

## Mixed phenotype acute leukemia contains heterogeneous genetic mutations by next-generation sequencing

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### ABSTRACT

Mixed phenotype acute leukemia (MPAL) is an uncommon manifestation of acute leukemia. The aim of this study is to further characterize the genetic landscape of *de novo* cases of MPAL that fulfill the 2016 World Health Organization (WHO) classification criteria for this entity. We identified 14 cases examined by next generation sequencing (NGS) using 28 ( $n = 10$ ), 53 ( $n = 3$ ) or 81 ( $n = 1$ ) gene panels: 7 cases with a B-cell/myeloid (B/My) immunophenotype, 6 T-cell/myeloid (T/My) immunophenotype, and 1 B-cell/T-cell (B/T) immunophenotype. A total of 25 distinct mutations were identified in 15 different genes in 9/14 (64%) patients. *FLT3*-ITD was the only recurrent mutation in 2 patients. B/My MPAL cases less commonly harbored mutations compared with T/My MPAL cases (43% vs. 100%,  $p = 0.07$ ). In contrast, B/My MPALs more commonly showed a complex karyotype compared to T/My MPALs (71% vs. 17%,  $p = 0.1$ ). With NGS and karyotype combined, most (93%) MPAL cases had mutations or cytogenetic abnormalities. With a median follow-up of 12.5 months, there were no significant differences in median overall survival (OS) between patients with B/My or T/My MPAL (17.8 and 6.5 months, respectively,  $p = 0.81$ ) or between patients with MPAL with versus without gene mutations (6.5 and 13.3 months, respectively,  $p = 0.86$ ). Our data suggest that the distinguishing cases of MPAL according to immunophenotype has value because the underlying mechanisms of leukemogenesis might differ between B/My and T/My MPAL.

### INTRODUCTION

Our capacity to characterize and classify acute leukemia has evolved greatly over recent decades, in part due to advances in technology applied to the study of these neoplasms. Since the advent of immunohistochemistry and flow cytometry, most patients with acute leukemia can be readily assigned to either myeloid, B- or T-lymphoid lineage [1, 2]. However, unusual cases also have been described with confounding immunophenotypes, expressing markers of more than one lineage. In 1981, McGraw *et al.* reported the first case of mixed phenotype acute leukemia (MPAL), followed by a number of other reports, although designated inconsistently using a

number of terms in the literature. Reports of MPAL cases prompted the first classification proposal by Catovsky and colleagues in 1991, and followed by subsequent revisions linked to the advent of newer, more specific markers [3–7]. The description of the clinical characteristics, outcomes and various clinicopathologic correlations of MPAL have increased over the last three decades [8–15]. The latest version by the World Health Organization (WHO) included more stringent diagnostic criteria and further delineation of the heterogeneity of cases of MPAL [16, 17].

Mixed phenotype acute leukemia (MPAL) is currently defined as a leukemia in which the blasts express antigens of more than one lineage to such a degree that it

is not possible to assign the leukemia to any single lineage with certainty. These cases can be further subdivided into bilineal and biphenotypic. In bilineal MPAL, two distinct blast populations with different immunophenotypes are present. In contrast, biphenotypic MPAL is characterized by one blast cell population expressing markers of more than one lineage [16, 17]. MPAL cases can express either B or T cell antigens together with myeloid markers (B/My or T/My, respectively). Less frequently, rare neoplasms express B and T cell antigens (B/T) or B, T and myeloid antigens (B/T/My) [18, 19].

Despite the progress described above, relatively little is known about the frequencies and types of genetic mutations in MPAL. Of the studies that have assessed MPAL cases for genetic mutations, few have used NGS methods that can assess a large number genes designed to detect common mutations in acute leukemia, including AML and B and T-ALL.

## RESULTS

We identified 14 patients with MPAL, who were examined by an NGS panel. There were 8 (57%) men and 6 (43%) women with a median age of 61 years (range, 19–89 years). There were similar numbers of patients with a B-cell/myeloid (B/My) immunophenotype (7/14, 50%) and a T-cell/myeloid (T/My) immunophenotype (6/14, 43%). There was one patient with a B-cell/T-cell (B/T) immunophenotype. The immunophenotype for each patient is shown in Supplementary Table 4.

