Co-existence of *PHF6* and *NOTCH1* mutations in adult T-cell acute lymphoblastic leukemia

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Abstract. T-cell acute lymphoblastic leukemia (T-ALL) results from the collaboration of multiple genetic abnormalities in the transformation of T-cell progenitors. Plant homeodomain finger protein 6 (*PHF6)* has recently been established as a key tumor suppressor, which is mutated in T-ALL; however, the clinical significance of *PHF6* mutations has not been fully determined in adult T-ALL. In the present study, amplification of the *PHF6* exons was performed, followed by DNA sequencing to identify the genomic mutations and examine the expression of *PHF6* in adult patients with T-ALL. The correlation between *PHF6* mutations and clinical features was also analyzed using a χ^2 test, and between *PHF6* mutations and survival curve using the Kaplan-Meier methods. *PHF6* mutations were detected in 27.1% of the Chinese adults with T-ALL (16/59), 10 of which were found to be novel mutations. A significantly lower expression level of *PHF6* was observed in T-ALL patients with PHF6 mutations compared with those without mutations. Of the observed mutations in *PHF6*, 6/16 were frame-shift mutations, indicating a *PHF6* dysfunction in those patients. Of note, *PHF6* mutations were found to be significantly associated with older age, lower hemoglobin levels, higher frequency of CD13 positivity and higher incidence of splenomegaly or lymphadenopathy. Furthermore, *PHF6* mutations were found to be significantly correlated with Notch homolog 1, translocation-associated (Drosophila) (*NOTCH1)* mutations. The patients with T-ALL with co-existence of the two mutations had a significantly shorter event-free survival and a poor prognosis. The present results indicated that *PHF6* is inactivated in adult T-ALL, due to its low expression and mutations. The present data indicated the synergistic effect of *PHF6* and *NOTCH1* mutations, as well as their co-existence, on the oncogenesis of adult T-ALL, and their potential as a prognostic marker for the disease.

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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) comprises an aggressive malignancy, in which multiple genetic abnormalities collaborate in the transformation of T-cell progenitors. Abundant genetic alterations in T-ALL have been identified by whole-genome sequencing and DNA copy number analysis of candidate genes, including deletions and/or sequence mutations of the cyclin-dependent kinase inhibitor 2A/B, lymphoid enhancer binding factor 1, Notch homolog 1, translocation-associated (Drosophila) (*NOTCH1*), F-box and WD repeat domain containing 7 (*FBXW7*), phosphatase and tensin homolog (*PTEN*), neuroblastoma RAS viral oncogene homolog, Wilms tumor 1, plant homeodomain finger protein 6 (*PHF6*), interleukin 7 receptor and runt related transcription factor 1 genes (1-4).

PHF6 encodes a PHD factor containing four nuclear localization signals and two PHD zinc finger domains, and has a proposed role in the control of gene expression (5). Inactivating mutations of the *PHF6* gene were originally found to be associated with a form of syndromic X-linked mental retardation, Börjeson-Forssman-Lehmann syndrome (6). In addition, *PHF6* has been identified as a novel key tumor suppressor gene in T-ALL. *PHF6* mutations were detected

in adult and pediatric patients with T-ALL, as well as also in adults with acute myeloid leukemia (7,8); however, the association between *PHF6* mutations and clinical outcome in these patients has not been fully determined.

NOTCH1 mutations have been shown to play an important role in the pathogenesis of T-ALL (9); however, the clinical significance of the co-existence of *NOTCH1* and *PHF6* mutations (*PHF6*^{mut}*NOTCH1*^{mut}) has not been sufficiently explored. The present study demonstrated the characteristics of *PHF6* mutations in adult Chinese patients with T-ALL, with 10/16 of the detected mutations being reported for the first time. In addition, the correlation between *PHF6*^{mut}*NOTCH1*^{mut} co-existence in T-ALL and event-free survival (EFS) was explored and found to be significant in this cohort of adult T-ALL patients.

