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PHF6 mutations in adult acute myeloid leukemia

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Author contributions

P.V.V. performed mutation analysis of *PHF6* and wrote the manuscript. J.P., O.A. and R.L. performed mutation analysis of *PHF6* on the ECOG patient series (E1900). C.L. and I.A. performed the isolation of murine hematopoietic stem cell (HSC) and myeloid progenitor populations. E.P. and A.M. provided samples and correlative data from ECOG. M.B., C.N. and A.P provided samples and correlative data from Hospital Central de Asturias. P.V., J.C. and F.S. provided samples and correlative data from University Hospital Leuven. R.L. and C.H. provided samples and correlative data from MSKCC. A.F. designed the studies, directed research and wrote the manuscript.

Disclosure of conflicts of interest

No conflicts of interest to disclose.

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Abstract

Loss of function mutations and deletions encompassing the *PHF6* gene are present in about 20% of T-cell acute lymphoblastic leukemias. Here we report the identification of recurrent mutations in *PHF6* in 10/353 adult acute myeloid leukemias (AML). Genetic lesions in *PHF6* found in AML are frameshift and nonsense mutations distributed through the gene or point mutations involving the second PHD-like domain of the protein. As in the case of T-ALL, where *PHF6* alterations are found almost exclusively in males, mutations in *PHF6* were 7 times more prevalent in males than in females with AML. Overall these results identify *PHF6* as a tumor suppressor mutated in AML and extend the role of this X-linked tumor suppressor gene in the pathogenesis of hematologic tumors.

Keywords

PHF6; mutations; AML

Introduction

Acute myeloid leukemia (AML) consists of a heterogeneous group of aggressive neoplasms that is characterized by clinical and genetic heterogeneity and shows an increasing incidence with age(1). Insights in the molecular genetic basis of AML initially came from the characterization of recurrent chromosomal rearrangements, including t(8;21), t(15;17), inv(16), and different 11q23 translocations (2). Such clonal chromosome aberrations are detectable in the leukemic blasts of approximately 55% of adults with AML and have been recognized as important prognostic factors. Moreover, the characterization of genes located in the breakpoints of these rearrangements identified critical fusion oncogenes involved in the pathogenesis of AML including *RUNX1-MTG8*/*AML1-ETO*, *PML-RARA*, *CBFB/SMMHC* and *MLL-AF9*. Subsequently, intense sequencing efforts of specific candidate genes, including *NPM1*, *FLT3*, *CEBPA*, *MLL*, *NRAS*, *WT1*, *RUNX1*, *NF1* and *TET2*, further broadened the spectrum of genetic lesions towards a wide variety of somatic mutations implicated in AML pathogenesis (2–5). Finally, sequencing of complete AML genomes (6, 7) revealed the presence of new somatically acquired mutations and led to the identification of recurrent mutations in the *IDH1* and *IDH2* genes (8, 9).

Recently, we identified the plant homeodomain finger 6 (*PHF6*) gene as a new tumor suppressor in T-cell acute lymphoblastic leukemia (T-ALL)(10). *PHF6* deletions and inactivating mutations are found in about 20% of T-ALL samples and are present almost exclusively in male leukemia cases(10). Notably, *PHF6* mutations were not identified in precursor B-lineage ALL samples suggesting that loss of *PHF6* might be restricted to lymphoid tumors of the T-cell lineage(10). In the past, detailed molecular characterization of T-ALL and AML revealed a number of common genetic lesions shared by these hematological tumors including the *CALM-AF10*(11, 12) and *SET-NUP214*(13, 14) gene fusions, *MLL* translocations(15) and somatically acquired mutations in *RAS*(16, 17), *WT1*(18, 19), *FLT3*(20–22) and *NF1*(23). Given these similarities, we hypothesized that mutational loss of *PHF6* might also be implicated in the pathogenesis of specific subtypes of AML. To address this question, we sequenced all coding exons of *PHF6* in a cohort of 353 AML patients. In addition, we used real-time quantitative DNA PCR to assess the presence of genomic *PHF6* deletions in 41 cases. The results of this analysis show the presence of

recurrent loss of function mutations in *PHF6* in AML and characterize the spectrum of associated genetic alterations cooperating with PHF6 loss in myeloid malignancies.

