Clinical diagnosis of adult patients with acute megakaryocytic leukemia

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Abstract. Acute megakaryocytic leukemia (AMKL) is a rare subtype of acute myeloid leukemia (AML), which is challenging to diagnose due to frequent myelofibrosis (MF) and a low percentage of blast cells. In the present study, clinical characteristics and experimental observations in 9 adult patients diagnosed with AMKL, who were recruited by the Sino-U.S. Shanghai Leukemia Co-operative Group, were analyzed in order to summarize the diagnostic experience and provide recommendations on diagnosing AMKL. All the patients were diagnosed according to the 2008 World Health Organization diagnostic criteria. The mean age of the patients with AMKL was 59 years (range, 53-68 years). A total of 8 patients had different degrees of anemia, and 2 patients had <5% marrow blasts present in the bone marrow; however, the percentage of positive cells with cluster of differentiation (CD)41 and CD61 expression was >20%, as demonstrated by flow cytometry. A total of 6 patients were positive for platelet-specific antigens, as indicated by immunocytochemistry. Furthermore, 7 patients presented with moderate or marked MF, as demonstrated by a bone marrow biopsy. Karyotypic analysis indicated that 6 patients had abnormal karyotypes. Only 1 patient exhibited the Janus kinase 2V617F mutation. Treatment efficiency was notably poor, with a median survival time of 6.0 months (range, 1.1-24.0 months). In conclusion, the diagnosis of AMKL requires a combination of the results of bone marrow smears and bone marrow biopsy, immunophenotype or immunohistochemistry. We recommend that routine immunophenotypic analysis should include the CD41 and CD61 markers for diagnosing acute leukemia when bone marrow morphology does not indicate the diagnosis.

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Introduction

Acute megakaryocytic leukemia (AMKL) is a rare type of acute leukemia that was first reported in 1931 by Von Boros (1). In subsequent years, patients were rarely diagnosed with AMKL due to its low incidence and a lack of accurate diagnostic criteria. In 1978, Breton-Gorius *et al* (2) utilized immunoelectron microscopy analysis with platelet peroxidase (PPO) to identify megakaryocytes and increase the accuracy of diagnosing AMKL. AMKL was then added to the French-American-British classification as acute myeloid leukemia (AML) M7 in 1985, which identified the diagnostic criteria as exhibiting >30% of blast cells of the bone marrow nucleated cells, which were demonstrated as a lineage derived from megakaryocytes by PPO staining (3). With the development and application of flow cytometry, the diagnosis of AMKL became more accurate. In 2008, the World Health Organization (WHO) produced precise criteria for diagnosing AMKL (4). In these criteria, AMKL was diagnosed by the presence of ≥20% blasts of the bone marrow nucleated cells, with >50% of the blasts being megakaryoblasts in the bone marrow; or positive platelet-specific antigens by bone marrow aspirate or biopsy, including factor VIII, cycle of differentiation (CD)41, CD42 or CD61, as determined by immunocytochemistry staining or immunophenotyping.

In the clinic, although there are now accurate diagnostic criteria, the diagnosis of AMKL is challenging due to a high incidence of myelofibrosis (MF), resulting in difficulty in differentiating AMKL from acute MF (5). Clinical diagnostic experience with this type of leukemia is also limited. When the percentage of blast cells in the bone marrow is <20%, immunohistochemical staining and immunophenotypic analysis are not performed; therefore, the diagnosis of AMKL may lack accuracy when depending solely on the information provided by cellular morphology (6). As a result, a comprehensive diagnosis is important for this disease.

In the present study, the clinical characteristics, experimental tests and survival times of 9 adult patients with AMKL, who were recruited by the Sino-U.S. Shanghai Leukemia Cooperative Group between June 2003 and December 2010, were analyzed. Additionally, the diagnostic experience was summarized and diagnostic recommendations for AMKL were provided.

Patients and methods

Patients. The Sino-U.S. Shanghai Leukemia Cooperative Group (School of Public Health, Fudan University) diagnosed 623 patients (Median age is 51 years old, and ranged from 18 to 88 years, the percentage of males were 56.5%) with AML between June 2003 and December 2010, and conducted follow-ups to determine their survival time. Of these patients, 9 (1.4%) were diagnosed with AMKL. These patients were diagnosed with AMKL according to the 2008 WHO classification criteria (4) as follows: i) The bone marrow aspirate exhibited a blast cell infiltrate comprising ≥20% of all cells, and >50% of the blast cells were identified as megakaryoblasts; ii) the expression of CD41, CD42 and/or CD61 was positive, as demonstrated by flow cytometry with monoclonal or polyclonal platelet-specific antibodies; and iii) bone marrow aspiration was frequently accompanied by MF, and in cases with fibrosis, a bone marrow biopsy was required, and the cell of origin was required to be identified as part of the megakaryocyte lineage. This was indicated by positive immunocytochemical staining for platelet-specific antigens, including factor VIII, CD41, CD42 and CD61. Patients were be diagnosed with AMKL if they met at least one of the diagnostic criteria. In the present study, all the patients with AMKL were administered one, two or more intravenous (IV) courses of a standard induction regimen that was comprised of 60 mg/m^2 daunorubicin or 12 mg/m^2 idarubicin per day for 3 consecutive days, plus 200 mg/m^2 cytarabine that was continuously administered by IV infusion for 7 days. Following one course of a standard induction, a number of patients had no response and succumbed shortly after. Additionally, a number of patients had complete remission or partial remission and continued the regimen, receiving two or more courses of a standard regimen.