Materials and methods

Patients and samples. Bone marrow (BM) samples were collected from 79 adult patients (age, 14-62 years) with ALL (57 males and 22 females; 59 T-ALL and 20 B-ALL samples) at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) between May 2008 and 2014. The diagnosis of ALL was made based on the molecular, immunophenotypic, morphologic and cytogenetic criteria established by the World Health Organization Diagnosis and Classification of ALL (2008) (10). Informed consent was obtained from all individuals prior to their participation to the study, in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board of Nanjing Medical University.

Mutation analysis of PHF6, NOTCH1, FBXW7, PTEN and Janus kinase 1 (*JAK1*). Mutation analysis was performed for *PHF6* exons 2-10. Genomic DNA was isolated with a QIAamp DNA Blood Mini kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. DNA fragments spanning the above *PHF6* exons were amplified by polymerase chain reaction (PCR) using AmpliTaq Gold (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and exon-specific primers (5). DNA sequencing was performed on the purified PCR products. In addition, exon 26/N-terminal region of the heterodimerization domain (HD-N), exon 27/C-terminal region of the heterodimerization domain (HD-C), exon 28 and exon 34/proline-glutamic acid-serine-threonine (PEST) domain of *NOTCH1* were amplified for mutation screening, as previously described (9). Exons in *FBXW7, PTEN* and *JAK1* were also screened as previously reported (11,12).

Cytogenetic and molecular analysis. Conventional cytogenetic analysis was performed using unstimulated short-term cultures at the time of diagnosis, according to the International System for Human Cytogenetic Nomenclature recommendations (13). For each sample, ≥20 BM metaphase cells were analyzed.

Immunophenotypic analysis was performed by flow cytometry on fresh BM samples. The cell-surface antigen was considered positive when fluorescence intensity of ≥20% of cells exceeded the fluorescence of negative control.

Statistical analysis. Patients were divided into high or low *PHF6* expression groups [the median (0.0020925) was used as cut-off value based on SPSS 17.0]. For quantitative parameters, the overall differences between the cohorts were evaluated using the Mann-Whitney U-test. For qualitative parameters, the overall group differences were analyzed using the χ^2 test. Survival analysis was calculated using the Kaplan-Meier method. Experimental data are presented as the mean ± standard error. Determinations of statistical significance were performed using a Student *t*-test for comparisons of two groups or using analysis of variance (ANOVA) for comparing multiple groups. Statistical analysis was performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

PHF6 mutations in adult T-ALL. PHF6 mutations were detected in 27.1% of the Chinese adults with T-ALL (16/59). The identified mutations located in exons 2 and 4-10. The most common locations for mutations were in exon 9 and exon 8, in which the mutation rate reached 25.0 and 18.8%, respectively.

The majority of the mutations detected were nonsense mutations (7/16, 43.8%), followed by insertion (4/16, 25.0%) and missense mutations (3/16, 18.75%), a deletion (1/16, 6.3%), and insertion/deletion mutations (1/16, 6.3%) (Fig. 1 and Table I). No *PHF6* mutations were identified in the 20 DNA samples from patients with B-ALL (data not shown), suggesting that *PHF6* mutations in lymphoid tumors could be restricted to T-ALL. Of note, 10/16 (62.5%) *PHF6* mutations identified in the present study were novel mutations (Table I). In addition, 6/16 (37.5%) mutations were frame-shift mutations, which may result in the deletion of the gene.

PHF6 expression and its association with mutations. PHF6 mRNA expression was detected in 46 patients whose cDNA samples were available. The *PHF6* expression was divided into high and low expression groups (G1-2 vs. G3-4). A significant correlation was observed between low *PHF6* expression levels and high frequency of splenomegaly and lymphadenopathy in adult T-ALL [73.9 (17/23) vs. 37.5% (9/24) (P=0.012) and 69.6 (16/23) vs. 29.2% (7/24) (P=0.006), respectively] (Fig. 2), suggesting that low *PHF6* expression levels may be associated with the markers of leukemic cell proliferation, which involved extramedullary infiltration in T-ALL. Furthermore, it was observed that *PHF6* expression was significantly lower in patients with T-ALL with *PHF6* mutations, as compared with those with *PHF6* wild-type (WT) (0.00423 vs. 0.06464; P=0.035) (Fig. 3), which further indicated *PHF6* mutations could result in loss of function mutations.

