Clinical, immunophenotypic, cytogenetic, and molecular genetic features in 117 adult patients with mixed-phenotype acute leukemia defined by WHO-2008 classification

Lingzhi Yan,[1]* Nana Ping,[1]* Mingqing Zhu,[1]* Aining Sun,[1] Yongquan Xue,[1] Changgeng Ruan,[1] Hans G. Drexler,[2] Roderick A. F. MacLeod,[2] Depei Wu,[1] and Suning Chen[1]

[1]Jiangsu Institute of Hematology, Key Laboratory of Thrombosis and Hemostasis of Ministry of Health, the First Affiliated Hospital of Soochow University, Suzhou, P.R. China; and [2]DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

ABSTRACT

Among 4,780 consecutive adult acute lymphoblastic/myeloblastic leukemia patients, we identified 117 (2.4%) patients with mixed-phenotype acute leukemia fulfilling WHO 2008 criteria; these were classified as: B-lymphoid+myeloid (n=64), T-lymphoid+myeloid (n=38), B+T-lymphoid (n=14) and trilineage (n=1). Of 92 patients karyotyped, 59 were abnormal and were classified as: complex (22 of 92), t(9;22)(q34;q11) (14 of 92), monosomy 7 (7 of 92), polysomy 21 (7 of 92), t(v;11q23) (4 of 92), t(10;11)(p15;q21) (3 of 92), while STIL-TAL1 fusion was detected in one (T+My) patient. After investigating common acute leukemia-related mutations in 17 genes, 12 of 31 (39%) patients were found to have at least one mutation, classified with: IKZF1 deletion (4 of 31), and EZH2 (3 of 31), ASXL1 (3 of 31), ETV6 (2 of 31), NOTCH1 (1 of 31), and TET2 (1 of 31) mutations. Array-CGH revealed genomic deletions of CDKN2A (4 of 12), IKZF1 (3 of 12), MEF2C (2 of 12), BTG1 (2 of 12), together with BCOR, EBF1, K-RAS, LEF1, MBNL1, PBX3, and RUNX1 (one of 12 each). Our results indicate that mixed-phenotype acute leukemia is a complex entity with heterogeneous clinical, immunophenotypic, cytogenetic, and molecular genetic features.

Key words: mixed-phenotype acute leukemia, immunophenotype, cytogenetic, mutation, therapy.

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Introduction

In the majority of patients with acute leukemia, blast cells can be unequivocally assigned to a specific lineage, myeloid, B- or T-lymphoid. However, in approximately 2-5% of patients, lineage origin remains ambiguous even after comprehensive immunophenotyping by flow cytometry (FCM). Historically, a variety of terms have been used to describe these cases, such as mixed lineage leukemia, hybrid acute leukemia, bilineal leukemia, and biphenotypic leukemia.[1-5] The 2008 WHO classification of hematopoietic and lymphoid tumors (WHO-2008) modified the diagnostic criteria and introduced a new designation for this entity, this is now termed mixed-phenotype acute leukemia (MPAL).[6]

The clinical and laboratory features of mixed lineage leukemia, hybrid acute leukemia, bilineal leukemia, or biphenotypic leukemia have been described in a few retrospective studies.[1-5,7-13] However, given the dearth of reports so far, the clinical and laboratory features of patients with MPAL remain largely undefined. The only investigation reported to date involved 2 infants, 28 children and 68 adults with MPAL who were reassessed according to WHO-2008.[14]

Here, we retrospectively analyzed the clinical, immunophenotypic, cytogenetic, and molecular genetic features of patients with MPAL as defined under WHO-2008 criteria.

Design and Methods

Patients

The study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (FAHSU), P.R. China, according to the Declaration of Helsinki. It involved 4,780 adult (≥14 years of age) patients presenting with *de novo* acute leukemia at the FAHSU from January 1998 to August 2011; 117 (2.4%) cases, all ethnic Chinese, fulfilled WHO-2008 criteria for MPAL. Patients with AML-related translocations, chronic myeloid leukemia in blast crisis (CML-BC), myelodysplasia-related changes, or therapy-related AML were excluded from the study.

