The association of complex genetic background with the prognosis of acute leukemia with ambiguous lineage

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Acute leukemia with ambiguous lineage (ALAL) is a rare and highly aggressive malignancy with limited molecular characterization and therapeutic recommendations. In this study, we retrospectively analyzed 1635 acute leukemia cases in our center from January 2012 to June 2018. The diagnose of ALAL was based on either EGIL or 2016 WHO criteria, a total of 39 patients were included. Four patients diagnosed as acute undifferentiated leukemia (AUL) by both classification systems. Among the patients underwent high-throughput sequencing, 89.5% were detected at least one mutation and the median number of gene mutation was 3 (0–8) per sample. The most frequently mutated genes were NRAS (4, 21%), CEBPA (4, 21%), JAK3 (3, 16%), RUNX1 (3, 16%). The mutations detected in mixed-phenotype acute leukemia (MPAL) enriched in genes related to genomic stability and transcriptional regulation; while AUL cases frequently mutated in genes involved in signaling pathway. The survival analysis strongly suggested that mutation burden may play important roles to predict the clinical outcomes of ALAL. In addition, the patients excluded by WHO criteria had even worse clinical outcome than those included. The association of the genetic complexity of blast cells with the clinical outcomes and rationality of the diagnostic criteria of WHO system need to be evaluated by more large-scale prospective clinical studies.

Abbreviations

AL  Acute leukemia
ALAL  Acute leukemia with ambiguous lineage
ALL  Acute lymphoid leukemia
AML  Acute myeloid leukemia
AUL  Acute undifferentiated leukemia
BAL  Bi-phenotypic acute leukemia
CAR-T  Chimeric antigen receptor T-cell
CR  Complete remission
EGIL  European Group for the immunological characterization of leukemia
HSCT  Hematopoietic stem cell transplantation
MPAL  Mixed-phenotype acute leukemia
NGS  Next-generation sequencing
OS  Overall survival
PFS  Progression free survival
TKI  Tyrosine kinase inhibitors

Majority of acute leukemia (AL) can be accurately diagnosed and classified into acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL) according to morphologic, cytogenetic, and immunologic features1. However, less than 5% of patients can’t be clearly assigned to the well-established subsets of AL as lacking expression of lineage-specific markers, or presenting mixed immune phenotype more than one lineage. In the past two...
decades, the diagnostic criteria for such kinds of leukemia were controversial, and the nomenclature was constantly improving. The European Group for the immunological characterization of leukemia (EGIL) separately defines them as acute undifferentiated leukemia (AUL), which means no lineage related antigens are expressed, and bi-phenotypic acute leukemia (BAL) when either two separate blast populations are encountered or a single blast population demonstrating evidence of both myeloid and lymphoid differentiation concurrently. WHO (2008/2016) classification system collectively groups these rare disorders as acute leukemia with ambiguous lineage (ALAL), which may possibly arise from hematopoietic pluripotent stem cells. The new classification system proposes the diagnosis of mixed-phenotype acute leukemia (MPAL) and emphasizes the impact of CD19 for B-cell lineage, CD3/CD3 for T-cell lineage, and cytoplasmatic myeloperoxidase (MPO) for myeloid lineage, respectively. The diagnosis of ALAL largely depends on the immunophenotype, and cases with AML specific recurrent cytogenetic abnormalities are classified to AML as defined in WHO classification system. Based on the markers expressed on the blast cells, BAL/MPAL can be further divided in to B/M, T/M, B/T, and B/T/M subtypes. It has been widely recognized that the clinical outcome of ALAL is markedly adverse, and the overall survival (OS) ranges from 9 months to 3.5 years according to published data. Poor response to conventional chemotherapy and high rate of relapse may partly explain the unsatisfactory clinical outcome. Some studies reported ALL-like therapy might be superior to AML-based protocols, and long-term survival can only be improved by allogeneic stem cell transplantation. However, till now, no consistent treatment strategies are recommended as there is no distinctive biological characteristics have been identified. As previous studies documented, cytogenetic abnormalities were detected in 59–91% of patients with MPAL and 80–90% of patients with AUL. Recently, Alexander et al. reported the sequencing data of 159 pediatric patients with ALAL, and showed rearrangement of ZNF384 were appeared in nearly half of B/M MPAL. With the progressions in molecular genetics, high-throughput sequencing technology makes it possible to investigate this kind of disease more profoundly in molecular level. Several studies demonstrated the genetic landscape of MPAL and showed patients clustered to either AML-like or ALL-like MPAL based on methylation profiling could be benefited from corresponding therapies. However, the correlation between the genetic mutation and the clinical outcomes of ALAL was not fully elucidated.