Methods

Patient samples

Leukemic DNA and cryopreserved lymphoblast samples were provided by collaborating institutions in the US [Eastern Cooperative Oncology Group (ECOG) and Memorial Sloan-Kettering Cancer Center (MSKCC)], Spain [Hospital Central de Asturias, Oviedo] and Belgium [Department of Pediatric Hemato-Oncology, Leuven University Hospital, Leuven]. All samples were collected with informed consent and under the supervision of local IRB.

Sequence analysis

PHF6 mutations were analyzed by PCR amplification of *PHF6* exons 2–10 followed by direct bidirectional DNA sequencing as previously described(10). Sequence analysis of *IDH1*, *IDH2*, *TET2*, *ASXL1*, *FLT3*, *NPM1*, *CEBPA*, *WT1*, *KRAS* and *NRAS* was performed as previously described(25).

Sorting of hematopoietic stem cell (HSC), myeloid progenitor and lymphoid populations

Murine bone marrow, thymus and spleen cells were sorted using a Dako Cytomation Mo-Flo Fluorescence Activated Cell Sorter. Antibody staining was performed as previously described(26). The antibodies used for sorting included c-kit (2B8), Sca-1 (D7), Mac-1 (M1/70), Gr-1 (RB6-8C5), NK1.1 (PK136), TER-119, CD3 (145-2C11), CD4 (L3T4), CD8α (53-6.7), CD19 (1D3), IgM (II/41), IL7Rα (A7R34), CD25 (PC61), TCRβ (H57-597), CD34 (RAM34), FcgammaRII/III (2.4G2), CD150 (9D1) and were purchased from BD-Pharmingen or e-Bioscience. The bone marrow lineage cocktail included antibodies against Mac-1, Gr-1, NK1.1, TER-119, CD3 and CD19. Sorted hematopoietic stem cell populations included total LSK ($lin^-/sca\text{-}1^+/c\text{-}kit^+$), $CD150^-$ LSK and $CD150^+$ LSK. Myeloid progenitor populations included common myeloid progenitors (CMP, $lin^-/sca\text{-}1^-/c\text{-}kit^+/CD34^+/FcgammaRII/III^{low}$), granulocyte-macrophage progenitors (GMP, $lin^-/sca\text{-}1^-/c\text{-}kit^+/CD34^+/FcgammaRII/III^{high}$) and megakaryocyte-erythroid progenitors (MEP, $lin^-/sca\text{-}1^-/c\text{-}kit^+/CD34^+/FcgammaRII/III^-$). Lymphocyte populations included bone marrow pro B ($IgM^-/CD19^+/cKit^+/CD25^-$) and pre B cells ($IgM^-/CD19^+/cKit^-/CD25^+$), mature splenic B cells ($CD19^+/IgM^+$), thymic double negative 1 T cells (DN1, $CD4^-/CD8^-/cKit^+/CD25^-$), double negative 2 T cells (DN2, $CD4^-/CD8^-/cKit^+/CD25^+$), double negative 3 T cells (DN3, $CD4^-/CD8^-/cKit^-/CD25^+$), double negative 4 T cells (DN4, $CD4^-/CD8^-/cKit^-/CD25^{low}$), intermediate single positive (ISP, $CD4^-/CD8^+/TCR\beta^-$) and double positive T cells (DP, $CD4^+/CD8^+$) and finally splenic peripheral mature single positive CD4 T cells (SP-$CD4^+$, $CD4^+/CD8^-$) and single positive CD8 T cells (SP-$CD8^+$, $CD4^-/CD8^+$).