The combined AML+ALL regimen, MOAP/IOAP/DOAP was used to treat 24 cases as induction therapy: daunorubicin, idarubicin or

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Correspondence: Suning Chen, Jiangsu Institute of Hematology, the First Affiliated Hospital of Soochow University, 188 Shizi Street, Suzhou 215006, Jiangsu Province, P.R. China. Phone: international +086.0512.67780441. Fax: international +086.512.65113556. E-mail: yanmiz@vip.sohu.net or Dr. Depei Wu, Jiangsu Institute of Hematology, the First Affiliated Hospital of Soochow University, 188 Shizi Street, Suzhou 215006, Jiangsu Province, P.R. China. Phone: international + 086.0512.67780322. Fax: international + 0086.512.6511355. E-mail: wudepei@medmail.com.cn

mitoxantrone and cytarabine plus vincristine or vindesine and prednisone. The CAG regimen was administered to 16 patients, 10 at presentation and 6 refractory to the combined AML+ALL therapy. The CAG regimen consisted of Ara-C 10 mg/m^2 injected subcutaneously every 12 h (Days 1-14), aclarubicin 6 mg/m^2 infused intravenously (Days 1-8), and G-CSF administered subcutaneously at a dose of 200 μg/m^2/day. Eight patients with MPAL underwent allo-HSCT. Donors were double unit cord blood (n=3), matched unrelated (n=2), mismatched related (n=2) and matched sibling (n=1).

Immunophenotyping

FCM immunophenotyping was performed on blast populations using FACSCalibur instruments (BD Biosciences, San Jose, CA, USA). All cases were characterized by a panel of antibodies to leukocyte-associated markers, including surface CD2, CD3 (and cytoplasmic cyCD3), CD5, CD7, CD10, CD11b, CD11c, CD13, CD14, CD15, CD19, CD20, CD22, (cyCD22), CD33, CD34, CD45, CD64, CDw65, CD79a, (cyCD79a), CD117, HLA-DR, TdT, and myeloperoxidase (MPO). Surface antigen expression was considered positive when more than 20% of blasts showed a positive signal. For cytoplasmic antigens, the threshold was set at 10%.

Cytogenetic and molecular genetic analysis

R-banding karyotypic analysis was carried out on bone marrow (BM) or peripheral blood (PB) cells at diagnosis. A multiplex reverse transcription-polymerase chain reaction (RT-PCR) strategy was used in 44 patients with MPAL to detect 29 acute leukemia-related fusion genes, as previously described.[15]

Mutation analysis was perfomed in 31 MPAL patients for whom genomic DNA and RNA were available. A variety of acute leukemia-related mutations were evaluated: ASXL1, CBL, DNMT3A, ETV6, EZH2, FBXW7, FLT3-ITD, FLT3-TKD, IDH1, IDH2, IKZF1, KIT, NOTCH1, NPM1, PHF6, RUNX1, TET2, and WT1.

Array-based comparative genomic hybridization (array-CGH) analysis with Agilent 244k Human CGH Microarrays (Agilent Technologies, Santa Clara, California, USA) was performed in 12 MPAL patients for whom at least 1.5 μg genomic DNA was available. Data were visualized with Agilent Genomic Workbench Lite Edition 6.5 software.

Statistical analysis

Patients' characteristics were analyzed by χ^2 or Fisher's exact tests for univariate analysis. $P<0.05$ was considered significant. All calculations were performed using the SPSS software package (version 13.0).

Results and Discussion

Patients

Among 4,780 patients admitted to our institute since 1998, we identified 117 adult patients fulfilling current WHO-2008 criteria for MPAL (60 males and 57 females). The proportion of MPAL patients in this cohort (2.4%) was consistent with that of the 0.9-2.6% of acute leukemia cases according to the meta-analysis performed by Weinberg *et al.*[5] Median age was 35 years (range 14-81 years). Based on FAB criteria, 40 (34%) patients showed AML morphologies, mainly M1 and M5. A total of 51 patients (44%) were classified ALL-L1, the dominant subtype. The remaining 26 (22%) cases resisted classification by morphology and were categorized as acute undifferentiated leukemia. Main patients' characteristics are summarized in Table 1.