Diagnostic process. i) If the percentage of blast cells was >20% in the bone marrow of nucleated cells, and cell morphology was demonstrated to be megakaryoblasts, as demonstrated using a bone marrow smear, the diagnosis was AMKL. For this diagnosis, the results of flow cytometry or immunocytochemical staining were required to increase the accuracy of the diagnosis. ii) If the bone marrow aspiration could not indicate a diagnosis of AMKL, detection methods of flow cytometry and immunocytochemical staining were vital, and the final diagnosis was frequently determined by positive platelet-specific antigens. iii) If the bone marrow aspiration diagnosis was not successful due to MF, a bone marrow biopsy was the primary test method, and the final diagnosis was determined by immunocytochemical staining for factor VIII, CD41, CD42 or CD61.

Cell morphology, bone marrow cellularity and cytochemical staining. Bone marrow aspirates and biopsies were obtained from the posterior iliac crest. Bone marrow smears were conducted by obtaining 0.2 ml marrow aspirate from each patient, in order to perform cytomorphological classification and cytochemical staining, including myeloperoxidase, periodic acid-Schiff, α naphthol-acetate esterase, Sudan black B and non-specific esterase staining, according to the International Committee for Standardization in Hematology (7). Bone marrow cellularity of the aspirate and biopsy were examined by routine light microscopy (x100 magnification) and assessed with the following grading: Definite hypercellularity, normal cellularity, moderate hypocellularity and severe hypocellularity (8).

Flow cytometry immunophenotyping. An additional 3 ml bone marrow aspirate was used for flow cytometry analysis. Mononuclear cells (MNCs) were isolated from those samples using lymphocyte separation medium (Ficoll®-paque Plus; GE Healthcare Life Sciences, Chicago, IL, USA). The cells were suspended in PBS (GE HealthCare Life Sciences) with 3% bovine serum albumin (A8020; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and was adjusted to 1x10^6 MNCs/ml and an aliquot of 100 μl was labeled with 5 or 20 μl fluorescein conjugated monoclonal antibodies. The applied monoclonal antibodies were directed against the following surface antigens: CD34 PE (550761, 1:5), CD45 PerCP (564105, 1:20), human leukocyte antigen DR related antigen (HLA-DR APC, 560896, 1:5), platelet-specific glycoproteins, CD41 FITC (555469, 1:5) and CD61 PE (555754, 1:5), B-cell antigens, CD10 PE (561002, 1:20) and CD19 FITC (560994, 1:5), T-cell antigens (CD2 PE (555327, 1: 20), CD3 APC (555335, 1:5), CD4 APC-CY7 (561839, 1:20) and CD7 FITC (555360, 1:5), myeloid antigens, CD13 APC (561698, 1:20), CD14 PE-CY7 (560919, 1:20), CD15 FITC (555401, 1:5), CD33 PE (555450, 1:5), CD64 APC (561189, 1:20), CD117 PE (561682, 1:20) and myeloperoxidase (556035, 1:20, BD Biosciences). All monoclonal antibodies were obtained from BD Biosciences (San Jose, CA, USA). Subsequently, the cell suspension was incubated with these antibodies in the dark for 15 min at 4˚C, then washed in PBS with 5% EDTA and centrifuged (200 x g) for 5 min at 4˚C three times. Following this, the cells were suspended in 500 μl PBS for further use. The immunophenotyping was performed with the BD FACS Canton flow cytometer using FCS Express 3.0 software (De Novo Software, Glendale, CA, USA). Gating was set with CD45 and 90˚ light-scatter parameters in order to exclude erythrocytes, platelets and subcellular debris. A total of 1x10^4 cells was acquired and the percentage of cells expressing the marker was calculated.

Immunohistochemical staining. The samples from the bone marrow biopsy were fixed in B5 stationary liquid (10% formaldehyde) (mercuric chloride 6.0 g, anhydrous sodium acetate 1.25 g, distilled water 90 ml and formaldehyde 10 ml) for 2 h at room temperature, followed by processing in 70% absolute ethyl alcohol, decalcification in Decal (EDTA bisodic salt) for 2.5 h at room temperature and ordinary processing with paraffin embedding. The paraffin-embedded samples were placed on slides (Dako ChernMate Capillary Gap slides; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and stored in a warm incubator at 56˚C. After 2 h, the samples were rinsed with water and washed with dimethylbenzene, and then, washed by graded concentration of absolute ethyl alcohol (100, 95, 90, 80 and 70%). Then, the samples were sealed in 3% H_2O_2 for 10 min at room temperature and washed in distilled water, three times at room temperature. Subsequently, the samples underwent optimized antigen retrieval procedures, the 0.01 M sodium citrate buffer (pH 6.0) (C1010; Beijing Solarbio