Quantitative real time PCR

RNA preparation of sorted cell population was achieved using the RNeasy plus mini kit (QIAGEN) according to manufacturer's protocol. cDNA was generated with the ThermoScript RT-PCR system (Invitrogen) and analyzed by quantitative real-time PCR using the SYBR Green RT-PCR Core Reagents kit (Applied Biosystems) and the 7300 Real-Time PCR System (Applied Biosystems). *Phf6* expression levels were calculated using *Gapdh* as reference. PCR primers sequences are available upon request.

Real-time quantification of DNA copy number

Real-time quantitative DNA PCR analysis was performed to screen AML cases for the presence of genomic *PHF6* deletions using the FastStart Universal SYBR Green Master Mix

(Roche) and the 7300 Real-Time PCR System (Applied Biosystems) as previously described(10) using *TIE2* as control gene. Data were analyzed using the comparative ddCT method (Applied Biosystems).

Statistical analysis

The Fisher's exact test was used to compare the frequency of *PHF6* mutations between male and female AML patients.

Results

PHF6 mutations in adult AML

PHF6 was recently identified as a novel X-linked tumor suppressor gene recurrently mutated and deleted in pediatric and adult T-ALL(10). To evaluate if *PHF6* inactivation might also contribute to the pathogenesis of AML, we sequenced all coding exons of *PHF6* and used real-time quantitative DNA PCR to assess the presence of genomic *PHF6* deletions in AML samples. DNA sequencing analysis of *PHF6* in AML revealed the presence of *PHF6* mutations in 10/353 (3%) AMLs analyzed. Most *PHF6* mutations present in AML were characteristically loss of function alleles with 3 nonsense and 4 frameshift truncating mutations (Figure 1a,b). In addition, we identified 3 missense mutations located in the N-terminal region (A40G) and the second PHD2 domain (H302Y and H329L) of PHF6 (Figure 1a,b). DNA copy number analysis of the *PHF6* locus failed to detect any genomic *PHF6* deletions in 41 AML (22 male and 19 female) cases analyzed.

Cooperative genetic lesions in *PHF6* mutated adult AML

PHF6 mutated AML cases in this series, corresponded to FAB subtype's M0, M1 and M2, or presented as a secondary AML (Table 1). At the genetic level, AML is a heterogeneous disease characterized by the accumulation of acquired somatic genetic lesions that cooperate in the transformation of myeloid progenitor cells. In order to identify genetic defects that might cooperate with *PHF6* inactivation in the pathogenesis of AML, we sequenced *IDH1*, *IDH2*, *TET2*, *ASXL1*, *FLT3*, *NPM1*, *CEBPA*, *WT1*, *KRAS* and *NRAS*, in *PHF6* mutated AML samples. This analysis revealed mutations affecting *IDH2*, *ASXL1*, *FLT3*, *CEBP*α and *NRAS* as additional genetic events that may cooperate with *PHF6* inactivation in the pathogenesis of AML (Table 1).

Phf6 expression in murine HSC, myeloid progenitor and lymphoid populations

PHF6 is highly conserved among vertebrates(28) and shows ubiquitous expression in a wide variety of human tissues(10, 28). The presence of recurrent mutations in *PHF6* in AML suggests a possible role of this tumor suppressor gene in the control of myeloid development. In order to evaluate *Phf6* expression in hematopoietic stem cells (HSCs) and myeloid progenitors, we performed quantitative RT-PCR analysis of sorted mouse myeloid progenitor and lymphoid cell populations. These analyses revealed ubiquitous but slightly lower expression levels of *Phf6* transcripts in HSC and myeloid cell progenitor populations as compared to different subsets of lymphoid cells (Figure 2). Within the myeloid progenitor populations, we noticed higher *Phf6* levels in LSK progenitors compared to CMP and GMP populations (Figure 2). The murine thymocyte populations at different stages of development showed a similar pattern of variable *Phf6* expression as previously identified in human T-cell subsets(10). Finally, in the murine B-cell populations, we noticed a marked upregulation of *Phf6* transcripts in pre-B cells compared to both pro-B and mature B cells.