Immunophenotype

Immunophenotyping data (Table 1) showed that 64 of 117 cases (55%) had combined B+My, 38 (33%) combined T+My, 14 (12%) combined B+T, and one (0.9%) a trilineage (My+B+T), immunophenotype. MPAL cases with B+T immunophenotypes were more commonly seen in males; 11 of 14 (79%), when compared to 23 of 38 (61%) with T+My (P=0.376) and 26 of 64 (41%) with B+My immunophenotypes (P=0.01). Immunophenotyping data are summarized in Table 1.

Our immunophenotyping data revealed significant gender differences in MPAL subtypes, with more male in B+T and T+My groups but female predominance among B+My patients. This contrasts with data reported by Matutes *et al.*[14] Further studies are needed to clarify this and, in particular, whether ethnic or genetic factors might contribute to these discrepancies in gender distribution of MPAL cases.

It is presumed that MPAL arises from multipotent stem cells, capable of differentiating into myeloid and lymphoid progenitors during the development of acute leukemia. In this study, a marker of early hematopoietic cells, CD34, was strongly positive in 82% cases, reinforcing the view that MPAL cells originate at early stages of hematopoietic differentiation.

Cytogenetics

Of the 92 patients with available karyotypic data, 33 (36%) had no detectable chromosomal abnormality, while the remaining 59 (64%) showed abnormal karyotypes. Complex karyotypic abnormalities (≥3 aberrations) described the most prolific subclass, found in 22 cases (24%). The t(9;22)(q34;q11)/BCR-ABL1 fusion was present in 14 patients (15%), all with B+My phenotypes. Monosomy 7 was detected in 7 of 92 cases (7.6%). Polysomy 21 was unexpectedly found in 7 of 92 (7.6%) cases. Trisomy 8 occurred in 5 (5.4%) cases, all with B+My phenotypes. t(v;11q23)/MLL rearrangements were seen in 4 (4.3%) patients, of whom 2 had t(11;19)(q23;p13), while one patient each carried t(4;11)(q21;q23) and t(9;11)(p22;q23), respectively. t(10;11)(p15;q21) was present in 3 (3.3%) cases, including 2 with T+My and one with B+T phenotypes. In 3 cases, translocations not previously reported in leukemia were found: t(7;9)(q32;p24) with B+My phenotype, t(2;9)(q13;q34) with B+T phenotype, and der(9)t(9;11)(p21;q12) with T+My phenotype. A novel fusion between NUP98 and IQCG gene was identified in a MPAL patient with T+My phenotype harboring t(3;11)(q29q13;p15)del(3)(q29), as previously described.[16] STIL-TAL1 fusion via 1p32 microdeletion was identified by multiplex RT-PCR analysis in one case with B+My immunophenotype. Cytogenetic data are summarized in Table 1.

Of the patients for whom cytogenetic data were available, 64% showed abnormal karyotypes. Similar to previous reports, the cytogenetic groups most commonly described in MPAL or biphenotypic leukemia, namely those with complex karyotypes, t(9;22)(q34;q11) and t(v;11q23), were also detected in our study in a substantial proportion of MPAL cases: 24%, 15%, and 4.3% of patients, respectively.

Importantly, we identified four recurrent cytogenetic changes for the first time in a significant number of MPAL

patients: monosomy 7 in 7.6%, polysomy 21 in 7.6%, t(10;11)(p15;q21) in 3.3%, and 1p32 deletion effecting STIL-TAL fusion in 1.1%. Monosomy 7 is one of the most frequent chromosome abnormalities observed in patients with myelodysplastic syndromes and acute myeloid leukemia (AML), in which it is associated with a relatively poor prognosis.[17] It may also be found in patients with chronic myeloid leukemia in blast crisis (CML-BC) or Philadelphia positive acute lymphoblastic leukemia (ALL). Acquired isolated trisomy 21 is a frequent cytogenetic abnormality in

Table 1. Clinical, morphological, immunological and cytogenetic characteristics of 117 cases with MPAL.