Cooperative genetic lesions of PHF6 mutations in adult T-ALL. The mutations in *NOTCH1, FBXW7, PTEN* and *JAK1* were also screened in this cohort of patients with T-ALL. It was found that the frequency of *PHF6*^{mut}*NOTCH1*^{mut} co-existence was significantly higher in patients with *PHF6* mutations than in those with *PHF6* WT (75.0 vs. 44.2; P=0.035), suggesting an association between *PHF6* and *NOTCH1* mutations in this cohort (Table II). No significant associations were observed between *PHF6* and *FBXW7, PTEN* or *JAK1* mutations (Table II).

The domains involving *PHF6*^{mut}*NOTCH1*^{mut} co-existence were further analyzed. The most commonly mutated domains

Table I. Plant homeodomain finger protein 6 mutations in adult T-cell acute lymphoblastic leukemia.

Patient ID	Mutation (nucleotide)	Exon	Type of mutation	Mutation (amino acid)	Previously reported
PHF6mu 1#	c.631A>T	7	Nonsense	p.K211X	N
PHF6mu 2#	c.479_480insA	6	Frame-shift	p.K161fs	N
PHF6mu 3#	c.820C>T	8	Nonsense	p.R274X	Y
PHF6mu 4#	c.93_94insG+94_95insCTA	2	Frame-shift	p.L32fs	N
PHF6mu 5#	c.517A>T	6	Nonsense	p.K173X	N
PHF6mu 6#	c.821G>A	8	Missense	p.R274Q	Y
PHF6mu 7#	c.731_732delTG	8	Frame-shift	p.L244fs	N
PHF6mu 8#	c.955C>T	9	Nonsense	p.R319X	Y
PHF6mu 9#	c.385C>T	5	Nonsense	p.R129X	Y
PHF6mu 10#	c.346C>T	4	Nonsense	p.R116X	Y
PHF6mu 11#	c.134delG+insCC	2	Frame-shift	p.C45fs	N
PHF6mu 12#	c.586_587insA	7	Frame-shift	p.R196 K	N
PHF6mu 13#	c.971T>C	10	Missense	p.L324P	N
PHF6mu 14#	c.957_958insGT	9	Frame-shift	p.G320fs	N
PHF6mu 15#	c.903C>A	9	Nonsense	p.Y301X	Y
PHF6mu 16#	c.905A>C	9	Missense	p.H302P	N

N, no; Y, yes.