Two patients (cases #5 and #7) with B/My MPAL had *BCR-ABL1* rearrangement and one patient (case #2) with B/My MPAL had *KMT2A (MLL)* rearrangement. One patient (case #11) with T/My MPAL had *KMT2A* rearrangement. In this cohort the median white blood cell (WBC) count was 4,600/microliter (range, 1,000–271,200/microliter); the median hemoglobin (Hb) was 9.5 g/dL (range, 5.5–12.8 g/dL); the median platelet count was 76,000/microliter (range, 18,000–275,000 microliter); the median peripheral blood (PB) blast percentage was 15.5% (range, 0–97%); and the median bone marrow (BM) blast percentage was 78.5% (range, 13–92%). BM blasts were higher in patients with T/My than in patients with B/My MPAL ( $p = 0.04$ ) (Table 1). Otherwise, there were no differences were observed in WBC, Hb, and platelet count, and PB blasts between.

Mutations were detected in 9 (64%) patients; in 5 (36%) patients no mutations were identified. A total of 25 distinct mutations were found involving: *ABL1*, *ASXL1*, *DNMT3A*, *EGFR*, *FLT3*, *GATA1*, *IDH1*, *IDH2*, *JAK2*, *NOTCH1*, *NRAS*, *RUNX1*, *TET2*, *TP53* and *WT1* (Figure 1). Internal tandem duplications in *FLT3* (*FLT3-ITD*) were the only recurrent mutation ( $n = 2$ ). The median mutant allelic frequency was 38.1% (range, 1.6–99%). When mutations were present they affected at least 2 genes in 6 of 9 patients. Two patients (cases #5 and

#7) with *t(9;22)/BCR-ABL1* rearrangement did not have any mutations. Two patients with *KMT2A* rearrangement had mutations (cases #2 and #11). The data suggest that B/My MPAL less commonly harbors mutations than T/My MPAL (43% vs. 100%,  $p = 0.07$ ). Mutant allelic frequencies were similar between the B/My and T/My subtypes (38.1% and 38%, respectively,  $p = 0.76$ ). Two patients (cases #4 and #5) had NGS panels performed subsequently, after therapy. In patient 4, the same *TP53* splice mutation (c.559+1G > A) was detected on 28-gene NGS panel, 3 months apart. The allele frequency seen in the first panel was 38.1% (manual blast count: 52%) and then 11.4% (manual blast count: 13%) in the follow-up panel. No additional mutations were detected at time of subsequent NGS testing. In patient 5, no mutations were detected using a 53-gene NGS panel or using 28-gene panel 8.5 months later.

Conventional cytogenetics showed at least one chromosomal aberration in 10 (71%) patients, including 7 with 3 or more abnormalities (Figure 1 and Table 2). Five of 7 (71%) patients with a B/My immunophenotype had a complex karyotype. In contrast, only 1 (17%) patient with a T/My immunophenotype had a complex karyotype ( $p = 0.1$ ). Thirteen of 14 (93%) patients had either cytogenetic aberrations or gene mutations. Two patients with a B/My immunophenotype also harbored *t(9;22) (q34;q11.2)/BCR-ABL1*, both detected by qualitative multiparametric reverse-transcriptase PCR, quantitative real-time PCR, fluorescence *in situ* hybridization (FISH) and conventional karyotyping. The 2 patients with *t(9;22)* harbored a complex karyotype. Two patients with a B/My and T/My immunophenotype, respectively, also showed *t(v;11q23)*; *KMT2A* was rearranged detected by FISH and conventional karyotyping.

The treatment regimens used for these patients were heterogeneous (Table 2); 8 patients were treated with a hybrid approach (AML-targeted therapy with vincristine and dexamethasone), 3 patients received ALL-targeted therapy (cyclophosphamide, vincristine, doxorubicin, and dexamethasone or its variant), and 2 patients were treated with AML-targeted therapy (clofarabine and cytarabine). The treatment regimen was unknown in one patient. Five patients underwent stem cell transplant by the time of last follow-up.

Excluding 2 patients without follow-up information, the median follow-up duration was 12.5 months (range, 2.9 to 54.5 months). The median overall survival (OS) was 7.7 months (range, 2.9 to 50.1 months). Patients with B/My MPAL had longer overall survival (median OS, 17.8 months) compared to patients with T/My MPAL (median OS, 6.5 months), but this difference was not significant ( $p = 0.81$ ) (Figure 2A). A similar trend was seen in patients in whom gene mutations were identified (OS, 6.5 months) compared to patients without any detectable mutations (OS, 13.3 months) but this difference was not significant ( $p = 0.86$ ) (Figure 2B).