Characteristics	Total (n=117) n. (%)	B+My (n=64) n. (%)	P	T+My (n=38) n. (%)	P	B+T (n=14) n. (%)	P	My+B+T (n=1) n. (%)
Sex			0.011		0.17		0.029	
Male	60 (51.3)	26 (40.6)		23 (60.5)		11 (78.6)		0
Female	57 (48.7)	38 (59.4)		15 (39.5)		3 (21.4)		1 (100)
Age, years			0.62		0.15		0.029	
Median (range)	35 (14-81)	34(14-81)		41(15-70)		23 (15-51)		16
WBC, $\times 10^9$/L			0.985		0.64			
Median (range)	5.4 (0.8-278.7)	3.6(1.7-278.7)		5.2(0.8-168.6)		20.2(6.0-126.1)		5.49
Missing	27	13		7		7		0
HB, g/L			0.533		0.86			
Median (range)	81 (39-135)	76(44-134)		80(39-135)		79 (42-117)		120
Missing	30	15		8		7		0
PLT, $\times 10^9$/L			0.776		0.97			
Median (range)	57 (13-295)	51(23-295)		58(13-219)		60 (22-240)		122
Missing	30	15		8		7		0
BM blast, %								
Median (range)	65 (3.5-95)	53(3.5-92.5)	0.039	85(4-95)	0.02	63 (13.5-94)	0.834	46.5
FAB classification								
AML	40 (34.2)	24 (37.5)		16 (42.1)		0		0
ALL	51 (43.6)	30 (46.9)		9 (23.7)		12 (85.7)		0
AUL	26 (22.2)	10 (15.6)		13 (34.2)		2 (14.3)		1 (100)
Cytogenetics	n=92	n=50		n=30		n=11		n=1
Normal	33	17		13		3		0
t(9;22)/BCR-ABL	14	14		0		0		0
Polysomy 21	7	2		5		0		0
Monosomy 7	7	6		1		0		0
Polysomy 8	5	5		0		0		0
t(v;11q23)/MLL-R	4	3		1		0		0
t(10;11)(p15;q21)	3	0		2		1		0
Del(1p32)/SIL-TAL1	1	1		0		0		0
Complex karyotype*	22	8		9		4		1
Immunophenotype								
NLS markers								
CD34	96/117(82.1)	56/64(87.5)		31/38(81.6)		8/14(57.1)		1/1(100)
HLA-DR	66/117(56.4)	40/64(62.5)		23/38(60.5)		2/14(14.3)		1/1(100)
Myeloid markers								
cyMPO	103/117(88.0)	64/64(100)		38/38(100)		0/14		1/1(100)
CD33	92/117(78.6)	54/64(84.4)		31/38(81.6)		6/14(42.9)		1/1(100)
CD13	90/117(76.9)	51/64(79.7)		30/38(78.9)		8/14(57.1)		1/1(100)
CD117	24/82(29.3)	10/50(20.0)		11/24(45.8)		3/7(42.9)		0/1(100)
CD11b	3/4(75.0)	-		3/4(75.0)		-		-
CD14	14/117(11.9)	12/64(18.8)		2/38(5.3)		0/14		0/1
CD15	33/79(41.8)	28/48(58.3)		5/23(21.7)		0/7		0/1
B-lymphoid markers								
cyCD79a	65/74(87.8)	50/52(96.2)		0/7		14/14(100)		1/1(100)
cyCD22	4/4(100)	4/4(100)		-		-		-
CD10	40/117(34.2)	31/64(48.4)		1/38(2.6)		7/14(50.0)		1/1(100)
CD19#	71/117(60.7)	60/64(93.8)		0/38		11/14(78.6)		0/1
CD20	12/117(10.3)	10/64(15.6)		0/38		2/14(14.3)		0/1
T-lymphoid markers								
cyCD3	53/117(45.3)	0/64		38/38(100)		14/14(100)		1/1(100)
CD2	24/117(20.5)	4/64(6.3)		12/38(31.6)		8/14(57.1)		0/1
CD7	62/117(52.9)	11/64(17.2)		37/38(97.4)		13/14(92.9)		1/1(100)