PHF6 mutations are characteristically present in male patients with in AML

One of the most notorious features of *PHF6* mutations in T-ALL is that they are almost exclusively found in male patients with this disease (10), which may explain in part the 3:1 higher prevalence of T-ALL in males than in females. Notably, although to a less extent than in T-ALL, AML is also more frequently found in males with a male to female ratio of 1.3 to 1. Analysis of the gender distribution in *PHF6*-mutated AML patients demonstrated that genetic alterations in *PHF6* are 7 times more frequent in male (9/195; 4.6%) than in female (1/158; 0.6%) AML patient samples ($P<0.05$, Figure 3).

Discussion

The *PHF6* tumor suppressor gene encodes a plant homeodomain (PHD) protein containing 4 nuclear localization signals and 2 imperfect PHD zinc finger domains(27) with a proposed role in transcriptional regulation and/or chromatin remodeling(27, 28). Inactivating mutations in *PHF6* cause the Börjeson-Forssman-Lehman syndrome(29) (BFLS), a relatively uncommon type of X-linked mental retardation that mainly affects males, and shows milder clinical features in affected carrier females(30). A recent report described a male BFLS patient that developed T-ALL, suggesting that BFLS represents a cancer predisposition syndrome(31).

In this study, we evaluated if mutational loss of *PHF6* might also be implicated in the pathogenesis of adult AML and identified *PHF6* mutations in ~3% (10/353) of adult AML samples analyzed. *PHF6* mutated primary AML cases were predominantly immature leukemias (FAB subtypes M0–M2), however, they showed definite AML immunophenotypes. Only in one case, retrospective analysis of one of the *PHF6* mutated AML M0 samples, showed weak cytoplasmic CD3 positivity, together with 8% myeloperoxidase positive blasts and CD15/CD33 expression. Moreover, the presence of additional cooperative mutations affecting prototypical AML associated oncogenes and tumor suppressor genes such as *IDH2*, *ASXL1*, *FLT3*, *CEBPα* and *NRAS* (Table 1), in *PHF6* mutated AML cases, further confirms the true myeloid nature of these samples.

Nonsense and frame-shift *PHF6* mutations accounted for 70% (7/10) of all *PHF6* mutations identified in our series and were distributed throughout the complete *PHF6* gene (Figure 1a,b). Missense mutations accounted for the remaining 30% (3/10) of *PHF6* lesions and mainly involved the second plant homeodomain (PHD)-like zinc finger domain of the PHF6 protein (Figure 1a,b). This includes an amino acid substitution (A40G) in the N terminus region of PHF6, a variant that was unique among 546 hematologic tumors analyzed in our lab. However, no remission material was available to test the somatic origin of this change. Thus, this particular variant may correspond to a novel point mutation disrupting the tumor suppressor function of PHF6 or alternatively correspond to a previously unreported polymorphism. Notably, the other two missense mutations found in AML involved residues R319 and H329 located in the second PHD-like domain of PHF6 (Figure 1a,b, Table 1), which have been previously found mutated in T-ALL(10), further strengthening the idea that the second PHD-like domain may mediate critical tumor suppressor functions of PHF6(10).

Overall, our results identify *PHF6* as an X-linked tumor suppressor gene that is mutated in a fraction of both *de novo* as well as secondary adult AMLs. *PHF6* mutations occur at a lower frequency in AML compared with T-ALL, but target mainly male patients in both hematological malignancies. The prognostic impact of *PHF6* mutations in AML will need to be assessed in larger cohorts of patients collected on multi center clinical trials. In addition, these results suggest the possibility that *PHF6* mutations might occur in male patients with other myeloid malignancies, such as myelodysplasia or myeloproliferative disorders which should be the addressed in future studies.