Better characterizing this subset of leukemia will shed new light in overcoming this special entity of disease. In this study, we retrospectively analyzed the patients with de novo acute leukemia from January 2012 to June 2018 in Tongji Hospital, Tongji Medical Collage of Huazhong University of Science and Technology and aimed to examine a wide spectrum of gene mutations in patients with ALAL with defined under WHO or EGIL criteria, to determine their clinical relevance with clinical outcomes of such dire leukemia and the clinical applicability of these two diagnostic systems.

Results

Basic characteristics of patients enrolled in this study. In all 1635 patients with acute leukemia admitted to our center over a period of more than five years, 39 patients (2.4%) satisfied the criteria of EGIL, and 30 (1.8%) patients were diagnosed as AUL or MPAL according to the WHO standards. For all 39 patients, mean age was 39 years and 23 (59%) of them were male. The median value of WBC count on presentation was $1 \times 10^9$, hemoglobin 80 g/L, platelet $71 \times 10^9$, and bone marrow blasts 80% (Table 1). There was no significant difference regarding to the baseline and clinical characteristics between the patients included (WHO 2016, n = 30) and excluded (EGIL-WHO, n = 9) by WHO criteria, except that patients excluded by WHO system had a lower level of platelet count ($p = 0.039$) and did not express MPO ($p = 0.001$). According to the FAB criteria, 14 (35.9%) patients were classified as ALL, and 5 (12.8%) patients displayed an AML morphology, and others were classified as AUL (7.7%), mix phenotype leukemia (2.6%), or inclusive (41%) (Supplementary Table 1). The clinical diagnosis of ALAL can hardly be realized by FAB diagnosis system.

Four AUL cases (10%) expressed no lineage specific markers but CD34, HLA-DR with or without TdT. Among the remaining cases, 16 (41%) cases had a myeloid and B-lymphoid phenotype (B-M), 16 (41%) for a myeloid and T-lymphoid phenotype (T-M), 2 (5.1%) for a B-T-lymphoid phenotype (B-T), and 1 cases (2.6%) with evidence of tri-lineage concomitant expression (myeloid, B, and T lymphoid) (B-T-M; Supplementary Table 1).

Among all, 32 patients underwent karyotyping, and 17 (53.1%) of them were abnormal. Complex karyotypes were observed in 9 (28.1%) patients with a high degree of heterogeneity as shown in Table 2. The most frequent abnormal karyotype is t (9; 22) (q34; q11.2), resulting in the BCR-ABL1 fusion gene, appeared in 7 patients. Interestingly, 6 patients were B-M MPAL while one of them was T-M MPAL. MLL rearrangement was observed in a patient diagnosed as T-M MPAL (Table 2). In addition to BCR-ABL and SET/CAN, which were previously reported, we also detected E2A-PBX1 fusion gene in a B-M MPAL patient, in which two lineage markers were detected in one cluster of blast (Table 2).

Genetic testing. Genetic testing was conducted in 27 cases. Nineteen out of them were tested by 173-gene panel (Supplementary Table 2) in next-generation deep sequencing platform, and 8 of them, who admitted in hospital in earlier time, were tested by Sanger sequencing. 17 cases (89.5%) were detected at least one mutation by high-throughput sequencing system, while only 2 patients were positive mutated by screening limited hot
spot mutation of AML by Sanger sequencing. The two mutated genes detected by first-generation sequencing were the CEBPA in No. 36 patient and the FLT3-ITD repetitive tandem sequence in No. 42 patient, respectively. Based on the data of the 19 patients who had been sequenced in the next-generation platform, 53 high-confidence mutations were detected in 33 genes in 17 patients. The two patients with no detectable mutations were both B-M phenotype. The median number of gene mutation was 3 (0–8) per sample. The most frequently mutated genes were NRAS (4, 21%), CEBPA (4, 21%), JAK3 (3, 16%), RUNX1 (3, 16%). Mutations in genes such as DNMT3A, ETV6, IDH2, KMT2D, KRAS, NOTCH1, PHF6, TP53, and WT1 were detected in two different patients. CEBPA mutations were detected in 3 patients with M-T phenotype and one with B-T phenotype. Notably, the mutations in NRAS gene (reoccurring in 4 cases) were concentrated at its 12th/13th amino acid site.

We divided genes into the following categories according to diagnostic system and gene attribution such as transcription factor, chromatin regulation, epigenetics, cell apoptosis, signaling pathway (RTK-RAS, NOTCH, MAPK-ERK, PI3K/AKT, JAK-STAT), and others. Fifteen patients (2 AUL and 13 MPAL) satisfied both WHO and EGIL criteria, and two patients satisfied the EGIL criteria, but excluded by WHO classification system. The results showed that mutations detected in MPAL cases enriched in transcription factor, chromatin regulation and epigenetic, all related to genomic stability and transcriptional regulation; while AUL cases frequently mutated in genes in signal pathway regulation such as RTK-RAS, NOTCH, and PI3K/AKT (Fig. 1A). The mutation profile of the 2 patients excluded by WHO diagnostic criteria were analyzed separately, and the limited data suggested that the genetic background of these cases were even more complex than those included patients (Fig. 1A). There was no significant difference in the distribution of B/M, B/T, and T/M gene mutations (Fig. 1B). The detailed information profile was shown in Fig. 1C.