Science & Technology Co., Ltd.) was heated in microwave oven to 95˚C, and kept the samples in boiled sodium citrate buffer for 15 min. After that, it was cooled at room temperature. Then the samples were washed in distilled water for 3 min, two times at room temperature, and in PBS for 5 min, three times at room temperature, and were sealed with 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc., MA, USA) for 20 min at room temperature. The applied monoclonal antibodies (Dako; Agilent Technologies, Inc.) were against factor VIII (ab236284, 1:200, Abcam, Cambridge, MA, USA), CD42 (ab134087, 1:250, Abcam) and CD61 (ab7166, 1:250, Abcam), and were incubated with the samples overnight at 4˚C. Subsequently, the sections were washed in Tris-buffered saline (TBS) three times for 10 min each at room temperature, and then incubated with biotinylated rabbit anti-goat secondary antibody (1:250; Vector Laboratories, Inc., Burlingame, CA, USA) for 60 min at room temperature, followed by washing with TBS three times for 10 min each. Following this, the sections were processed with avidin-biotin complex (TA-015-BB, Thermo Fisher Scientific Inc.) reagent for 30 min at room temperature, incubated in 3,3'diaminobenzidine solution (710008, Sera Care Inc, MA, USA) for 30 min in the dark and counterstained with hematoxylin (0701; Beijing Solarbio Science & Technology Co., Ltd.). Subsequently, the sections were dehydrated in an ascending graded series of absolute ethyl alcohols (70, 80, 90, 95 and 100%), cleared in xylene and cover-slipped with neutral balsam. These samples were observed in a light microscope (Nikon 801; x60 and x100) and the imaging Software NIS-Elements F 3.00, SP7 (Build 547) (both Nikon Corporation, Tokyo, Japan). The stain intensity was classified as -, +, ++, +++. -, No staining is observed. + positive staining is observed in less than 25% cells. ++ positive staining in 25-49% cells. +++ extensive positive staining in more than 50% cells.

Gomori silver impregnation staining. The Gomori silver impregnation staining kit (G1800; Beijing Solarbio Science & Technology Co., Ltd.) were used. The stationary process, paraffin embedding and dehydration procedure were the same as described previously. The oxidizing agent Gomori was added to the samples for 5 min at room temperature, washed with running water for 30 sec. Subsequently, the samples were bleached by oxalic acid solution for 2 min, then washed by running water for 2 min, by distilled water for 1 min, all the process was performed at room temperature. After that, Samples were stained with ammonium ferric sulfate solution (5%) for 5 min, washed by tap water for 1 min, by distilled water for 2 min. Silver ammonia solution was used to stain samples for 3 min, then distilled water was used to wash the solution for 1 min. Reducing agent of Gomori was added to the samples for 1 min and washed by running water for 10 min at room temperature. Subsequently, the sections were dehydrated in an ascending graded series of absolute ethyl alcohols (70, 80, 90, 95 and 100%), cleared in xylene and cover-slipped with a neutral balsam.

Evaluation of MF. MF was graded according to the European Consensus Grading system (9) as follows: MF-0, scattered linear reticulin with no intersections; MF-1, loose network of reticulin with numerous intersections, particularly in perivascular areas; MF-2, diffuse and dense increases in reticulin with extensive intersections; and occasionally with only focal bundles of collagen and/or focal osteosclerosis MF-3, diffuse and dense increases in reticulin with extensive intersections, with coarse bundles of collagen, often associated with significant osteosclerosis.

Detection of the t(9;22) mutation. Blood samples were obtained from the posterior superior iliac spine by bone marrow aspiration and collected in a heparinized tube. Blood samples (1 ml) were cultured in a culture dish containing 6 ml RPMI-1640 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 1% antibiotics and 100 μl 10 mg/ml phytohemagglutinin for each patient. After 24 h at 37˚C, 50 μl colchicine (0.1 mg/ml) was added in culture dish and incubated at 37˚C for 60 min. Subsequently, the samples were centrifuged at 200 x g for 10 min at room temperature, the supernatant was removed and then 5 ml 0.075 M KCl was added. Following this, the samples were incubated for 20 min at 37˚C, followed by centrifugation at 200 x g for 10 min at room temperature. The supernatant was discarded and cells were resuspended in 1 ml cold fixative (methanol: Acetic acid, 3:1), incubated for 0.5 h at room temperature, and washed three times at 200 x g for 5 min each at room temperature. Finally, cells were suspended in 3 drops of the fixative. Slides were prepared by dropping a suspension of the pellet onto clean glass slides and dried at 37˚C for 10 min. Giemsa staining was performed for 30 min at room temperature, then it was examined under a light microscope (x100 magnification), followed by G-banding for analysis. Primarily, 20-30 good-quality (The mitotic phase was complete and independent and the chromosome of intact cells was observed) metaphases were screened. Chromosome abnormalities were described according to the standard of The International System for Human Cytogenetic Nomenclature (10).

Detection of the Janus kinase (JAK2)V617F mutation. Total DNA was isolated from bone marrow MNCs using the QIAamp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocols. DNA quality was assessed using a Bio-Rad Experion electrophoretogram instrument (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The purity of the DNA was determined by measuring the optical density of the 260/280 ratio using a NanoDrop spectrophotometer. DNA samples were then stored at -20˚C for further use. All DNA samples were amplified in the C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc.) using the Takara Premix Taq™ HS polymerase chain reaction (PCR) kit, UNG plus (Takara Bio, Inc., Otsu, Japan). In this system, a 50-μl reaction mixture contained the following components: 0.25 μl Takara TaqHS DNA polymerase, 5 μl 10X PCR buffer for UNG plus, 4 μl dU plus dNTP mixture, 0.5 μl UNG, 1 μl each 20 pmol/μl primer, 2 μl genomic DNA (200 ng) and 34.25 μl diethyl pyrocarbonate-treated water. The mixture was amplified at 94˚C for 4 min and subsequently subjected to 36 cycles of 94˚C for 45 sec, 60˚C for 45 sec and 72˚C for 45 sec, and then extended at 72˚C for 8 min. Patients and controls were genotyped by a DNA tetra-primer Amplification Refractory Mutation Screening (ARMS) assay (11), a method that uses

