristics of 38 primary T-ALL samples showing *PHF6* inactivation**

| | | | | | | | ***PHF6* lesion** | | |
|---|---|---|---|---|---|---|---|---|---|
| **ID** | **Sex** | **Age** | **WBC (×10$^9$/l)** | **Immuno-phenotype** | **Genetic subtype** | ***NOTCH1*** | **Type of alteration** | **Predicted protein** | **Germline/somatic** |
| | *PHF6* deletions | | | | | | | | |
| 1 | M | Ped | 77 | Cortical | *TLX3* | mut | Deletion (0.55 Mb) | | NA |
| 2 | M | Ped | 46 | Pre-T | *TLX3* | wt | Deletion (0.23 Mb) | | NA |
| 3 | M | Ped | 31 | Pre-T | *TLX3* | NA | Deletion (1.50 Mb) | | NA |
| 4 | M | Ped | 2 | Pre-T | unknown | NA | Deletion (0.27 Mb) | | NA |
| 5 | M | Ped | NA | Cortical | *HOXA* | NA | Deletion (1.90 Mb) | | Somatic |
| 6 | M | Ped | NA | Cortical | unknown | NA | Deletion (0.20 Mb) | | Somatic |
| 7 | M | Ped | NA | Cortical | *TLX1* | NA | Deletion (0.08 Mb) | | Somatic |
| 8 | M | Adult | NA | Pre-T | Unkn | NA | Deletion (0.11 Mb) | | NA |
| | *PHF6* mutations | | | | | | | | |
| 9 | M | Ped | 185 | Cortical | *TLX3* | wt | Nonsense | p.G122X | NA |
| 10 | M | Ped | 417 | Pre-T | *TLX3* | mut | Nonsense | p.R116X | NA |
| 11 | F | Ped | 280 | Pre-T | *TLX1* | mut | Frameshift | p.F172fs | NA |
| 12 | M | Ped | 405 | Pre-T | *TLX3* | mut | Frameshift | p.Y303fs | NA |
| 13 | M | Ped | 159 | Pre-T | *TLX1* | mut | Nonsense | p.K235X | NA |
| 14 | M | Ped | 500 | Pre-T | *TLX3* | mut | Frameshift | p.A41fs | NA |
| 15 | M | Ped | 347 | Cortical | *HOXA* | mut | Nonsense | p.K274X | NA |
| 16 | M | Ped | 129 | Cortical | unknown | mut | Frameshift | p.D333fs | NA |
| 17 | M | Ped | 174 | Cortical | *TLX3* | mut | Nonsense | p.R225X | NA |
| 18 | M | Ped | 27 | Cortical | *TLX1* | wt | Nonsense | p.R116X | NA |
| 19 | M | Ped | 310 | Cortical | *TAL1* | mut | Missense | p.C283R | NA |
| 20 | M | Ped | 189 | Cortical | *TAL1* | mut | Frameshift | p.C28fs | NA |
| 21 | M | Adult | 170 | Cortical | *TLX3* | wt | Frameshift | p.H44fs | NA |
| 22 | M | Adult | 21 | Cortical | *TLX1* | mut | Frameshift | p.H43fs | Somatic |
| 22 | M | Adult | 21 | Cortical | *TLX1* | mut | Frameshift | p.H43fs | Somatic |

| | | | | | | | ***PHF6* lesion** | | |
|---|---|---|---|---|---|---|---|---|---|
| **ID** | **Sex** | **Age** | **WBC (×10$^9$/l)** | **Immuno-phenotype** | **Genetic subtype** | ***NOTCH1*** | **Type of alteration** | **Predicted protein** | **Germline/somatic** |
| 23 | M | Adult | NA | Pre-T | *TLX3* | mut | Frameshift | p.T98fs | Somatic |
| 24 | M | Adult | 14 | Pre-T | *TLX3* | wt | Frameshift | p.Y105fs | NA |
| 25 | M | Adult | 28 | Mature | *TLX3* | wt | Frameshift | p.S158fs | NA |
| 26 | M | Adult | NA | Cortical | *TLX3* | mut | Missense | p.C215Y | NA |
| 27 | M | Adult | NA | Pre-T | Unkn | mut | Missense | p.C215F | NA |
| 28 | M | Adult | 31 | Cortical | Unkn | mut | Missense | p.C215Y | NA |
| 29 | M | Adult | NA | Mature | *TLX3* | mut | Missense | p.T300A | Somatic |
| 30 | M | Adult | 21 | Cortical | *TLX1* | wt | Missense | p.A311P | NA |
| 31 | M | Adult | 30 | Mature | Unkn | wt | Missense | p.C280Y | NA |
| 32 | M | Adult | 23 | Cortical | Unkn | wt | Missense | p.H329R | Somatic |
| 33 | M | Ped | NA | NA | *TAL1* | NA | Nonsense | p.R257X | Somatic |
| 34 | M | Ped | NA | NA | *TLX1* | NA | Frameshift | p.S191fs | Somatic |
| 35 | M | Adult | NA | NA | *TLX1* | NA | Missense | p.C215F | Somatic |
| 36 | M | Adult | NA | NA | *TLX1* | NA | Nonsense | p.Y303X | NA |
| 37 | M | Adult | NA | NA | Unkn | NA | Nonsense | p.R274X | NA |
| 38 | M | Adult | NA | NA | Unkn | NA | Frameshift | p.H135fs | NA |

Ped, pediatric; NA, not available; mut, mutated; wt, wild-type.