Survival analyses. Survival analyses were used to compare the prognosis of clinical subsets and types of treatment. Up to January 16, 2019 (mean follow up time 428 days), 34 patients received chemotherapy, while

| Characteristics                        | EGIL (n = 39) | WHO 2016 (n = 30) | EGIL-WHO (n = 9) | p value* |
|---------------------------------------|---------------|-------------------|-----------------|---------|
| Sex, m/f                              | 23/16         | 18/12             | 5/4             | 0.812   |
| Age, y                                | 39 ± 16.6     | 39 ± 14.9         | 38 ± 22.4       | 0.935   |
| WBC, ×10^9/L                          | 11 (3–40)     | 10 (3–39)         | 37 (2–107)      | 0.588   |
| HR, g/L                               | 80 (62–106)   | 86 (64–105)       | 68 (42–124)     | 0.501   |
| PLT, ×10^9/L                          | 71 (35–131)   | 79 (44–145)       | 29 (7–80)       | 0.039   |
| BM blast (%)                          | 80 (55–92)    | 76 (51–90)        | 92 (75–97)      | 0.069   |

| Karyotype abnormality, N (%)          |               |                   |                 |         |
| Abnormal                              | 17 (53.1)     | 15 (60.0)         | 2 (28.6)        | 0.141   |
| Ph+                                   | 7 (21.9)      | 7 (28.0)          | 0 (0)           | 0.113   |
| MLL rearrangement                     | 1 (3.1)       | 0 (0)             | 1 (14.3)        | 0.055   |
| Complex                               | 9 (28.1)      | 8 (32.0)          | 1 (14.3)        | 0.357   |

| Immunophenotype profile, N/tested     |               |                   |                 |         |
| CD34+                                 | 36/39         | 27/30             | 9/9             | 0.323   |
| MPO+                                  | 19/39         | 19/30             | 0/9             | 0.001   |
| CD13+                                 | 30/38         | 22/29             | 8/9             | 0.402   |
| CD11c+                                | 9/17          | 7/13              | 2/4             | 0.893   |
| CD14+                                 | 5/29          | 3/21              | 2/8             | 0.495   |
| CD64                                  | 6/25          | 5/17              | 1/8             | 0.356   |
| CD33+                                 | 24/34         | 17/25             | 7/9             | 0.581   |
| CD117+                                | 24/36         | 16/27             | 8/9             | 0.102   |
| CD19+                                 | 17/38         | 15/29             | 2/9             | 0.120   |
| CD20+                                 | 4/23          | 4/19              | 0/4             | 0.313   |
| CD22+                                 | 10/13         | 8/10              | 2/3             | 0.631   |
| CD10+                                 | 18/32         | 15/24             | 3/8             | 0.217   |
| CD79a+                                | 17/36         | 14/27             | 3/9             | 0.335   |
| CD5+/cCD3+                            | 20/37         | 14/28             | 6/9             | 0.383   |
| CD2+                                  | 9/23          | 6/16              | 3/7             | 0.809   |
| CD5+                                  | 11/24         | 9/16              | 2/8             | 0.148   |
| CD7+                                  | 25/38         | 18/29             | 7/9             | 0.386   |
| Patients with mutations, N/tested     | 17/19         | 15/17             | 2/2             | 0.608   |

Table 1. Clinical and laboratory features of patients in this study. EGIL European Group for the Classification of Acute Leukemia, WHO World Health Organization, BM bone marrow. *p values were evaluated by comparing EGIL-WHO with WHO 2016.
### Table 2. Detailed information of the patients with cytogenetic abnormalities.