2 primer pairs to amplify the normal and mutant sequences plus a positive control band specifically in a single reaction. Primers included mismatches to maximize discrimination of the two alleles (in lowercase) and mutant/wild-type-specific bases (underlined). The PCR primers were as follows: Forward outer (FO), 5'-TCCTCAGAACGTTGATGGCAG-3'; reverse outer (RO), 5'-ATTGCTTTCCTTTTTCACAAGAT-3'; forward wild-type-specific (Fwt), 5'-GCATTTGGTTTTAAATTATGGAGTATaTG-3', and reverse-mutant-specific (Rmt), 5'-GTTTTACTTACTCTCGTCTCCACAaAA-3' (The subsequent amplicons were electrophoresed on a 1.5% agarose gel and visualized following staining with ethidium bromide. The samples were visualized with a gel imaging system (Tanon 2500R; Tanon Science & Technology Co., Ltd. Shanghai, China).

Results

Clinical characteristics and survival time. Among all the patients with AMKL, the white blood cell (WBC) count of 3 patients was normal, while 4 patients had a low WBC count and 2 patients had an increased WBC count. A total of 8 patients exhibited different degrees of anemia, 6 patients exhibited elevated lactate dehydrogenase levels, 6 patients exhibited hyperpyrexia and 6 patients exhibited bleeding from the skin or the mucosa. Peripheral blasts were observed in 6 patients, with a range of 2-30% (Table I).

The group consisted of 3 patients who exhibited marked splenomegaly. Additionally, patient no. 4 underwent splenectomy at diagnosis. A total of 2 patients exhibited superficial lymphadenopathy, 1 patient exhibited hepatomegaly (patient no. 6 underwent splenectomy due to trauma at diagnosis) and 1 patient exhibited sternal tenderness (Table I).

All patients were treated with cytarabine and daunorubicin/idarubicin. A total of 4 patients (patient nos. 3-6) achieved complete remission, 1 patient (patient no. 7) achieved partial remission and the remaining 4 patients exhibited no response. The median overall survival time of all patients was 6 months (range, 1.1-24.0 months).

Bone marrow smear and bone marrow cellularity. On the bone marrow smears, irregular forms of megakaryoblasts, pseudopodia or flocculence were frequently observed around the edge of the cytoplasm, which was occasionally accompanied by platelet aggregation (Fig. 1). Patient nos. 3 and 6 exhibited a notable increase in megakaryoblasts, as demonstrated by bone marrow aspiration, and the percentage of megakaryoblasts was 24 and 25%, respectively. For the other patients, it was not possible to accurately identify the cells as megakaryoblasts by observing cell morphology, therefore, the percentage of megakaryoblasts was reported as 0%. The bone marrow cellularity of these 2 patients (patient nos. 3 and 6) was moderately hypocellular or hypercellular, respectively (Table II). The percentage of bone marrow blasts in 2 patients (patient nos. 4 and 9) was <5%, while it was >30% in 4 patients and it ranged from 20-30% in 3 patients (Table II).

Bone marrow biopsy and immunohistochemical staining. The bone marrow cellularity was also evaluated with a bone marrow biopsy. A total of 6 patients exhibited moderate or severe hypocellular bone marrow cellularity. The bone marrow cellularity of patient nos. 1, 2, 6 and 8 was severely, moderately, severely and moderately hypocellular, respectively, as demonstrated by bone marrow biopsy (Table III); however, the cellularity was hypercellular or moderate hypercellular, as indicated by bone marrow aspiration (Table II). The cellularity of the other patients was consistent between the results of the bone marrow aspiration and bone marrow biopsy. Gomori silver staining demonstrated that 7 patients had moderate or marked MF, and the remaining 2 patients were negative for the condition. Additionally, 7 patients exhibited the expression of platelet-specific antigens, including factor VIII, CD61, CD41 or CD42, as demonstrated by immunohistochemical staining (Fig. 2; Table III).

Immunophenotype. Immunophenotypic analysis was performed on all patients. The blast cells of 6 patients primarily expressed myeloid-specific antigens, including CD13 and CD33. A total of 3 patients primarily expressed platelet-specific antigens, including CD41 and CD61. The blast cell percentages of patient no. 4 and patient no. 9 were <20% by bone marrow smear analysis; however, the percentage of CD41- and CD61-positive cells was >90%, as demonstrated by flow cytometry. In 5 patients, the percentage of CD34-positive cells was >20% (Table IV).

Karyotypic analysis. A total of 6 patients exhibited an abnormal karyotype. Additionally, in 5 patients (5/6 patients) complex karyotypes were observed. The karyotypes of patient nos. 3 and 5 contained the translocation t(9;22)(q34;11.2) (Table V).

JAK2V617F mutation. Primers FO and RO flanked the JAK2 exon 12 and generated a control 463-bp band in all patients. Primers Fwt and RO produced a 229-bp wild-type-specific product, and primers FO and Rmt generated a 279-bp mutant-specific product. Patient no. 6 was positive for the JAK2V617F mutation (Fig. 3).