**Table 1: Clinicopathologic features of 14 patients with mixed phenotype acute leukemia**

	Total	B/My <sup>†</sup>	T/My <sup>‡</sup>	B/T	P value († vs. ‡)
<b>Gender</b>					
<b>Male</b>	8	4	3	1	1
<b>Female</b>	6	3	3	0	
<b>M:F ratio</b>	1.3	1.3	1	N/A	
<b>Age</b>					
<b>Median (range)</b>	61 (19–89)	68 (28–89)	57 (19–76)	45	0.19
<b>WBC (K/uL)</b>					
<b>Median (range)</b>	4.6 (1–271.2)	4.4 (1.3–239.7)	4.9 (1–271.2)	4.8	0.95
<b>Hemoglobin (g/dL)</b>					
<b>Median (range)</b>	9.5 (5.5–12.8)	9.8 (8.2–12)	9.1 (5.5–12.8)	9.7	0.70
<b>MCV (fL)</b>					
<b>Median (range)</b>	92.5 (82–103)	92 (85–103)	95 (82–123)	89	0.62
<b>Platelets (K/uL)</b>					
<b>Median (range)</b>	76 (18–275)	76 (27–268)	105 (18–275)	20	0.86
<b>PB Blast %</b>					
<b>Median (range)</b>	15.5 (0–97)	7 (0–53)	16 (0–97)	34	0.60
<b>BM Blast %</b>					
<b>Median (range)</b>	78.5 (13–92)	52 (13–90)	84 (65–92)	84	0.04

B/My, B-cell and myeloid mixed phenotype acute leukemia; T/My, T-cell and myeloid mixed phenotype acute leukemia; B/T, B-cell and T-cell mixed phenotype acute leukemia; WBC, white blood cell count; MCV, mean corpuscular volume; PB, peripheral blood; BM, bone marrow.

## DISCUSSION

The study of ambiguous lineage in cases of acute leukemia continues to be a challenge. The precise cytogenetic and molecular events leading to co-expression of antigens of more than one cell lineage in MPAL are unknown. Cytogenetically, using the 2008 WHO criteria, no single cytogenetic abnormality was overrepresented in a cohort of 100 patients indicating that MPAL is not the result of unique recurrent genetic abnormalities [10]. The outcome of patients with MPAL is inferior to the outcomes of patients with AML or either B or T ALL. Clearly, improved or novel therapies are needed for patients with MPAL. Toward this end, limited preliminary exploration of genetic mutations has been published in patients with MPALs [11, 20–23].

In this study, we found that almost two thirds of cases of MPAL harbor mutations. While our NGS panels cover most genes frequently mutated in AML or ALL (see supplementary information), the true mutation frequency is likely higher because the NGS panels used in this study do not cover genes in cohesion-complex or spliceosome-complex. Accordingly, it is not surprising that a whole exome sequencing study detected mutations in 91% of MPAL cases assessed [23]. Similar to earlier literature, the mutation pattern in patients with MPAL is heterogeneous [11, 23]. Mutated genes involve a variety

of cellular functions including chromatin modification (*ASXL1*), DNA methylation (*DNMT3A*, *IDH* and *TET2*), tumor suppressors (*TP53* and *WT1*), transcription factors (*NOTCH1*, *RUNX1*, and *GATA1*) and activated signaling (*FLT3*, *EGFR*, *NRAS* and *JAK2*).

When a gene mutation is present in MPAL, co-mutations of other genes are common (71%). Mutant allelic frequencies in co-mutated genes demonstrate variance, consistent with the heterogeneous nature (i.e. immunophenotype) of MPAL (Figure 1). However, we cannot conclude that MPAL shows a hybrid mutational profile between AML and ALL because many mutations, even those regarded as myeloid-associated gene mutations, are present in both AML and ALL. Particularly, early precursor T-ALL is known to have mutations in *FLT3*, *RAS*, *DNMT3A* and *IDH1/2* [24, 25]. Similarly, *RUNX1* mutations are seen in B-ALL [26]. Interestingly, mutations in *NPM1* and *CEBPA*, which define unique genetic subtypes of AML, were not in the MPAL cases in this study. Absence of mutations in *NPM1* and *CEBPA* also has been observed in other studies [11, 23].