| Patient no | Gender | Age (years) | EGIL classification | 2016 WHO classification | Gene fusion | Chromosome karyotype | CR | OS (day) |
|------------|--------|-------------|---------------------|------------------------|-------------|---------------------|----|----------|
| 1          | Male   | 24          | BAL                 | MPAL, B-M, NOS         | E2A/PBX1    | 46, XY, [9]/43, XYh (1q), 6q-, -9, -11q-, -15, -17, -18, 19p-, -21 [1] | Yes | 478+    |
| 2          | Female | 42          | BAL                 | MPAL, BCR/ABL+         | BCR/ABL (P210) | 46, XX, t(9;22) (q34;q11) [20] | Yes | 115+    |
| 3          | Female | 24          | BAL                 | MPAL, B-M, NOS         | Neg         | 46, XX, t (2;5) (p21;p15), del (6) (q16-q22), t (9;4) (p24;q21) | Yes | 578     |
| 13         | Female | 56          | BAL                 | MPAL, T-B, NOS         | Neg         | 46, XY, dic(11)(q24;q12),del(12)(q22) [7]/46,x [3] | No  | 381     |
| 15         | Male   | 20          | BAL                 | MPAL, BCR/ABL+         | BCR/ABL (P190) | 46, XX, r (5) (p15q35), t (9; 22) (q34, q11) [14]/46, XY, t (9; 22) (q34, q11) [6] | Yes | 530+    |
| 16         | Female | 58          | BAL                 | MPAL, T-M, NOS         | SET/CAN     | Neg                 | No  | 360     |
| 19         | Male   | 26          | BAL                 | MPAL, T-B, NOS         | Neg         | Subtriploid karyotype | Yes | 602+    |
| 21         | Female | 29          | BAL                 | MPAL, BCR/ABL+         | BCR/ABL     | 46, XX [6]/[CP] 46, XX, 2q+, t (9;22) (q34; q11), 14p+ [8] | Yes | 579+    |
| 22         | Male   | 25          | BAL                 | T-ALL                  | Neg         | 43–47, XX, t (4;11) (q21; p15), +6, del (6) (q21), del (7), (q21), del (7) (q14), add (9) (p24), add (11) (p15), add (14) (q22), -17, (p13), +ace (p26) [46, XX, (11)] | No  | 83      |
| 25         | Female | 43          | BAL                 | MPAL, T-M, NOS         | Neg         | 47, XX, +21 [4]/46, XX [2] | Yes | 545+    |
| 26         | Female | 41          | BAL                 | MPAL, BCR/ABL+         | BCR/ABL (P210) | 46, XX, +11 [9]/46, XX, [1] | Yes | 2293+   |
| 29         | Female | 60          | BAL                 | B-ALL                  | Neg         | 47, XX, t(8;13), (q10; p10), +22 [10] | No  | 51      |
| 31         | Male   | 50          | BAL                 | MPAL, BCR/ABL+         | BCR/ABL (P190) | 47, XX, +11 [9]/46, XX, [1] | Yes | 850+    |
| 32         | Female | 36          | AUL                 | AUL                    | Neg         | 47, XX, +8 [3]     | No  | 382     |
| 36         | Male   | 18          | BAL                 | MPAL, BCR/ABL+         | BCR/ABL (P190) | 46, XX, t(4;11) (q21; p15), +6, del (6) (q21), del (7), (q21), del (7) (q14), add (9) (p24), add (11) (p15), +ace (p26) [46, XX, (11)] | No  | 565+    |
| 37         | Male   | 42          | BAL                 | MPAL, BCR/ABL+         | BCR/ABL (P190) | 46, XX, t (9; 22) (q34; q11), +10, +21, +der (22) t (9; 22) (q34; q11) [1]/53, idem, M31 + X, +1, +5, +8 [1]/46, xy [18] | No  | 731     |
| 44         | Male   | 44          | AUL                 | MPAL, T-M, NOS         | Neg         | 46, XY [6]/47, XY, +10 [14] | Yes | 351+    |
| 45         | Male   | 23          | AUL                 | AUL                    | Neg         | 47, XX, add (2) (q37), +4, t (10;11) (p12q21), -17, +mar (4)/47, idem, add (2) (q37) [1]/46, XX, [15] | Yes | 141     |

Our study provided the genetic landscape of ALAL by screening the hot-spots in 173 genes strongly associated with myeloid and lymphoid malignancies and evaluated the clinical characteristics with the dismal clinical outcomes.
Recent studies also emphasized the important roles of somatic mutations in the diagnoses and treatment strategy of ALAL. These genetic data and our study both revealed that MPAL shared mutations with both AML and ALL, which indicated it would probably originated from early hematopoietic stem cells. B/M phenotype and T/M phenotype shared genetic alterations in the category of transcription factor and epigenetics, which was consistent with our findings. However, we did not observe the tendency towards signaling pathway mutations of T/M and B/M cases as previous reported. Limited number of patients may be the key reason. In this study, we analyzed 4 patients with AUL and 15 acute leukemia with mix-phenotype (BAL/MPAL) by high throughput sequencing and the results demonstrated these two sub-types of ALAL possessed different mutation files. We anticipate that the genetic information revealed by high throughput sequencing will innovate the medical care of ALAL in the future.