*B+My: B lymphoid and myeloid; T+My: T lymphoid and myeloid; B+T: B lymphoid and T lymphoid; My+B+T: trilineage leukemia; *:≥3 aberrations (including specific anomalies with additional chromosomal anomalies); NLS: no lineage specificity; #:strong CD19 expression(≥20%).*

myeloid malignancies, and has been reported as an anomaly accompanying t(12;21)(p13;q22) in childhood B-cell ALL. Several studies in leukemia patients with trisomy 21 showed frequent mutations of RUNX1, often duplicated as a consequence of the trisomy.[18] However, mutations of RUNX1 were absent in all 4 MPAL patients screened for RUNX1 mutation in this study. t(10;11)(p15;q21), resulting in PICALM-MLLT10 fusion, is a recurrent chromosomal translocation seen in AML or ALL patients showing T+My or B+T phenotype. STIL-TAL1 fusion results from a 1p32 microdeletion and is detected in 10-25% of T-cell ALL. This is the first report indicting these four cytogenetic alterations in the pathogenesis of MPAL.

Frequencies and distribution of gene mutations

In the past decade, with the advent of the novel genomic microarray technologies and next-generation sequencing, numerous genetic mutations which escape conventional cytogenetic detection have increasingly been reported in patients with AML, ALL, and other hematologic malignancies. However, as yet no studies have tried to delineate the distribution and clinical significance of these genetic mutations in patients with MPAL.

In the present study, we evaluated a variety of acute leukemia-related mutations. After excluding known polymorphisms and silent mutations, a total of 12 mutations (39%) were documented (Table 2) including: IKZF1 deletions in 4 of 31 (13%) patients, and mutations affecting EZH2 in 3 of 31 (9.7%), ASXL1 in 2 of 31 (6.5%), and ETV6, NOTCH1, and TET2 mutations in one of 31 (3.2%) patients each. Although mutations of CBL, DNMT3A, FBXW7, FLT3, IDH1, IDH2, KIT, NPM1, PHF6, RUNX1, and WT1 have been frequently detected in patients with AML or ALL, none were found in this study. Collectively, our findings suggest that MPAL may carry a spectrum of molecular aberrations that differ from other acute leukemias.

With respect to karyotype, cytogenetic abnormalities were detected in 10 of 11 patients with available data. Among these, IKZF1 deletions were found exclusively in 4 of 8 (50%) patients with t(9;22)(q34;q11)/BCR-ABL1 fusion and B+My phenotype. To evaluate the clinical impact of gene mutations on patients with MPAL, we compared the prognosis of 24 MPAL patients undergoing mutation screening. However, no significant difference on overall survival (P=0.158) between patients with and without gene mutations was found.

Array-based comparative genomic hybridization analysis of 12 MPAL samples

Genomic microarrays provide powerful tools enabling global high-resolution analysis of submicroscopic chromosome deletions, amplifications, and unbalanced chromosome rearrangements. They are widely used in the molecular genetic studies of hematologic malignancies and have helped identify many important cryptic leukemia-related genetic alterations, such as IKZF1 deletion, MYB duplication, LEF1 deletion, SET-NUP214 fusion, PAX5 deletion, and TET2 mutation. This is the first molecular genomic study of MPAL or biphenotypic leukemia. We performed array-CGH analysis on 12 MPAL samples and found that all had one or more genomic abnormalities (*Online Supplementary Table S1*). A total of 68 genomic alterations were detected, with a mean of 5.7 genomic alterations per sample. We identified new cryptic copy number changes in MPAL containing interesting potential candidate genes such as deletions of the CDKN2A (4 of 12), IKZF1 (3 of 12), MEF2C (2 of 12), together with one each (8.3%) of BCOR, EBF1, KRAS, LEF1, MBNL1, PBX3 and RUNX1.

Among these, deletion of CDKN2A and mutations of KRAS are found in a wide variety of solid tumors and hematologic malignancies. The *BTG1*, *EBF1* and *IKZF1* genes are associated with B-cell development and are frequently deleted in patients with B-cell precursor ALL.[19,20] Mutations of *BCOR* and *RUNX1* genes have been repeatedly found in patients with AML.[21,22] Deletion of LEF1 and transcriptional deregulation of MEF2C have already been identified in T-ALL.[23,24] All of the cryptic gene lesions listed above are described here for the first time in MPAL patients.