About 70% of MPAL patients have chromosomal aberrations and often have a complex karyotype. Thirteen of 14 (93%) patients had cytogenetic aberrations and/or gene mutations, demonstrating genetic instability in MPAL. There appear to be some differences between B/My and T/My MPAL. B/My MPAL is less commonly

**Table 2: Conventional karyotype and treatment in each patient with mixed phenotype acute leukemia**

Patient	Age	Sex	NGS	Phenotype	Conventional karyotyping	Treatment
1	28	F	28	B/My	46,XX[20]	CIA, vincristine and dexamethasone
2	38	M	28	B/My	46,XY,t(11;19)(q23;p13.3)[20]	CIA, vincristine and dexamethasone, followed by SCT
3	89	M	28	B/My	49,XY,del(5)(q23q31),del(20)(q11.2q13.3),+21,+21,+21[9]/46,XY[11]	Unknown
4	79	F	28	B/My	46~55,XX,t(1;7)(q25;q35),del(5)(q13q33),+8,+11,+i(11)(q10), idic(11)(p11.2)x2,add(12)(p12),-15,-16,+19,+r,+3~4mar[cp17]/46,XX[3]	Fludarabine, cytarabine, vincristine and dexamethasone
5	55	M	53	B/My	46,XY,t(9;22)(q34;q11.2)[9]/47,idem,+der(22)t(9;22)[2]/48,idem,+21,+der(22)t(9;22)[9]	FIA, hyper-CVAD with dasatinib
6	76	F	28	B/My	47,X,del(X)(q22q27),+21[7]/47,XX,+10[2]/46,XX[11]	Hyper-CVAD with inotuzumab
7	85	M	28	B/My	45,X,-Y,inv(1)(p13p36.1)[1]/44,idem,-7,t(9;22)(q34;q11.2)[3]/44,idem,-5,-7,t(9;22),+mar[5]/44,idem,-6,-7,t(9;22),+mar[11]	Hyper-CVAD, dasatinib, rituximab, decitabine
8	76	F	28	T/My	46,XX[20]	Clofarabine and cytarabine
9	57	M	28	T/My	46,XY,t(6;14)(q25;q32)[4]/46,XY[16]	CIA with sorafenib, followed by SCT
10	65	M	53	T/My	46,XY,del(4)(p16)[1]/47,XY,+14[1]/46,XY[18]	Idarubicin, cytarabine, vincristine and dexamethasone, followed by SCT
11	34	F	28	T/My	47,XX,+4,t(11;19)(q23;p13.3)[13]/46,XX[7]	CIA, vincristine and dexamethasone, followed by SCT
12	67	M	81	T/My	46,XY[20]	CIA, vincristine and dexamethasone with plan for future SCT
13	19	F	28	T/My	45,XX,-9,-15,del(16)(p11.2p12.2),+der(?)t(?)q32)[10]/45,idem,add(17)(q25)[10]	CIA, vincristine and dexamethasone
14	45	M	53	B/T	50,XY,dup(1)(p22p36.1),+4,+10,-15,+21,+22,+mar[12]/50,idem,del(11)(q12),add(19)(q13.1)[3]/46,XY[5]	Hyper-CVAD, followed by SCT

NGS, next generation sequencing panel used; Hyper-CVAD, cyclophosphamide, vincristine, doxorubicin, dexamethasone, methotrexate and cytarabine; SCT, stem cell transplant; FIA, fludarabine, idarubicin and cytarabine; CIA, clofarabine, idarubicin and cytarabine.

associated with gene mutations than T/My MPAL (43% vs. 100%,  $p = 0.07$ ). In contrast, B/My MPAL cases more commonly had a complex karyotype (although not statistically significant). Although the statistical power of this study is insufficient, the data suggest that B/My and T/My cases have disparate pathogenetic mechanisms.

The results in this study and those in the literature show that genomic aberrations in MPAL are complex. Indeed, the few studies reported thus far contain differing results when it comes to genetic mutations seen in MPAL. It is not surprising that there exists such heterogeneity in

MPAL considering that the definition of the entity is that of ambiguous lineage. In addition, the pathogenesis of MPAL is not well understood. It is possible that competing transcription factors antagonize the functions of each other to promote expression of one lineage over the other. Alternatively, dysregulation and aberrant expression of transcription factors that govern cell differentiation occur on the basis of the genomic and epigenetic alterations. The timing and level of expression of specific transcription factors may therefore affect lineage determination [27, 28]. For example, reduced expression of human PAX5 during

the development of early lymphoid progenitors committed to B lineage has been associated with biphenotypic cells and acute leukemia [29, 30]. It seems that a high level of PAX5 expression is critical for the development of common lymphoid progenitors along the B-cell pathway, whereas low levels may result in a mixed phenotype. Along similar lines, it seems that the fate of early T-cell lineage progenitors is dependent on the Notch receptor signaling pathway, without which myeloid differentiation may occur [31]. This suggestion is consistent with the study by Eckstein *et al.* as well as this study which identified *NOTCH* mutations to only be present in T/My MPAL [23].