Another important point of view of our study is the deep exploration of the diagnostic system of ALAL. EGIL classification guideline was first proposed in 1995 and adopted in WHO criteria in 2001. Updated WHO 2008/2016 classification implicated significant modification to the EGIL score system and quite a number of patients were eliminated outside ALAL diagnosis, just as we found in our study. This part of patients was diagnosed as AML or ALL, and received corresponding treatment. However, whether the remarkable change is suitable and beneficial for the patients is still controversial, and the conflict focuses on the part of patients excluded by the new WHO system. In this study, we found the patients excluded by WHO system had an even worse prognosis (Log rank p = 0.016 for PFS and Log rank p = 0.016 for OS), which was consistent with a previous reported clinical study. In spite of this, we also found that the patients, satisfied the EGIL criteria but excluded by WHO diagnose system, shared the similar basic characteristics and immunophenotype with the patients included as showed in Table 1. Of the 9 patients excluded by WHO from ALAL, 2 cases underwent high throughput sequencing, and the results showed both of them carried completed genetic mutations distributed in genes like PTEN, KRAS, JAK3, KMT2C, WT1, ETV6, TP53, JAK3, RUNX1, NOTCH1, PHF6, and CREBBP. One of them showed no response to conventional chemotherapy, and unfortunately died 83 days after the diagnose. This is the first study to investigate the genetic background of this part of patients, however, the number of patients is limited to describe the whole picture of these patients. As the diagnosis significantly

![Figure 1. Landscape of somatic mutations in ALAL by next-generation sequencing. (A) Landscape of mutations detected in acute undifferentiated leukemia (AUL, n = 4), mixed-phenotype acute leukemia (MPAL, n = 13) and cases who were satisfied EGIL but excluded by WHO (n = 2). (B) Landscape of mutations detected in MPAL, including myeloid/B-lymphoid phenotype (MPAL B/M, n = 4), B-lymphoid/T-lymphoid (MPAL B/T, n = 3), and myeloid/T-lymphoid phenotype (MPAL T/M, n = 6). Y-axis represents the ratio of patients with mutations and the patients performed next-generation genetic testing. Columns with different colors represent genes belong to different categories according their attributes. (C) Detailed information of the 17 cases who detected mutations. Each column represents a case and each raw represents a gene. The top bar graph shows the number of mutations detected in each sample. The row directly underneath that graph show the immunophenotypes and Different colors of squares represent various mutation type.](https://doi.org/10.1038/s41598-021-03709-7)
dictates the therapeutic decision making, the clinical appliance of the WHO 2008/2016 criteria needs to be in more prospective clinical studies.

**Figure 2.** Outcomes of patients with ALAL by different clinical subsets. (A,B) Kaplan–Meier curve of PFS (A) and OS (B) stratified by tumor mutation burden/person by EGIL criteria. (C,D) Kaplan–Meier curve of PFS (C) and OS (D) stratified by tumor mutation burden/person by WHO 2016 criteria. (E,F) Kaplan–Meier curve of PFS (C) and OS (D) stratified by different classification systems (green curves indicating patients included in WHO 2016 criteria and red curves indicating patients who were included by the EGIL classification but excluded by the WHO 2016 classification).
The role of genetic (cytogenetic and molecular) markers in guiding chemotherapy strategies as well as targeted therapy is inexperienced. According to the WHO2016 criteria, t (9; 22)/Ph+ was considered as a separate entity. And the Ph chromosome was revealed as the most common cytogenetic abnormality, with the incidence ranging from 17–41% in MPAL especially with B-M phenotype12. All Ph+ patients should be confirmed as soon as possible due to the benefit probability from TKI20,21. In our study, we not only screened out 7 abnormal karyotyping patients with Ph+ resulting in BCR/ABL fusion gene, but also further detected 1 case with T315I mutation. Therefore, it is evident for us to make a better choice on the utility of TKIs. New molecular information in combination with cytogenetic characteristics will be valuable for the identification of genetic changes in ALAL, and undoubtedly play an important role in precision medicine of ALAL.

IDH2 gene mutations frequently include R140Q and R172K22. Surprisingly among our 19 available samples for NGS, we found 2 cases (AUL: n = 1, MPAL, T/M, NOS: n = 1) harboring IDH2 R140Q mutation. Recently, novel selective inhibitors of IDH1/2 mutations, ivosidenib and enasidenib, have been approved for patients with relapsed or refractory IDH1/2-mutated AML22. There is a proof that a patient with IDH1-mutated AUL achieved molecular complete remission following ivosidenib23. This might be a promising step to open the door for targeted therapy in IDH-mutated ALAL24.

Interestingly, we first found E2A/PBX1 in MPAL, B/M, NOS, which has not been reported in ALAL before. To our knowledge, it is one of the most common translocations in pediatric B-ALL. In adult, although not exceptional, it appears approximately 3%25. The prognostic significance of E2A/PBX1 fusion gene in ALAL is acquired further research.

Given that the druggable genetic target is still limited, analyzing the aberrant or specific antigen expression on leukemia cells seems to be another potential strategy. We tried to detect some canonical surface markers which can be targeted by off-the-shelf chimeric antigen receptor T-cell (CAR-T) immunotherapy. Strikingly, we definitely found 11 patients (47.8% of tested, data not shown) expressed CD123 on the surface of the blast cells. It has been reported that CD123, as a more specific marker for AML cells, is expressed at low levels on normal hematopoietic stem/progenitor cells26. An CD123 antibody–drug conjugate IMGN632 had been approved by FDA for blast plasmacytoid dendritic cell neoplasm (BDPCN), and CD123 CAR-T therapy exhibited a good effect in patients who are no longer responding to standard therapies26–28. It is potentially considered as a novel magic bullet for the therapeutics of ALAL with CD123 expression.