Discussion

AMKL is a rare subtype of AML comprising only ~1% of all AML cases (12,13). In China, a limited number of patients had been diagnosed with AMKL due to its low incidence. In the present study, only 1.4% of patients with AML were diagnosed with AMKL. In the clinic, AMKL may not be considered due to its low incidence. When clinicians diagnose AMKL, bone the marrow biopsy frequently exhibits proliferation of abnormal megakaryoblasts and extensive fibrosis (3). Immunohistochemical staining and flow cytometry frequently express platelet-specific antigens, including factor VIII, CD41, CD42 or CD61; however, no specific cytogenetic abnormalities are detected in patients with AMKL, including abnormalities of chromosomes 3 and 8, and translocations, such as t(8;17), t(1;5) and t(10;17), which have previously been reported in adult patients with AML-M7 (14-17).

In the clinic, patients may be diagnosed with AMKL upon finding >20% of blasts and >50% megakaryoblasts among all blasts, as determined by bone marrow aspiration; however, the diagnosis of AMKL is frequently challenging due to a high

Table I. Characteristics of patients with acute megakaryocytic leukemia.

Patient no.	Sex	Age, years	WBC count ($x10^9/l$)	RBC count ($x10^{12}/l$)	Plts ($x10^9/l$)	Hb, g/l	LDH, IU/l	Peripheral blasts, %	Fever	Bleeding	Splenomegaly	Hepatomegaly	Lymphadenopathy	Sternal tenderness	Induction regimen	Survival time, months
1	M	53	2.58	1.36	6.68	48.3	773	5	+	+	-	-	+	-	DA	3
2	M	58	2.53	1.68	42.4	50.3	138	15	+	+	-	-	-	+	DA	2
3	F	68	5.02	3.77	324	96.6	1392	30	+	+	6 cm below the rib	-	-	-	DA	24
4	M	66	13.7	1.7	460	73.3	1451	0	-	+	Splenectomy for splenomegaly, 1 year ago at diagnosis	-	-	-	Hu, DA	20
5	F	66	6.39	2.47	57.4	81.3	619	0	-	+	4 cm below the rib	-	-	-	IA	10
6	M	54	35.0	1.88	173	55.1	987	10	-	-	Splenectomy for trauma, 10 years ago at diagnosis	5 cm below the rib	-	-	Hu, IA	13
7	M	58	2.04	3.84	181	121	180	2	+	-	-	-	+	-	DA	6
8	M	53	3.62	2.0	118	56.3	476	2	+	-	-	-	-	-	DA	3
9	F	60	6.71	3.21	72.4	77.1	190	0	+	+	-	-	-	-	IA	1.1

+, positive; -, negative; DA, daunorubicin and cytarabine; IA, idarubicin and cytarabine; Hu, hydroxyurea; M, male; F, female; WBC, white blood cell; RBC, red blood cell; Plts, platelets; Hb, hemoglobin; LDH, lactase dehydrogenase.

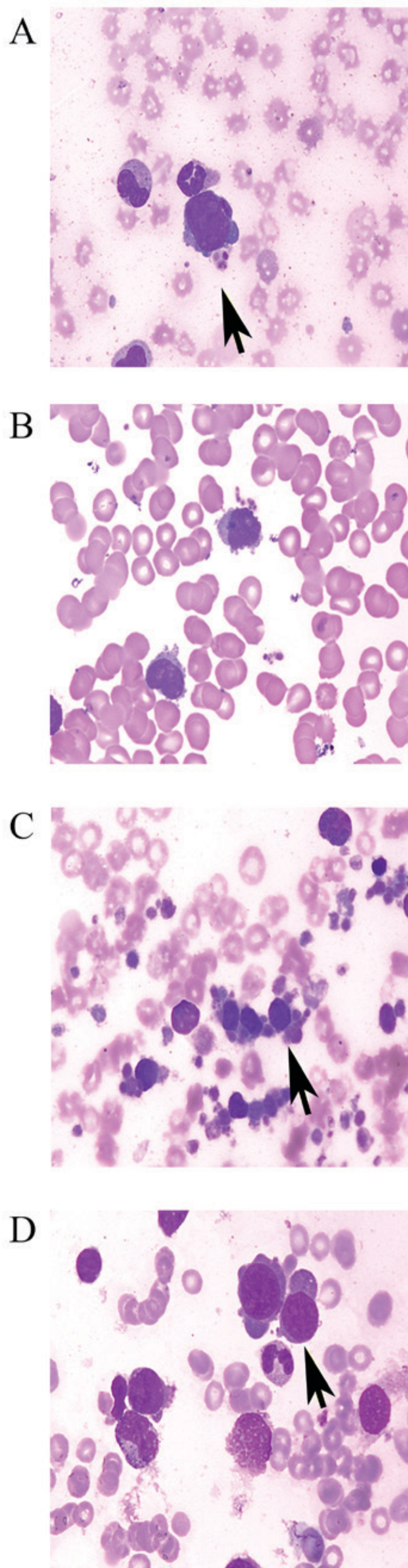

Figure 1. Cell morphology observation of bone marrow smears. Megakaryoblasts often displayed different cell morphology in patients with AMKL (A) Megakaryoblasts frequently present with a cytoplasmic budding and groups of aggregated platelets, which also present with (B) marginal flocculence and (C) production of large platelets. The amount of cytoplasm is variable, frequently with (C) a pale blue coloration or (D) mauve staining pattern of the granules. The nucleus is round, oval or irregular in shape, and fine-granular heterochromatin is also observed.