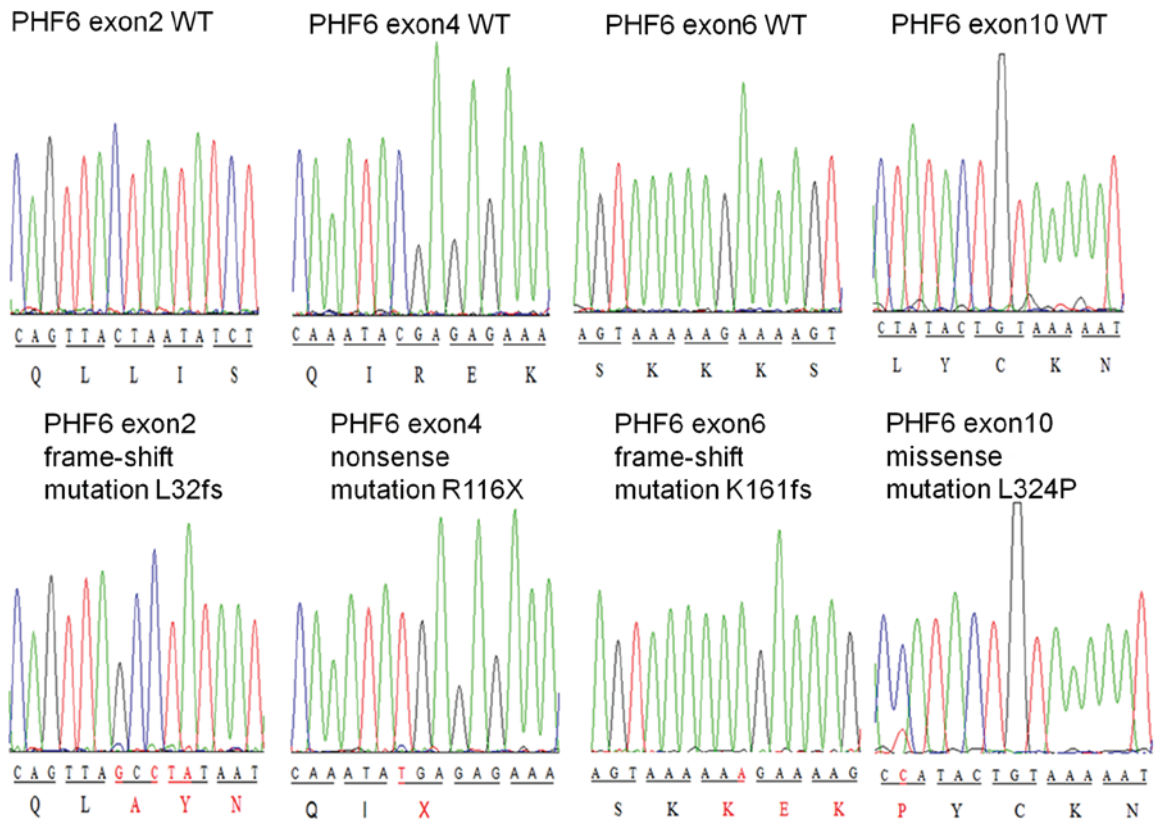

Figure 1. Representative DNA sequencing chromatograms of T-cell acute lymphoblastic leukemia genomic DNA samples showing mutations in exons 2, 4, 6 and 10 of *PHF6*. *PHF6*, plant homeodomain finger protein 6; WT, wild-type.

in *NOTCH1* co-existing with *PHF6* mutations in same patient were the HD-N domain (6/12, 50.0%), followed by HD-C (2/12, 16.7%), PEST (2/12, 16.7%), HD-C + PEST (1/12, 8.3%) and HD-N + HD-C 1/12 (8.3%) (Table III). These data indicated that the HD domain (particularly the HD-N) of *NOTCH1* may contribute to the synergistic oncogenic effect of the two genes.

Table II. Association of *PHF6* mutation with clinical feature in adult T-cell acute lymphoblastic leukemia.

	PHF6		
Clinical features	WT (n=43)	Mutation (n=16)	P-value
Male, %	83.7	68.8	0.365
Age, median (range)	26 (14-62) years	39 (14-60) years	0.043
WBC, median (range)	47.9 (1.2-546.0) x10^9/l	19.0 (2.7-175.3) x10^9/l	0.060
Platelet, median (range)	56.0 (17.0-267.0) x10^9/l	53.0 (25.0-223.0) x10^9/l	0.871
Hemoglobin, median (range)	119.0 (56.0-171.0) g/l	99.5 (56.0-153.0) g/l	0.044
LDH , median (range)	1038.0 (131.0-8601.0) U/l	811.5 (294.0-5353.0) U/l	0.747
Marrow blasts, median (range)	75.0 (22.0-98.0)%	80.0 (27.0-99.0)%	0.615
Peripheral blasts, median (range)	80.0 (0-90.9)%	28.0 (5.0-94.0)%	0.080
Immunophenotype (≥20%)			
CD13	24.2	71.4	0.002
CD33	35.3	54.5	0.436
CD34	48.6	66.7	0.278
Other genetic abnormalities, %			
Complex Karyotype	11.8	7.7	1.000
NOTCH1 mutation	44.2	75.0	0.035
FBXW7 mutation	11.6	6.3	0.902
PTEN mutation	11.6	6.3	0.902
JAK1 mutation	7.0	12.5	0.880
Extramedullary infiltration, %			
Hepatomegaly	16.3	18.8	1.000
Splenomegaly	39.5	68.8	0.046
Lymphadenopathy	44.2	81.3	0.011

PHF6, plant homeodomain finger protein 6; WT, wild-type; WBC, white blood cells; LDH, lactate dehydrogenase; *NOTCH1*, Notch homolog 1, translocation-associated (Drosophila); *FBXW7*, F-box and WD repeat domain containing 7; *PTEN*, phosphatase and tensin homolog; *JAK1*, Janus kinase 1.