The prognosis of patients with MPAL is poor. Although patients were not uniformly treated in this study, we evaluated if mutational status could help to identify patients with a worse outcome. Although not significant, patients with B/My MPAL had a longer median overall survival than patients with T/My MPAL (17.8 and 6.5 months, respectively,  $p = 0.81$ ) (Figure 2A).

MPAL patients with mutations also appeared to have a worse OS compared to MPAL patients without mutations (6.5 vs. 13.3 months,  $p = 0.86$ ) (Figure 2B). A larger-scale cohort study is needed to better assess these possible associations.

## MATERIALS AND METHODS

### Diagnostic criteria and patients

We searched for all MPAL cases tested using an NGS panel over 4 years (2012–2016). Acute undifferentiated or unclassifiable leukemia and early thymocyte precursor T-ALL were not included. Molecular data were collected. Clinical, laboratory, cytogenetic and bone marrow findings were also reviewed.

All diagnoses of MPAL were based on the 2008 and 2017 WHO classification criteria. In brief, lineage was designated as follows: 1) myeloid lineage: positive

Patient #	Karyotype	ABL1	ASXL1	DNMT3A	EGFR	FLT3	GATA1	IDH1	IDH2	JAK2	NOTCH1	NRAS	RUNX1	TET2	TP53	WT1
1	Non-complex karyotype															
2	Non-complex karyotype					*										*
3	Complex karyotype		43.1					45.8					23.3			
4	Complex karyotype						43.8								38.1	
5	Complex karyotype															
6	Complex karyotype															
7	Complex karyotype															
8	Non-complex karyotype	37.3		34.6		55				51.5			17.2	48.8		
9	Non-complex karyotype		40.2			**						1.6				**
10	Non-complex karyotype								38							
11	Non-complex karyotype										41.7					
12	Non-complex karyotype															27.6
13	Complex karyotype														99	
14	Complex karyotype															

 B/My	 T/My	 B/T
 Non-complex karyotype	 Complex karyotype	
 Missense mutation	 Nonsense mutation	 Frameshift mutation
 Splice site mutation	 Multiple mutations	

**Figure 1: Immunophenotype, karyotype and mutations in each patient with mixed phenotype acute leukemia.** Patient #2 had two different *in-trans* mutations in *FLT3* and *WT1* genes; *FLT3* p.D835E (mutant allelic frequency, 45%), *FLT3* p.I836fs (4.8%), *WT1* p.R370fs (58.6%) and *WT1* p.V371fs (35.6%). Patient #9 also had multiple mutations in *FLT3* and *WT1* genes; *FLT3*-ITD (29%), *FLT3* p.D835V (11%), *WT1* p.R380fs (8.3%) and *WT1* p.R434fs (38.2%). B/My, mixed phenotype acute leukemia with B-lymphoblast and myeloblast phenotypes; T/My, mixed phenotype acute leukemia with T-lymphoblast and myeloblast phenotypes; B/T, mixed phenotype acute leukemia with B-lymphoblast and T-lymphoblast. \*: *FLT3* p.D835E (26.4%), *FLT3* p.I836fs (5%), *WT1* p.R370fs (58.6%), *WT1* p.V371fs (35.6%) \*\*: *FLT3*-ITD (29%), *FLT3* p.D835V (11%), *WT1* p.R434fs (38.2%), *WT1* p.R380fs (8.3%).

myeloperoxidase (flow cytometry, immunohistochemistry or cytochemistry) or monocytic differentiation (at least 2 of the following: NSE, CD11c, CD14, CD64, lysozyme); 2) T-lineage: positive cytoplasmic CD3 (flow cytometry with antibodies to CD3 epsilon chain) or surface CD3; 3) B-lineage: strong positive CD19 with strong expression of either CD79a, cytoplasmic CD22 or, CD10. Alternatively, a weak CD19 with strong expression of at least 2 of the following: CD79a, cytoplasmic CD22, or CD10.