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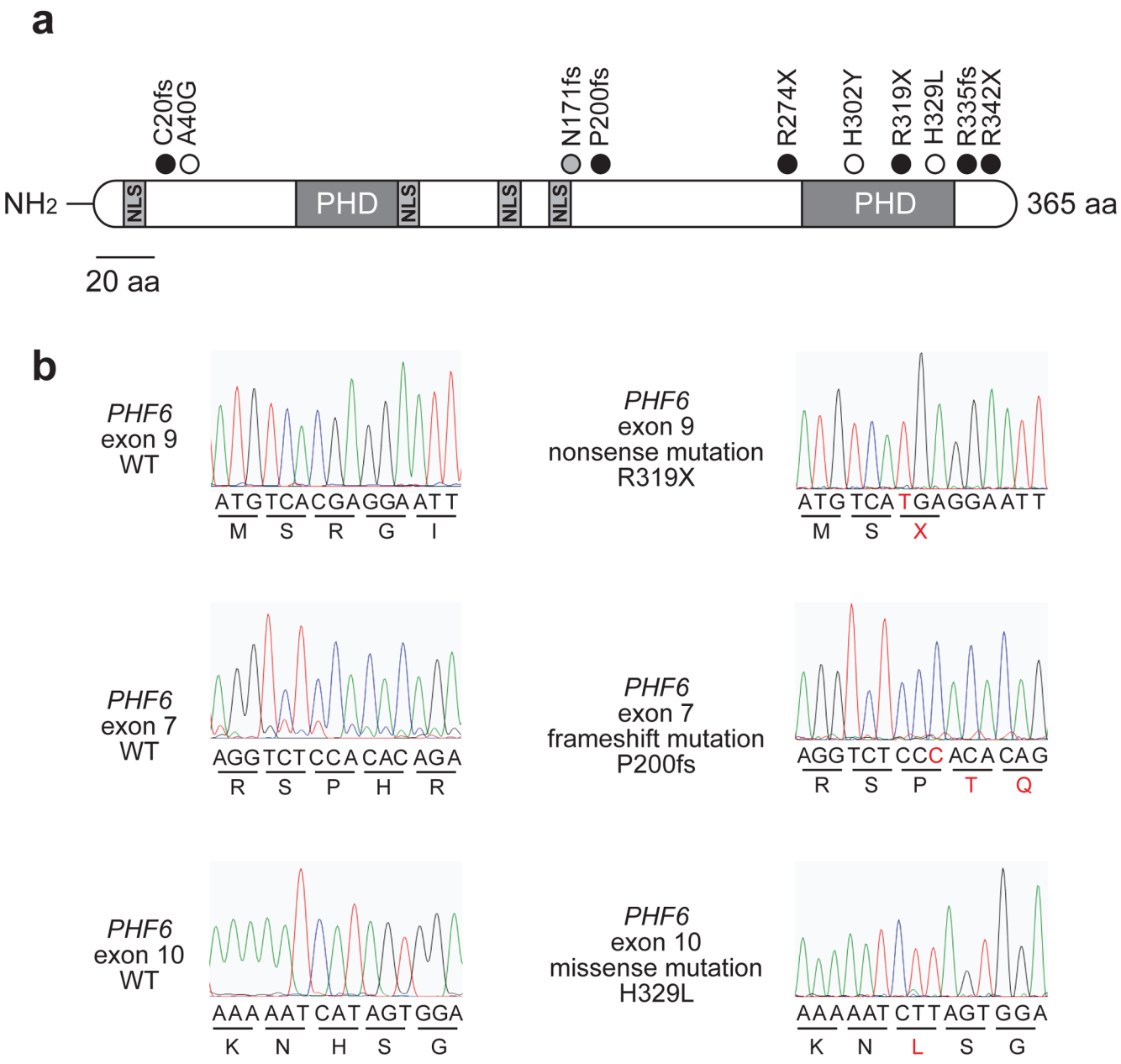

Figure 1. *PHF6* mutations in AML

(A) Schematic representation of the PHF6 protein depicting the location of 4 nuclear localization signals and 2 imperfect PHD zinc finger domains. Overview of *PHF6* mutations identified in primary AML samples. Filled circles represent nonsense and frameshift mutations, whereas missense mutations are depicted as open circles. The circle filled in gray indicates a frameshift *PHF6* mutation identified in a female AML sample. (B) Representative DNA sequencing chromatograms of AML DNA samples showing mutations in exons 7, 9 and 10 of *PHF6*.