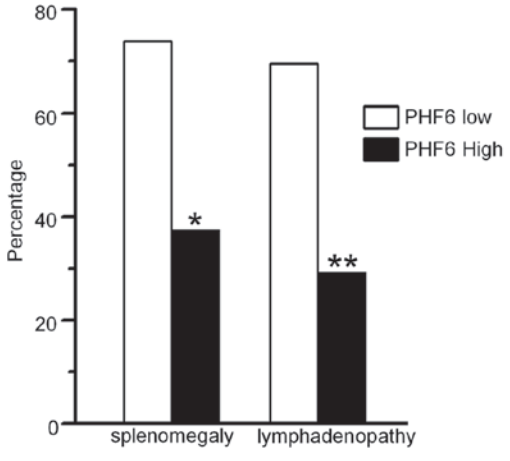

Figure 2. Association of *PHF6* expression with clinical features. *PHF6* expression was divided into two (low and high) groups (Q1-2 vs. Q3-4). The correlation was analyzed with χ^2 test. *P<0.05; **P<0.001. *PHF6*, plant homeodomain finger protein 6.

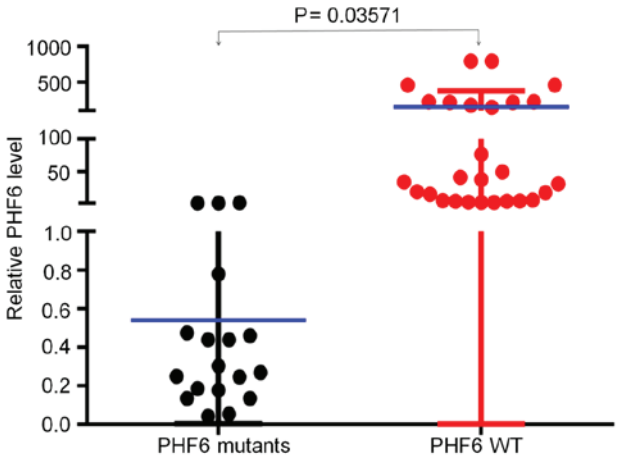

Figure 3. *PHF6* expression in patients with *PHF6* mutations. *PHF6* mRNA level was detected in 46 patients by quantitative polymerase chain reaction. *PHF6*, plant homeodomain finger protein 6; WT, wild-type.

Correlation of PHF6 mutations with clinical features. The association between *PHF6* mutations and clinical characteristics was analyzed in the patients with T-ALL. No gender differences were observed in the incidence of *PHF6* mutations in this cohort of adult Chinese patients with T-ALL.

Table III. Correlation of *PHF6* mutations with *NOTCH1* mutations in adult T-cell acute lymphoblastic leukemia.

	PHF6 mutations			*NOTCH1* mutations			
Patient ID	Nucleotide	Exon	Amino acid	Nucleotide	Exon	Amino acid	Domain
PHF6mu 1#	c.631A>T	7	p.K211X	c.7355C>A	34	p.A2452E	PEST
PHF6mu 2#	c.479_480insA	6	p.K161fs	c.4732_4734delGTG	26	p.V1578delV	HD-N
PHF6mu 3#	c.820C>T	8	p.R274X	c.4732_4734delGTG c.5094C>T	26	p.V1578delV p.D1698D	HD-N
PHF6mu 4#	c.93_94ins G+94_95insCTA	2	p.L32fs	c.5126T>C	27	p.L1709P	HD-C
PHF6mu 6#	c.821G>A	8	p.R274Q	c.4815_4817delinsAGC CGGGGGGGA c.7541_7542insC	26	p.F1606AGGD p.E2515fs*1	HD-N
PHF6mu 8#	c.955C>T	9	p.R319X	c.4799T>A	26	p.L1600Q	HD-N
PHF6mu 9#	c.385C>T	5	p.R129X	c.4721T>C	26	p.L1574P	HD-N
PHF6mu 10#	c.346C>T	4	p.R116X	c.5033T>C c.7400C>A	27 34	p.L1678P p.S2467*	HD-C PEST
PHF6mu 13#	c.971T>C	10	p.L324P	C.4845_4846insCCT c.5114C>T	26 27	p.1615_1616insP p.A1705 V	HD-N HD-C
PHF6mu 14#	c.957_958insGT	9	p.G320fs	c.7368_7369insTA	34	p.L2457fs*21	PEST
PHF6mu 15#	c.903C>A	9	p.Y301X	c.4776_4777insGAA TCCAACCCTCCC	26	p.F1592LNPTLP	HD-N
PHF6mu 16#	c.905A>C	9	p.H302P	c.5033T>C	27	p.L1678P	HD-C