Table 2. Characteristics of 12 MPAL patients with gene mutation(s).

Case (n.)	Sex	Age	Phenotype	Cytogenetics	Gene	Mutation	Exon	Type of mutation	Protein level
1	F	21	B+My	46,XX,t(4;11)(q21;q23),6q-,9q-[3]/46,XX[17]	TET2	c.3278_3283delCCAA	3	frameshift	p.Thr1093fs
2	F	64	T+My	46,XX[20]	EZH2	c.444_446delC	4	frameshift	p.Pro108fs
3	M	60	T+My	46,XY,+4,der(9)t(9;11)(p21;q12),-11[10]/46,XY[2]	EZH2	c.1532A_T	11	missense	p.Arg470Trp
4	M	69	B+My	45,XY,-7[14]/46,XY[6]	EZH2	c.1819G_A	15	missense	p.Arg566His
5	M	27	B+My	92,XXYY[12]/46,XY[8]	ASXL1	c.2813G_T	13	nonsence	p.Glu796X
6	M	27	B+My	47,XY,+8[8]/46,XY[2]	ASXL1	c.2240C_T	13	missense	p.Arg605Trp
7	M	26	T+My	NA	ETV6	c.547insert TCC	3	frameshift	p.Tyr104fs
8	M	39	T+My	46,XY,der(12)t(12;17)(q10;q10), der(17)t(1;17)(p10;q10)[12]/46,XY[8]	NOTCH1	c.5238T_G	27	missense	p.Val1720Gly
9	F	25	B+My	46,XX,t(9;22)(q34;q11)[10]/46,XX[3]	IKZF1	deletion	-	frameshift	-
10	M	31	B+My	46,XY,t(9;22)(q34;q11) [20]	IKZF1	deletion	-	frameshift	-
11	M	51	B+My	46,XY.[20](FISH: BCR-ABL: 88% positive)	IKZF1	deletion	-	frameshift	-
12	M	19	B+My	46,XY.[20] (RT-PCR:BCR-ABL positive)	IKZF1	deletion	-	frameshift	-

NA: not available.

Treatment and clinical response of MPAL patients

For the moment, the optimal therapy for patients with MPAL has still not been defined. There is little consensus as to whether induction therapy should follow AML and/or ALL chemotherapy, and as to whether HSCT might be effective. We compared the relative efficacies of the combination of AML+ALL therapy and of the CAG regimen in induction therapy on 34 MPAL patients at presentation. MOAP/IOAP/DOAP was administered to 24 MPAL patients, of whom 14 (58%) achieved complete remission (CR), while 2 died from severe infection. The remaining 10 patients received induction therapy according to the CAG regimen of whom 7 (70%) achieved CR. There was no significant difference in CR rates between the two groups (P=0.802). However, interestingly, 5 of the 6 patients (83%) who failed to respond to MOAP/IOAP/DOAP therapy achieved CR after receiving the CAG regimen. Furthermore, the overall survival rate in 8 patients with MPAL receiving allo-HSCT was longer than that in the 13 patients receiving consolidation chemotherapy (22.0 *vs*. 9.0 months; P=0.004). Therefore, it seems patients receiving allo-HSCT had the better outcomes. However, owing to the limited numbers of patients who received allo-HSCT in this study, further investigation involving more cases is needed to determine its role in MPAL therapy.

In summary, this study demonstrates that adult MPAL is a complex entity with heterogeneous clinical, immunophenotypic and genetic characteristics. We observed widespread differences in the cytogenetic and molecular features of adult MPAL *versus* AML or ALL. We also identified gene mutations or cryptic copy number alterations housing potential oncogene targets. Further genetic studies with the novel genomic technologies, such as next-generation sequencing, may help define the leukemogenic mechanisms of patients with MPAL. In addition, it will be necessary to conduct multi-center co-operative studies to determine optimal regimens for induction and consolidation therapy and to define the role of HSCT in MPAL.

Authorship and Disclosures

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