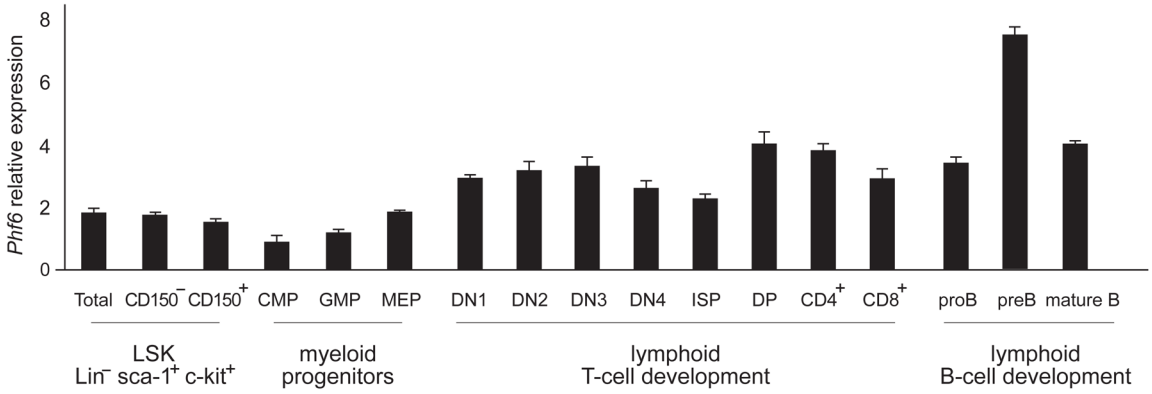

Figure 2. *Phf6* expression in HSC and myeloid progenitor populations

Quantitative RT-PCR analysis of murine *Phf6* expression normalized to *Gapdh* in HSC, myeloid progenitor and lymphoid populations. Error bars indicate s.d.