PHF6, plant homeodomain finger protein 6; *NOTCH1*, Notch homolog 1, translocation-associated (Drosophila); PEST, exon 28 and exon 34/proline-glutamic acid-serine-threonine; HD-N, heterodimerization domain; HD-C, C-terminal region of the HD-N.

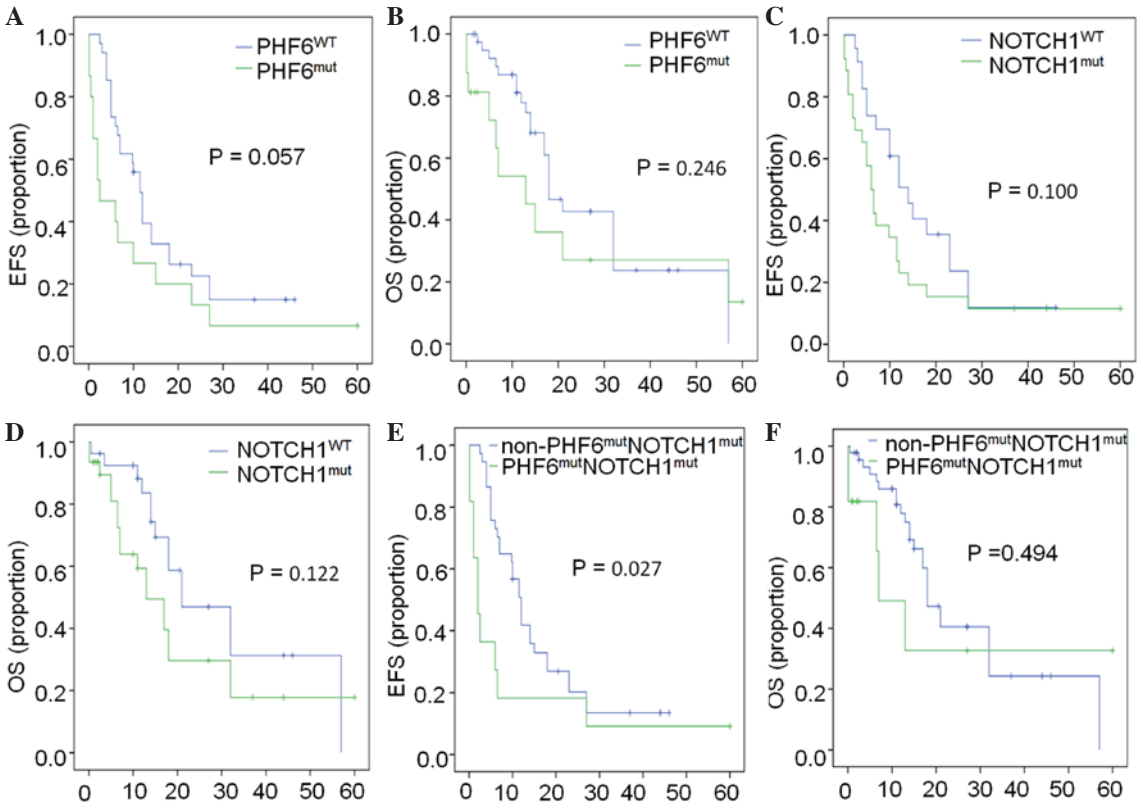

Figure 4. Effect of *PHF6*^{mut} on EFS and OS. (A-B) Comparison of (A) EFS and (B) OS between patients with *PHF6*^{mut} and *PHF6*^{WT} mutations; (C-D) comparison of (C) EFS and (D) OS between patients with *NOTCH1*^{mut} and *NOTCH1*^{WT}; (E and F) comparison of (E) EFS and (F) OS between patients with *PHF6*^{mut}*NOTCH1*^{mut} and non-*PHF6*^{mut} *NOTCH1*^{mut}. EFS, event-free survival; OS, overall survival; *PHF6*, plant homeodomain finger protein 6; *NOTCH1*, Notch homolog 1, translocation-associated (Drosophila); mut, mutation; WT, wild-type.