MPALs were grouped in the following subtypes: 1) B/myeloid, not otherwise specified (NOS), meeting diagnostic criteria for assignment to both B and myeloid lineage, in which the blasts lack genetic abnormalities involving *BCR-ABL1* or *KMT2A*; 2) T/myeloid, NOS, meeting diagnostic criteria for assignment to both T and myeloid lineage, in which the blasts lack genetic abnormalities involving *BCR-ABL1* or *KMT2A*; 3) MPAL with t(9,22)(q34;q11.2); *BCR-ABL1* meeting diagnostic criteria for MPAL in which the blasts also have the (9,22) translocation or the *BCR-ABL1* rearrangement; 4) MPAL with t(v;11q23); *KMT2A* rearranged meeting diagnostic criteria for mixed phenotype acute leukemia in which the blasts also have a translocation involving the *KMT2A* gene; and 5) MPAL meeting diagnostic criteria for assignment to both B- and T- lineage. Due to the limited number of cases of MPAL with t(9;22) and MPAL with t(v;11q) we did not analyze these separately, but grouped them by immunophenotype.

### Next generation sequencing

Clinically validated 28-gene, 53-gene or 81-gene panels were used to assess mutational status in all patients as described previously (see supplementary Table 1, 2 and 3 for assessed genes/codons in each panel) [32]. Adequate coverage was defined as  $\geq 250$  reads for

each exon. The analytical sensitivity of the platforms is variable for different genes but is generally 1–3%. Only mutations identified in both forward and reverse reads were considered positive.

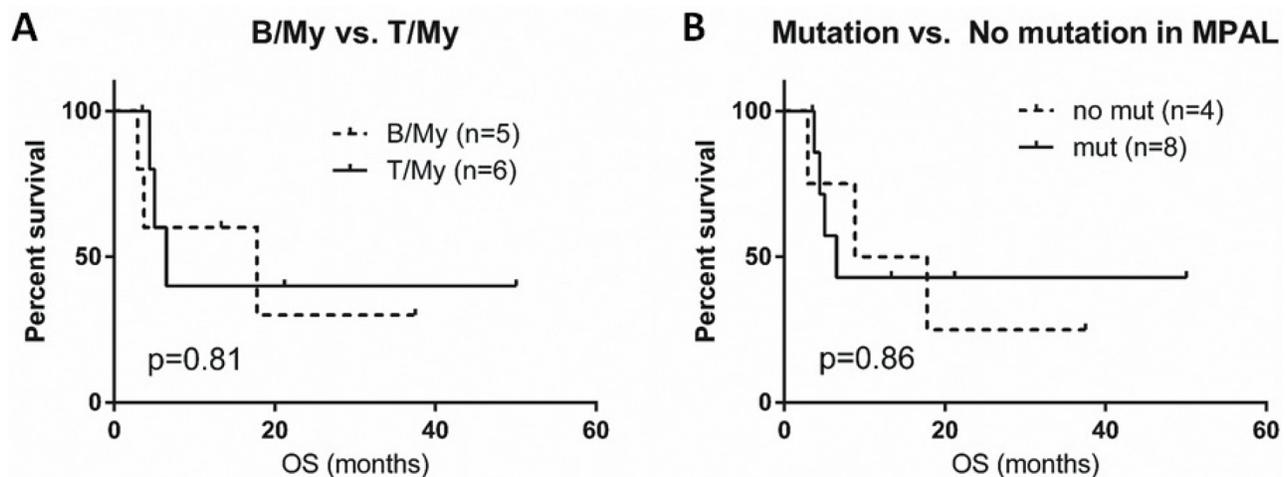
### *FLT3* and *CEBPA* analyses

PCR-based DNA analysis was performed to detect internal tandem duplications (ITD) and codon 835/836 point mutations in *FLT3*. A multiplex PCR using fluorescently-labeled primers was performed, followed by detection and sizing of PCR products using capillary electrophoresis. For detecting point mutations in codons 835/836, restriction enzyme digestion of the PCR products was performed prior to capillary electrophoresis. The lower limit of detection (analytical sensitivity) of this assay is approximately 1% of mutant DNA in a background of wild type DNA.