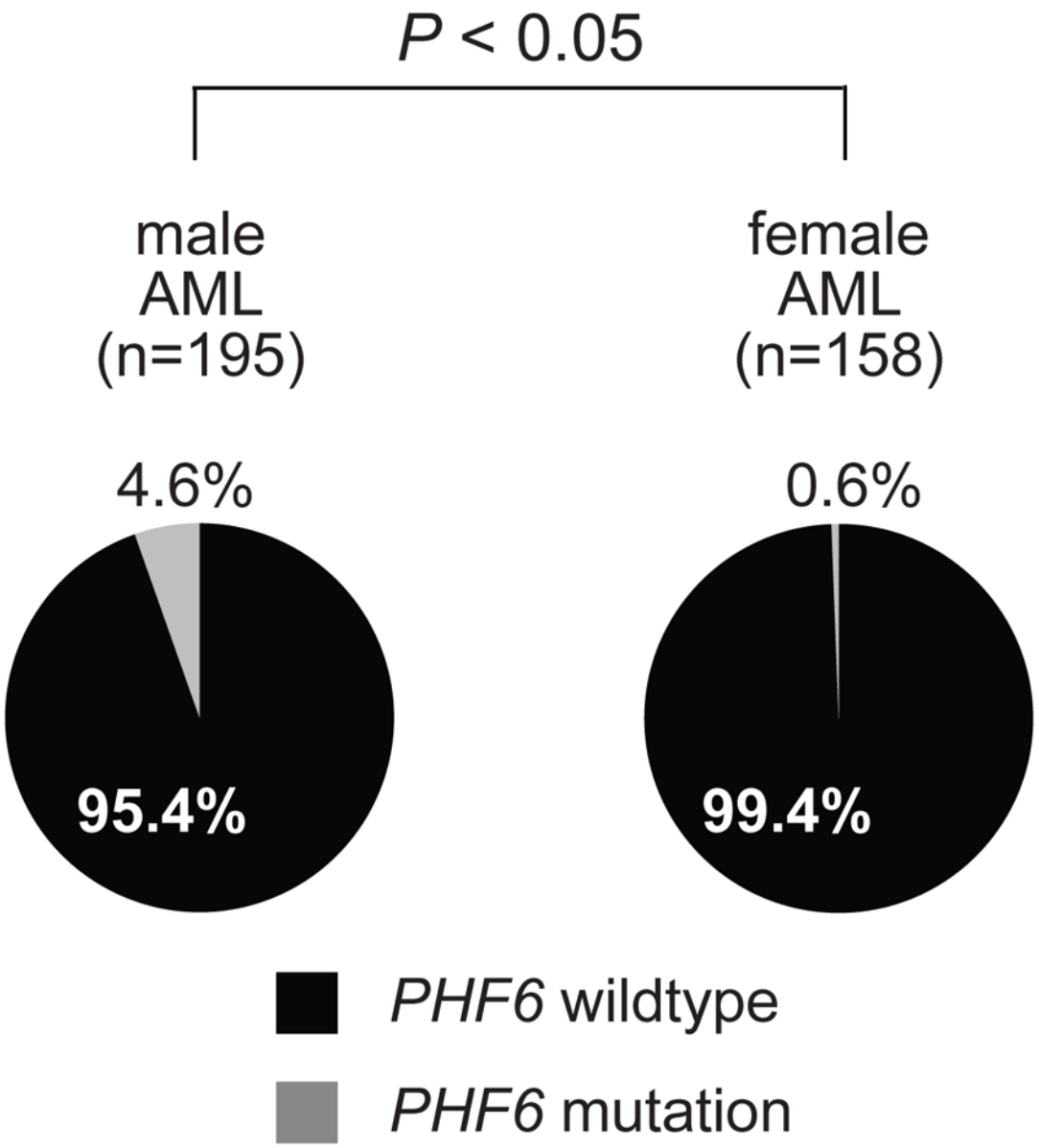

Figure 3. Gender distribution of *PHF6* mutations in AML

Differential distribution of *PHF6* mutations in AML samples from male and female patients.

Table 1

Characteristics of 10 primary AML samples showing *PHF6* inactivation

					***PHF6* lesion**		**Cooperative gene mutations**									
ID	**Sex**	**Age (yr)**	**FAB**	**Cytogenetics**	**Type of alteration**	**Predicted protein**	***IDH1***	***IDH2***	***TET2***	***ASXL1***	***FLT3***	***NPM1***	***CEBPA***	***WT1***	***KRAS***	***NRAS***
1	M	60	M0	normal	Missense	H329L	WT	WT	WT	WT	**TKD**	WT	WT	WT	WT	WT
2	M	45	M1	normal	Nonsense	R274X	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
3	M	72	2nd	tri(8)	Frameshift	R335fs	WT	WT	WT	**A637fs**	WT	WT	WT	NA	NA	NA
4	M	66	M2	tri(8)	Nonsense	R342X	WT	**R140Q**	WT	WT	WT	WT	WT	NA	NA	NA
5	M	65	2nd	normal	Missense	H302Y	WT	WT	WT	WT	WT	WT	WT	NA	NA	NA
6	M	41	M2	tri(8)	Frameshift	C20fs	WT	WT	WT	WT	**ITD**	WT	WT	NA	NA	NA
7	M	74	2nd	del(5q),-7,-20	Missense	A40G	WT	WT	WT	WT	**ITD**	WT	**H195fs**	NA	NA	NA
8	M	NA	M0	NA	Frameshift	P200fs	WT	WT	WT	**E1192X**	WT	WT	WT	WT	WT	WT
9	F	NA	M0*	NA	Frameshift	N171fs	WT	WT	WT	WT	WT	WT	WT	WT	WT	**G12V**
10	M	NA	M0	NA	Nonsense	R319X	WT	**R172K**	WT	**E558X**	WT	WT	**P39H**	WT	WT	WT

2nd, secondary AML; TKD, tyrosine kinase domain mutation; ITD, internal tandem duplication

*Biphenotypic T-myeloid leukemia

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