Some previous studies have explored the factors affecting the clinical prognosis of ALAL. From the results of meta-analyses with large sample size, significant survival benefit can be obtained from starting with ALL therapy followed by allogeneic hematopoietic stem cell transplantation irrespective of definition system12,20,29–31. The study of pediatric patients with ALAL also suggested that the ALL-like treatment scheme could benefit more. In this study, apart from immunophenotype, complex karyotype, treatment, mutation complexity is exclusively associated PFS and OS in ALAL, we observed the clue that the mutation burden in blast cells predicted higher risk of relapses and deaths. Due to the limitation of sample size, prospective studies with larger sample size will provide more powerful evidence.

Collectively, as the ALAL appears heterogeneous, it seems reasonable to establish stratification system of diagnosis and prognosis, especially based on the genetic features. Elucidating the genetic heterogeneity implicated in the process of leukemogenesis will provide insights into the pathogenesis and improve the management of the unusual subtype of acute leukemia.

Methods

Study design and participants. A retrospective analysis was performed based on data from 1635 de novo acute leukemia patients in Tongji Hospital (Wuhan, China) from January 2012 to June 2018. Standard procedures for diagnosis were performed, including morphology examination, multi-parameter flow cytometry analysis, molecular detection and karyotype analysis. According to EGIL or 2016 WHO criteria, 39 (2.4%) patients were enrolled in this study14,15. A total of 35 patients were designated as BAL according to EGIL scoring system and/or as MPAL if 2016 WHO criteria were fulfilled and 4 patients were diagnosed as AUL. The clinical characteristics, treatments and prognostic information were objectively collected from the medical records and validated by the physicians in charge or the telephone follow-up to the patients. AML-like therapy is defined as a “7 + 3” induction approach, followed with middle/high dose of Cytarabine for consolidation. ALL-like regimen refers to a standard treatment regimen of acute lymphoblastic leukemia with VDCLP as induction regimen and successive schedules such as hyper CVAD/MA as consolidation. AML + ALL like therapy is defined as a combination of ALL and AML drugs. AML + ALL-like are further divided into regimen based on AML (a schedule with “7 + 3” as induction followed with drugs in ALL treatment) and based on ALL (a schedule with DVCCLP as induction followed with drugs in AML treatment). The detailed information of therapy were provided in Supplementary Table 1.

The study design was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology according to the Declaration of Helsinki and all patients gave written informed consents to participate in this study.

Targeted gene next-generation sequencing and mutation calling. Among the 19 patients underwent NGS, 3 patients carried gene sequencing reports when they were admitted to our hospital, the data of next-generation sequencing was obtained from their medical record. For the others, samples from 15 patients were sequenced in Ion Personal Genome Machine and 1 was sequenced in Illumina NextSeq 500/550 platform in our center. The panel designed for variants detection covered the main mutation hotspots of a total of 173 genes which were the most frequently detected in myeloid and lymphoid malignancies (Supplementary Table 2). Genomic DNA was isolated from tumor blasts from bone marrow samples with the QiAamp DNA Blood Kit

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(Quagen GmbH, Hilden, Germany). Quality verified DNA extracted from tumor cells of patients was diluted to 5 ng/μl and prepared for library construction with the Ion AmpliSeq Library Kit 2.0 (Applied Biosystems, Foster City, CA, USA). The blast percentage of the samples ranged from 54.4 to 98.0%, with a median of 82.8% (Supplementary Table 1). The concentration of library was measured by Qubit 3.0. All consumable items and reagents used in Library pooling, Emulsion PCR and Enrichment of template were provided in Ion PGM Hi-Q View OT2 Kit and used according to the manufacturer's instructions. The next-generation sequencing was conducted in the Ion torrent PGM (Applied Biosystems, Foster City, CA, USA) with Ion 318 chip. The procedure of transferring raw signal to base and reads alignment were conducted in the local server by default settings. The VCF (Variant Call Format) files containing the raw mutation information was uploaded to Ion Reporter server for further analysis. After getting MAF (Mutation Annotation Format) files, we filtered mutations by conditions: (1) MAF (Minor Allele Frequency) value < 0.01; (2) Mutation reads number > 10; (3) Coding region mutation; (4) Non-synonymous mutation; (5) Not recorded in our false positive mutation database; (6) If the frequency was above 1% in any study of TopMed (freeze 8), gnomAD (v2.1.1), ExAC (v.0.3.1), The PACE Study (phase II), 3.5KPv2, 1000Genomes (phase 3), KOREAN population from KRDBG (v1) and Korean Genome Project (phase I), and detected variant allele frequency was in 0.4–0.6 or 0.9–1.0, the mutation would be annotated as germline SNP and removed. IGV32 software (v2.6.3) was used to screening all mutations in BAM and filtering typical false positive mutations caused by targeted sequencing primers as described in McCall et al's work32. CEBPA, NPM1 and FLT3 were additionally sequenced by Sanger sequencing since of low coverage in NGS. Copy number variations (CNV) were calculated using CODEX2 (v1.3.0)34. Mutation burden was calculated by (aberrant CNV number + mutation number)/panel size (Mb).