PCR-based DNA sequencing analysis was performed to assess *CEBPA* (CCAAT/enhancer binding protein-alpha). Mutations in this gene have been implicated as a favorable prognostic factor in AML. The lower limit of detection (analytical sensitivity) of the assay is 10–20% mutation bearing cells in the sample tested.

### *BCR-ABL1* analysis

Quantitative real-time PCR analysis was performed on reverse-transcribed RNA for the *BCR-ABL1* fusion transcripts. This multiplex assay is designed to detect common *BCR-ABL1* fusion transcripts e13a2(b2a2), e14a2(b3a2) and e1a2. The fusion transcripts are differentiated based on the size of the PCR product using capillary electrophoresis. *BCR-ABL1* and *ABL1* transcript levels are detected simultaneously and quantitative results are expressed as the percent ratio of *BCR-ABL1* to *ABL1* transcript levels.



**Figure 2: Survival graphs in patients with mixed phenotype acute leukemia (MPAL).** (A) Overall survival (OS) curve comparison for B/My and T/My MPAL. (B) OS curve comparison for MPAL patients with any mutations and patients without detectable mutations. B/My, mixed phenotype acute leukemia with B-lymphoblast and myeloblast phenotypes; T/My, mixed phenotype acute leukemia with T-lymphoblast and myeloblast phenotypes; MPAL, mixed phenotype acute leukemia.

## Flow cytometric immunophenotyping

Flow cytometric immunophenotyping was performed in all patients as described previously. [33] The markers assessed included: CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD15, CD19, CD20, CD22, CD25, CD33, CD34, CD36, CD38, CD41, CD45, CD52, CD56, CD58, CD64, CD79a, CD81, CD123, human leukocyte antigen (HLA)-DR, terminal deoxynucleotidyl transferase (TdT), myeloperoxidase (MPO), cytoplasmic CD3 and cytoplasmic immunoglobulin M (IgM).

## Cytogenetic analysis

Conventional chromosomal analysis was performed on G-banded metaphase cells prepared from unstimulated 24- and 48-hr BM aspirate cultures using standard techniques described previously [34]. Twenty metaphases were analyzed. The karyotype was documented according to the International System for Human Cytogenetic Nomenclature [35].

Fluorescence *in situ* hybridization for detection of *BCR-ABL1* was performed using a LSI fusion probe from Abbott Molecular, Inc (Abbott Park, IL). A total of 200 interphases are analyzed.

Fluorescence *in situ* hybridization for detection of *KMT2A* rearrangement was performed using a LSI dual color, break-apart probe from Abbott Molecular, Inc (Abbott Park, IL) which hybridizes to band 11q23 (spectrum green on the centromeric side and spectrum orange on the telomeric side of the gene breakpoint).

## Statistical analysis

Comparison among categorical variables and numerical variables was carried out by using the Fisher exact test and Mann-Whitney test, respectively. Overall survival was calculated from the date of diagnosis to the date of death or the last date of follow-up, whichever occurs earlier. Patients who underwent allogeneic stem cell transplant were censored. Survival probability was determined using the Kaplan–Meier method, with differences compared by the log-rank test. Statistical analysis was performed using GraphPad Prism (GraphPad software, San Diego, CA) with significance set at a *p*-value < 0.05 (two-sided).

## Abbreviations

Mixed Phenotype Acute Leukemia (MPAL); Next generation sequencing (NGS); Overall survival (OS); Acute leukemia (AL); World Health Organization (WHO); Acute myeloid leukemia (AML); Acute lymphoblastic leukemia (ALL); B cell and myeloid immunophenotype (B/My); T cell and myeloid immunophenotype (T/My); B

cell and T cell immunophenotype (B/T) ; B cell, T cell and myeloid triple immunophenotype (B/T/My); White blood cell (WBC); Hemoglobin (Hb); Peripheral blood (PB); Bone marrow (BM).

## Author contributions

A.E.Q. and C.Y.O. designed the study, A.E.Q, Z.H., L.J.M., and C.Y.O. wrote and revised the manuscript; A.E.Q and C.Y.O. collected and interpreted the data; A.E.Q and C.Y.O. performed statistical analysis; A.E.Q., M.J.R, K.P.P, R.L, S.L, Z.Z, C.C.Y, R.K.S, S.A.W, J.L.J, L.J.M., C.Y.O. provided study patients and related data; all authors revised and approved the final manuscript.

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## CONFLICTS OF INTEREST

None.

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None.

## REFERENCES

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