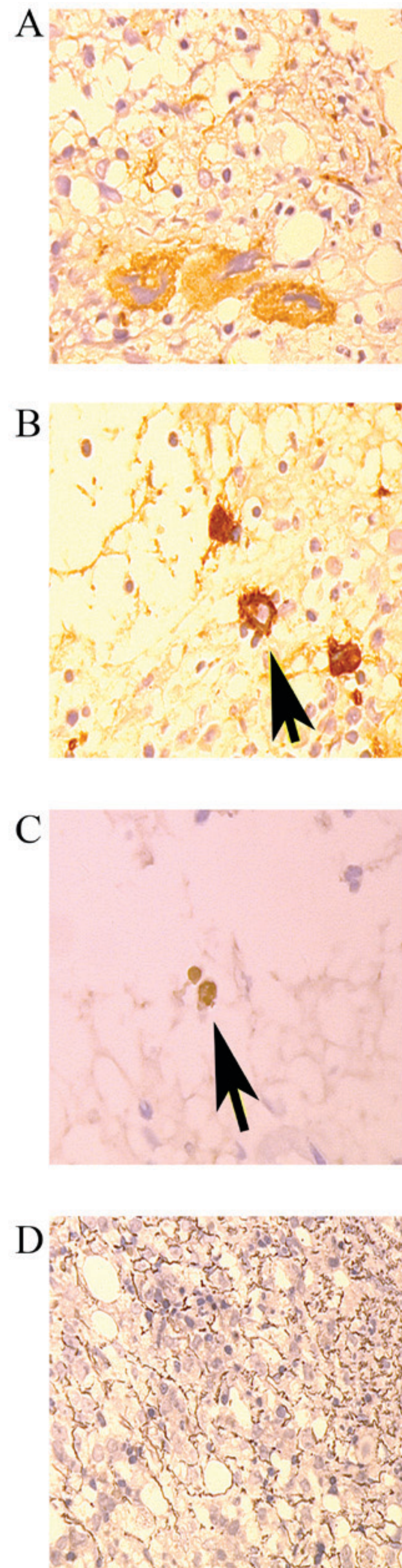

Figure 2. Immunohistochemical staining of bone marrow biopsy. (A) Immunocytochemical staining for factor VIII (magnification, x60) demonstrates that the expression of the marker is frequently positive. (B) The cell indicated by the arrow displays distinct blebs of the cytoplasm or pseudopod formation, and glycoprotein IIb/IIIa (CD41) (magnification) is positive. (C) The two cells indicated by the arrow are large cells present with irregular shapes, the small cell has a high nuclear-cytoplasmic ratio resembling lymphoblasts. The glycoprotein IIIa (CD61) (magnification, x100) staining of these cells is also positive. (D) Gomori staining frequently reveals myelofibrosis in patients with AMKL (magnification, x60). AMKL, acute megakaryocytic leukemia; CD, cluster of differentiation.

Table II. Bone marrow smear and cytochemical staining.

Patient no.	Bone marrow cellularity	Marrow blasts, %	Megakaryoblasts, %	No. of megakaryocytes	POX	PAS	NAE	SBB	NCE
1	Hypercellular	30.0	0	12	-	+++	+++	N/A	N/A
2	Hypercellular	60.5	0	6	-	+++	++~+++	-	-
3	Moderately hypocellular	28.0	24	>500	-	+++	++	-	-
4	Severely hypocellular	2.0	0	38	++	N/A	N/A	++	++
5	Hypercellular	31.0	0	89	-	-	+++	N/A	N/A
6	Hypercellular	29.0	25	>300	N/A	N/A	N/A	N/A	N/A
7	Hypercellular	59.0	0	21	-	+++	++	-	-
8	Moderately hypercellular	22.0	0	14	-	++++	++	-	-
9	Hypercellular	3.5	0	143	N/A	N/A	N/A	N/A	N/A

N/A, not performed; POX, Peroxidase; PAS, Periodic Acid- Schiff; NAE, Alpha-Naphythol/Acetate Esterase; SBB, Sudan Black B; NCE, naphythol AS-D chloroacetate esterase.

Table III. Bone marrow biopsy and immunohistochemical stain.

Patient no.	Bone marrow cellularity	MF	Immunohistochemical stain
1	Severely hypocellular	0	$FVIII^{+}/CD61^{+}$
2	Moderately hypocellular	0	$FVIII^{+++}/CD42^{+}/CD61^{+}$
3	Moderately hypocellular	2	$FVIII^{+}/CD61^{+}$
4	Severely hypocellular	3	N/A
5	Hypercellular	3	$FVIII^{+}/CD42^{+}/CD61^{+}$
6	Severely hypocellular	2	N/A
7	Hypercellular	3	$FVIII^{+}/CD42^{+}/CD61^{+}$
8	Moderately hypocellular	2	$FVIII^{+}/CD41^{+}/CD42^{+}/CD61^{+}$
9	Hypercellular	3	$FVIII^{+}/CD61^{+}/CD42^{-}$

FVIII, factor VIII; N/A, not performed; MF, marrow fibrosis; CD, cluster of differentiation.

incidence of MF, resulting in the failure of the bone marrow aspiration and inconsistency between aspiration and biopsy. In the present study, the bone marrow cellularity of patient nos. 1, 2, 6 and 8 was hypocellular to different degrees upon bone marrow biopsy; however, the cellularity was hypercellular upon bone marrow aspiration. Primarily, in other types of AML, if the bone marrow aspiration demonstrates hypocellular bone marrow cellularity, the result of the biopsy is frequently hypercellular; however, in AMKL, due to extensive MF, following bone marrow aspiration, the samples selected by biopsy are frequently insufficient, therefore the opposite result occurs.