Statistical analysis. The baseline characteristics were compared by independent sample t test, Chi square test, or non-parametric test when appropriate. OS was measured from the date of diagnosis to the date of last follow-up or death from any cause. For progression-free survival (PFS) calculation, the uncensored event was relapse of leukemia and the censoring times reflected the end of follow up without an event or lost to follow up. The Kaplan–Meier survival analysis method was used to evaluate the prognosis determinants. The estimated survival values in different subsets were compared using the log-rank test and p < 0.05 were considered statistically significant. All analyses were performed using SPSS 21.0 (SPSS, Chicago, IL, USA).

Ethics approval and consent to participate. The study design was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology according to the Declaration of Helsinki and all patients gave written informed consents to participate in this study.

Consent for publication. All authors are aware of and agree to this submission.

Data availability The raw data of next-generation sequencing are available at SRA (the sequence read archive) database of NCBI website (Bioproject: PRJNA646819).

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References
1. Bene, M. C. et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia 9, 1783–1786 (1995).
2. van Dongen, J. J. Proposals for immunological classification of acute leukemias. Leukemia 9, 2149–2150 (1995).
3. van den Ancker, W. et al. Acute leukemias of ambiguous lineage: Diagnostic consequences of the WHO2008 classification. Leukemia 24, 1392–1396. https://doi.org/10.1038/leu.2010.119 (2010).
4. Vardiman, J. W. et al. The 2016 revision to the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: Rationale and important changes. Blood 114, 937–951. https://doi.org/10.1182/blood-2009-03-209262 (2009).
5. Arber, D. A. et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 127, 2391–2405. https://doi.org/10.1182/blood-2016-03-643544 (2016).
6. Yan, L. et al. Clinical, immunophenotypic, cytogenetic, and molecular genetic features in 117 adult patients with mixed-phenotype acute leukemia defined by WHO-2008 classification. Haematologica 97, 1708–1712. https://doi.org/10.3324/haematol.2012.064845 (2012).
7. Weinberg, O. K., Seetharam, M., Ren, L., Alizadeh, A. & Arber, D. A. Mixed phenotype acute leukemia: A study of 61 cases using the 2016 revision of the WHO classification. Am. J. Clin. Pathol. 142, 803–808. https://doi.org/10.1309/AJCPVYUPOTUYOIBS (2014).
8. Zheng, C. et al. What is the optimal treatment for biphenotypic acute leukemia? Haematologica 94, 1778–1780. https://doi.org/10.3324/haematol.2009.014829 (2009) (author reply 1780).
9. Hoesch, S. et al. Acute leukemias of ambiguous lineage in adults: Molecular and clinical characterization. Ann. Hematol. 92, 747–758. https://doi.org/10.1007/s00277-013-1694-4 (2013).
10. Matutes, E. et al. Mixed-phenotype acute leukemia: Clinical and laboratory features and outcome in 100 patients defined according to the WHO 2008 classification. Blood 117, 3163–3171. https://doi.org/10.1182/blood-2010-10-314682 (2011).
11. Liu, Q. F. et al. Allo-HSCT for acute leukemia of ambiguous lineage in adults: The comparison between standard conditioning and intensified conditioning regimens. Ann. Hematol. 92, 679–687. https://doi.org/10.1007/s00277-012-1662-4 (2013).
12. Munker, R. et al. Mixed phenotype acute leukemia: Outcomes with allogeneic stem cell transplantation. A retrospective study from the Acute Leukemia Working Party of the EBM. Haematologica 102, 2134–2140. https://doi.org/10.3324/haematol.2017.174441 (2017).
13. Wolach, O. & Stone, R. M. Mixed-phenotype acute leukemia: Current challenges in diagnosis and therapy. Curr. Opin. Hematol. 24, 139–145. https://doi.org/10.1097/MOH.0000000000000322 (2017).
14. Manola, K. N. Cytogenetic abnormalities in acute leukaemia of ambiguous lineage: An overview. *Br. J. Haematol.* **163**, 24–39. https://doi.org/10.1111/bjh.12494 (2013).
15. Takahashi, K. et al. Integrative genomic analysis of adult mixed phenotype acute leukemia delineates lineage associated molecular subtypes. *Nat. Commun.* **9**, 2670. https://doi.org/10.1038/s41467-018-04924-z (2018).