Table IV. Association of *PHF6*^{mut}*NOTCH1*^{mut} co-existence with clinical feature in adult T-cell acute lymphoblastic leukemia.

	PHF6^{mut}*NOTCH1*^{mut} co-existence		
Clinical features	WT (n=47)	Mutation (n=11)	P-value
Male, %	83.0	63.6	0.311
Age, median (range)	26 (14-62) years	27 (14-60) years	0.456
WBC, median (range)	47.9 (1.2-546.0) x10^9/l	20.5 (2.9-175.3) x10^9/l	0.144
Platelet, median (range)	57.0 (17.0-267.0) x10^9/l	46.0 (25.0-223.0) x10^9/l	0.979
Hemoglobin, median (range)	122.0 (56.0-171.0) g/l	95.0 (56.0-117.0) g/l	0.007
LDH, median (range)	1038.0 (131.0-8601.0) U/l	861.0 (294.0-5353.0) U/l	0.921
Marrow blasts, median (range)	0.75 (0.22-0.98)%	0.85 (0.27-0.99)%	0.585
Peripheral blasts, median (range)	0.70 (0.00-0.99)%	0.40 (0.06-0.94)%	0.310
Immunophenotype (≥20%)			
CD13	29.7	66.7	0.094
CD33	35.1	57.1	0.501
CD34	50.0	62.5	0.800
Other genetic abnormalities, %			
Complex karyotype	10.8	10.0	1.000
FBXW7 mutation	12.8	0.0	0.483
PTEN mutation	12.8	0.0	0.483
JAK1 mutation	6.4	9.1	1.000
Extramedullary infiltration, %			
Hepatomegaly	14.9	18.2	1.000
Splenomegaly	38.3	81.8	0.009
Lymphadenopathy	44.7	90.9	0.006

PHF6^{mut}*NOTCH1*^{mut} co-existence, co-existence of plant homeodomain finger protein 6 and Notch homolog 1, translocation-associated (Drosophila) mutations; WT, wild-type; WBC, while blood cells; LDH, lactate dehydrogenase; *FBXW7*, F-box and WD repeat domain containing 7; *PTEN*, phosphatase and tensin homolog; *JAK1*, Janus kinase 1.

It was found that the *PHF6* mutations were significantly associated with older age, lower hemoglobin levels, higher frequency of CD13 positivity, and higher incidence of splenomegaly or lymphadenopathy, as compared with *PHF6* WT patients (Table II).

Since an association was found between *PHF6* and *NOTCH1*, *PHF6*^{mut}*NOTCH1*^{mut} co-existence was further analyzed in relation to the clinical features of the cohort. It was found that the patients with *PHF6*^{mut}*NOTCH1*^{mut} co-existence had lower hemoglobin levels, along with a higher incidence of splenomegaly or lymphadenopathy, as compared with patients without such co-existence (Table III).

In addition, the survival status of this population was analyzed. The EFS and overall survival (OS) in patients with *PHF6* mutations vs. *PHF6* WT were 2.5 vs. 11.5 months (P=0.057) and 13.0 vs. 18.0 months (P=0.246), respectively (Fig. 4A and B), while those in patients with *NOTCH1* mutations vs. *NOTCH1* WT were 6.0 vs. 14.0 months (P=0.100) and 13.0 vs. 21.0 months (P=0.122), respectively (Fig. 4C and D). These results indicated that there were no significant differences in the EFS and OS between patients with *PHF6* or *NOTCH1* mutations and patients with *PHF6* WT or *NOTCH1* WT; however, it was found that patients with *PHF6*^{mut}*NOTCH1*^{mut} co-existence had a significantly shorter EFS compared with that of patients without such co-existence (2.0 vs. 12.0 months, respectively; P=0.027). No differences in the OS were observed between patients with *PHF6*^{mut}*NOTCH1*^{mut} co-existence and those without (7.0 vs. 18.0 months, respectively; P=0.494) (Fig. 4E and F). The patients with *PHF6*^{mut}*NOTCH1*^{mut} co-existence also exhibited a higher rate of splenomegaly and lymphadenopathy compared with those without this co-existence (Table IV).