In the present study, 7 patients presented with moderate or extensive MF, which frequently results in 'dry taps' and diagnosis inaccuracy in the morphological examination of bone marrow; thus, in these 7 patients with MF, it was difficult to obtain the exact number of blast cells. Therefore, >20% blasts and >50% megakaryoblasts among the blasts were not essential diagnostic criteria for AMKL, and it was necessary to conduct a comprehensive diagnosis that depended on the results of bone marrow biopsy, flow cytometry and immunohistochemical staining. Furthermore, the diagnosis could not be confirmed under conditions where the percentage of blast cells was <20% (18). In the present study, the percentage of blast cells in patient no. 4 and patient no. 9 was <5%, and this outcome may be associated with severe MF, which is demonstrated with strong positive Gomori staining or dilution of bone marrow blood; however, flow cytometry demonstrated the percentage of blast cells was >20%, and the expression levels of CD41 or CD61 were significantly increased (both >90%). Immunohistochemical staining also demonstrated that the expression of factor VIII was notably positive in patient no. 9, therefore leading to a diagnosis of AMKL.

Due to the rare incidence of AMKL and cost-saving considerations, conventional antibody combinations for surface antigens in diagnosing AML do not include CD41 and CD61, which may result in a missed diagnosis of AMKL. The 9 patients in the present study were not tested for the antigens of CD41 and CD61 at first, which made the diagnosis challenging depending on the results of cell morphology only. Finally, only 4 patients (patient nos. 1, 4, 6 and 9) underwent detection of expression levels of CD41 and CD61 by flow cytometry, and these patients were diagnosed with AMKL. The remaining 5 patients did not have data available to determine the

Table IV. Immunophenotype of patients with acute megakaryocytic leukemia.

Patient no.	CD2, %	CD3, %	CD4, %	CD7, %	CD10, %	CD13, %	CD14, %	CD19, %	CD33, %	CD34, %	CD41, %	CD61, %	CD64, %	MPO, %	CD117, %	HLA-DR, %
1	2	6	39	44	2	63	19	2	88	0	17	15	24	28	12	46
2	0	0	0	0	57	44	0	0	54	0	N/A	N/A	0	84	17	15
3	2	0	2	3	2	10	5	0	62	62	N/A	N/A	22	29	9	60
4	0	0	1	0	0	0	1	0	2	22	99	99	0	0	0	5
5	0	0	30	78	2	89	2	0	95	88	N/A	N/A	10	4	0	9
6	0	2	7	0	0	3	0	0	17	24	92	92	0	5	8	10
7	0	0	0	0	0	0	0	0	89	0	N/A	N/A	0	2	13	52
8	0	0	0	2	39	32	0	1	80	0	N/A	N/A	64	81	12	16
9	0	0	0	0	0	0	0	0	2	40	98	98	0	6	3	3

N/A, not performed; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-antigen D related; MPO, myeloperoxidase.

expression levels of CD41 and CD61 antigens as they were not requested by the clinicians. In these 5 cases, the final diagnosis of AMKL depended on bone marrow biopsy and immunohistochemical staining. We suggest that when immunophenotypic analysis fails to diagnose AMKL, bone marrow biopsies should be analyzed by immunocytochemical staining for platelet-specific antigens, including factor VIII, CD41, CD42 or CD61. Compared with other types of leukemia, flow cytometry, bone marrow biopsy and immunocytochemical staining produce more notable results when patients are diagnosed with AMKL; thus, a comprehensive diagnosis in AMKL should be emphasized. We recommend that CD41 and CD61 analysis should be contained in the diagnosis of AMKL, if available.

Furthermore, clinical presentations may indicate the cause of AMKL and assist clinicians with diagnosing this type of disease accurately. In AMKL, 20-30% of patients frequently exhibit splenomegaly and hepatomegaly, without lymphadenopathy (12,13). In the present 9 patients, 3 exhibited splenomegaly, 1 exhibited hepatomegaly, possibly due to compensatory hepatomegaly (this patient underwent splenectomy for trauma 10 years ago prior to diagnosis), and 2 patients exhibited superficial lymphadenopathy. Patient no. 4 underwent splenectomy 1 year ago prior to diagnosis for unexplained splenomegaly and intolerable symptoms, and the diagnosis has now been confirmed as AMKL. It was considered that this patient may have had a history of myeloproliferative neoplasm (MPN). The patient was diagnosed with AMKL; however they may have had a history of MPN, meaning that AMKL may have transformed from MPN, therefore, they were diagnosed with secondary AMKL.