16. Alexander, T. B. et al. The genetic basis and cell of origin of mixed phenotype acute leukemia. *Nature* **562**, 373–379. https://doi.org/10.1038/s41586-018-0436-0 (2018).
17. Lao, Z. T. et al. Mutational and transcriptomic profiling of acute leukemia of ambiguous lineage reveals obscure but clinically important lineage bias. *Haematologica* **104**, e200–e203. https://doi.org/10.3324/haematol.2018.202911 (2019).
18. Weinberg, O. K. & Arber, D. A. Mixed-phenotype acute leukemia: Historical overview and a new definition. *Leukemia* **24**, 1844–1851. https://doi.org/10.1038/leu.2010.202 (2010).
19. Pomerantz, A. et al. Mixed-phenotype acute leukemia: Suboptimal treatment when the 2008/2016 WHO classification is used. *Blood* **125**, 233–241. https://doi.org/10.1056/brs.2016.51.4.233 (2016).
20. Wolach, O. & Stone, R. M. How I treat mixed-phenotype acute leukemia. *Blood* **125**, 2477–2485. https://doi.org/10.1182/blood-2014-10-551465 (2015).
21. Roberts, K. G. et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. *N. Engl. J. Med.* **371**, 1005–1015. https://doi.org/10.1056/NEJMoA1403088 (2014).
22. Dugan, J. & Pollyea, D. Enasidenib for the treatment of acute myeloid leukemia. *Expert Rev. Clin. Pharmacol.* **11**, 755–760. https://doi.org/10.1080/17512433.2018.1477585 (2018).
23. Patel, S. H. et al. Molecular complete remission following ivosidenib in a patient with an acute undifferentiated leukemia. *J. Natl. Compr. Cancer Netw.* **18**, 6–10. https://doi.org/10.6004/jncn.2019.7368 (2020).
24. Isa, R. et al. E2A-PBX1 fusion in adult acute lymphoblastic leukemia: Biological and clinical features. *Br. J. Haematol.* **120**, 484–487. https://doi.org/10.1046/j.1365-2433.2003.04113.x (2003).
25. Jordan, C. T. et al. The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia* **14**, 1777–1784. https://doi.org/10.1038/sj.leu.2401903 (2000).
26. Mardiros, A. et al. T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia. *Blood* **112**, 3138–3148. https://doi.org/10.1182/blood-2012-12-474056 (2013).
27. Loff, S. et al. Rapidly switchable universal CAR-T cells for treatment of CD123-positive leukemia. *Mol. Ther. Oncolytics* **17**, 408–420. https://doi.org/10.1016/j.mto.2020.04.009 (2020).
28. Li, Y., Sun, V., Sun, W. & Pawlowska, A. Blastic plasmacytoid dendritic cell neoplasm in children. *Hematol. Oncol. Clin. North Am.* **34**, 601–612. https://doi.org/10.1016/j.hoc.2020.01.008 (2020).
29. Stone, R. M. Transplantation after remission in mixed phenotype acute leukemia: A good idea. *Biol. Blood Marrow Transplant.* **22**, 971–972. https://doi.org/10.1016/j.bbmt.2016.04.001 (2016).
30. Maruffi, M., Sposto, R., Oberley, M. J., Kysil, L. & Orgel, E. Therapy for children and adults with mixed phenotype acute leukemia: A systematic review and meta-analysis. *Leukemia* **32**, 1515–1528. https://doi.org/10.1038/s41375-018-0058-4 (2018).
31. Wolach, O. & Stone, R. M. Optimal therapeutic strategies for mixed phenotype acute leukemia. *Curr. Opin. Hematol.* **27**, 95–102. https://doi.org/10.1097/MOH.0000000000000570 (2020).
32. Robinson, J. T. et al. Integrative genomics viewer. *Nat. Biotechnol.* **29**, 24–26. https://doi.org/10.1038/nbt.1754 (2011).
33. McCall, C. M. et al. False positives in multiplex PCR-based next-generation sequencing have unique signatures. *J. Mol. Diagn.* **16**, 541–549. https://doi.org/10.1016/j.jmoldx.2014.06.001 (2014).
34. Jiang, Y. et al. CODEX2: Full-spectrum copy number variation detection by high-throughput DNA sequencing. *Genome Biol.* **19**, 202. https://doi.org/10.1186/s13059-018-1578-y (2018).

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**Author contributions**

H.C. and G.W. were responsible for the design and overall coordination of the study, and manuscript revising. J.H. is responsible for data analyzing and manuscript writing. M.X. is responsible for the genetic testing and sequencing data interpretation. J.Z. is responsible for the follow-up and re-analyzing the follow up data, and the other authors took part in the clinical testing and data collection.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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