Discussion

To the best of our knowledge, this study is the second report of *PHF6* mutations in an Asian adult population with T-ALL. Of note, a higher incidence of *PHF6* mutations was observed in Asian adults with T-ALL in the present study (27.1%), compared with the previous one (18.6%) (8). A different study showed that the frequency of *PHF6* mutations in pediatric patients with T-ALL in the USA was 16% (5), which was lower than the frequency found in the present study (27.1%). This variance was possibly attributable to differences in age, area and race of the observed populations.

PHF6 has been reported to be a novel tumor suppressor in T-ALL (5). Of note, out of the 16 *PHF6* mutations identified in

this cohort of adult patients with T-ALL, 10 were novel mutations. Consistent with other reports (5,7,9), no *PHF6* mutations were detected in B-ALL samples, suggesting that *PHF6* inactivating mutations are restricted to lymphoid tumors of the T-cell lineage.

It has been reported that *PHF6* is an X-linked gene, and that *PHF6* mutations were almost exclusively found in T-ALL samples from male subjects (5); however, no significant differences were observed in *PHF6* mutations in relation to gender in this cohort of Chinese adults with T-ALL, which is consistent with another study conducted on a Chinese population (8,9), which suggests that ethnic factors may contribute to gender differences in the risk of PHF6 mutations, and this requires further investigation in larger cohorts.

In the present study, it was observed that patients with T-ALL exhibited significantly lower *PHF6* expression, and that low *PHF6* expression in T-ALL is associated with leukemic cell proliferation. In addition, 6 of the 16 mutations were found to induce a frame-shift, which may result in the deletion of the PHF6 protein and its eventual dysfunction. These data indicated that *PHF6* inactivation in T-ALL is a result of genetic abnormalities and/or low *PHF6* expression.

No associations were observed between *PHF6* and *NOTCH1* mutations in either pediatric (n=65) or adult (n=34) cohorts with T-ALL in the previous study (5). However, the significant correlation found between *PHF6* and *NOTCH1* mutations in the present study is consistent with another study on Chinese patients with T-ALL (8). *PHF6* serves a potential role in transcriptional regulation, but its effects on genomics are not fully understood. Both *PHF6* and *NOTCH1* mutations have a high incidence in T-ALL. Whether *PHF6* inactivating mutations could induce the genomic alterations of other genes in T-ALL, such as *NOTCH1,* requires further research.

In order to further explore the effect of *PHF6* mutations on clinical outcomes, the OS and EFS of patients with T-ALL were analyzed. Despite the fact that no significant differences were identified in the OS and EFS of patients with *PHF6* mutations compared with those with *PHF6* WT, a significantly shorter EFS was observed in patients with $PHF6^{mut}NOTCH1^{mut}$ co-existence. This result further indicated the synergistic effects of *PHF6* and *NOTCH1* mutations on the oncogenesis of T-ALL; therefore, $PHF6^{mut}NOTCH1^{mut}$ co-existence could serve as a prognostic marker for the disease and should be integrated into future prognostic models of adult T-ALL.

In conclusion, a high incidence of *PHF6* mutations was observed in Chinese adults with T-ALL. The low expression of *PHF6* was found to be associated with the markers of leukemic cell proliferation. A $PHF6^{mut}NOTCH1^{mut}$ co-existence was observed and shown to be correlated with a shorter EFS in patients with T-ALL. The present results indicated a synergistic effect of *PHF6* and *NOTCH1* mutations on the oncogenesis of adult T-ALL, suggesting that their co-existence could serve as a prognostic marker for the disease.

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