Notably, chromosomal karyotypic analysis may also indicate the cause of AMKL. Patient nos. 3 and 5 exhibited translocation of chromosomes 9 and 22, t(9;22), which primarily occurs in chronic myeloid leukemia (CML) (19). In patients with AMKL, the t(9;22) chromosomal translocation may be exhibited in the acute stages of CML (20). Patient nos. 3 and 5 had no prior history of CML, and it is possible that CML was not previously diagnosed; thus, the diagnosis may be the blast crisis of CML. In the clinic, primary AMKL with Philadelphia chromosome is rare; therefore, this type of disease is frequently considered as secondary AMKL (21). Secondary AMKL is frequently reported from the transformation of CML, essential thrombocytosis (ET) and primary MF (PV) (20,22,23). In the present study, 2 patients exhibited the Philadelphia chromosome (patient nos. 3 and 5), and patient no. 4 had a history of splenomegaly, which may be associated with MPNs. Furthermore, it was considered that the proportion of secondary AMKL was notably high. Additionally, all patients also underwent the detection of the JAK2 gene mutation, for which patient no. 6 was positive. This patient underwent a splenectomy due to trauma 10 years ago at diagnosis, and whether or not the spleen was enlarged at the time was unclear. However, JAK2V617F is frequently exhibited in ET and PV (11,24,25), but not in AMKL, according to at least one report (26). Thus, it was speculated that the transformed AMKL of this patient was secondary to MPN.

In previous reports, patients with AMKL have short survival times and a poor prognosis, with median survival times that range from 5-10 months (12,13,15,27). In the 9 patients in the present study, the median survival time

Table V. Karyotype of patients with acute megakaryocytic leukemia.

Patient no.	Karyotype
1	73,XYY,+2,der(5)t(5;19)(q10;q10),+der(5)t(5;19),-6,-7,+8,+8,-9,-11,-13,+15,+15,+15,-16,18,+19,+mar1x2[6]/ 74,idem,+add(5)(q10)[3]/73,idem,-6,+8,+11,der(8;17)(q10;q10)[3]/ 72,idem,der(8;19)(q10;p10),+13,-15,del(20)(q11.2q12)[2]/ 74,idem,t(8;8)(q13;p13),+13[3]/ 46,XY,der(4)t(4;9)(p16;q10),-9, der(20)(t9;20)(p10;q13.3),+mar2[2]/46,XY[5]
2	46,XY
3	46,XY,t(9;22)(q34;q11.2)[30]/92, idemx2[2]/184,idemx4[2]
4	46,XYdel(13)(q12q14)[20]
5	63,XXX,-4,-5,-7,-9,t(9;22)(q34;q11.2),+10,der(12;21)(p10;p10),-17,-18,-20,+21,der(22)t(9;22)[13]/ 63,sl,der(12;21),+add(12)(q11.1)[2]/65,sdl1,+12,-add(12),+21,+der(22)t(9;22)[5]
6	46,XY
7	46,XY
8	43,X,-Y,del(5)(q11.1),-13,add(14)(p11.1),add(15)(p11.1),add(16)(p13.3),-17,-18,-19,+mar1,+mar2[5]/46,XY[15]
9	44,XX,i(5)(p10),-7,-12,der(21)t(?;21)(?;q22)t(?;12)(?;q13)[10]/ 43,idem,-3,+add(12)(p11.1),add(14)(q32),-15,+21,-der(21)[10]

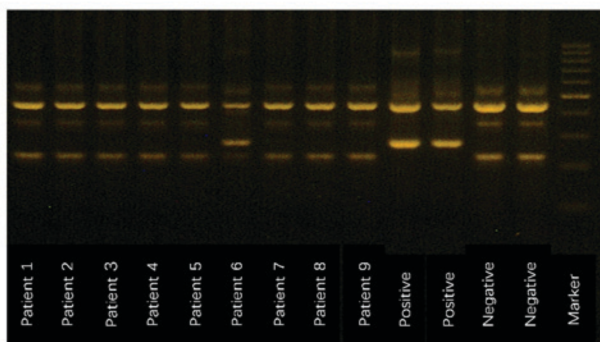

Figure 3. Amplification Refractory Mutation Screening assay to detect the JAK2V617F mutation in genomic DNA. Agarose electrophoresis analysis of the produced amplicons of the JAK2V617F gene mutation by reverse transcription-quantitative polymerase chain reaction. Patient no. 6 exhibited a mutant band, while other patients exhibited normal genotypes. JAK, Janus kinase.

was 6 months, which was consistent with the median survival time reported from other groups outside of mainland China (12,13,15,27). Thus, it was considered that the survival time may be associated with the high proportion of secondary AMKL, bone marrow fibrosis and a complex karyotype. If patients with AMKL receive a regimen of cytarabine combined with anthracycline, ~50% patients could achieve complete remission (15). Compared with traditional chemotherapy, allogeneic hematopoietic stem cell transplantation is a better choice in patients with complete remission, and benefits could be observed from this mode of therapy (28); however, the recurrence rate is high and the survival time is notably short (29). Novel treatment regimens require investigation for this type of disease in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JG and XW designed the experiments; GZ performed and analyzed most of the experiments; WW performed some experiments; GZ wrote the manuscript. All authors read and approved the final manuscript.

Ethical approval and consent to participate

All procedures performed in studies involving human participants were approved by the Ethics Committee of Huashan Hospital, Fudan University and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Informed consent was obtained from all individual participant included in the study.

Competing interests

The authors declare that they have no competing interests.

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