Furosemide-induced eradication of myeloblasts via the inhibition of tumor necrosis factor-α expression in a patient with acute biphenotypic leukemia: A case report

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Abstract. The present study reports the potential of furosemide therapeutic activity in acute myeloid leukemia. A 26-year-old man with acute biphenotypic leukemia was treated with furosemide for suspected pulmonary edema, which was later deemed to be an infiltration of leukemia cells. Notably, the myeloblast population was rapidly eliminated during furosemide therapy. Bone marrow specimens biopsied at different time points were used for immunohistochemical analysis of the expression levels of tumor necrosis factor-α (TNF-α) and its two receptors, TNF-α receptors 1 and 2. The expression of TNF-α and its receptors in the bone marrow was markedly suppressed by furosemide, along with the elimination of the myeloblasts. Thus, it was hypothesized that the growth of myeloblasts in the patient depended on autocrine and/or paracrine TNF-α stimulation, whereas furosemide disrupted this positive feedback loop. Therefore, furosemide is suggested as an effective therapeutic agent for acute myeloid leukemia, at least as an adjunct to standard chemotherapy and gene-targeted therapy.

Introduction

Tumor necrosis factor-α (TNF-α) is a major inflammatory cytokine, which was originally noted as an agent capable of inducing hemorrhagic necrosis of cancer and was subsequently found to have a paradoxical tumor-promoting function (1). Although the role of this highly pleiotropic cytokine in hematological malignancies remains controversial, due to the fact that its downstream signaling pathway can lead to either apoptosis or proliferation of hematopoietic progenitors, anti-TNF-α therapy has been suggested as a potential modality against acute myeloid leukemia (AML), based on the finding that the mean serum levels of TNF-α in patients with AML were higher than those in healthy people (2). Due to the development of various novel anti-TNF-α approaches that have shown considerable adverse effects, including headaches, rashes, anemia, transaminitis, infections and lymphoproliferative disorders (3,4), in the fields of autoimmune disease and oncology, researchers may neglect the fact that a common diuretic, furosemide, has a marked efficacy in reducing the action of TNF-α (5). The present study describes the case of a patient with acute biphenotypic leukemia whose myeloblasts may have been completely eliminated solely by furosemide treatment without the aid of chemotherapy via the suppression of certain presumed autocrine and/or paracrine TNF-α feedback loops.

Case report

Patient. A 26-year-old man who had been ill with SARS-CoV-2 infection in May 2023 still presented with a fever after the other symptoms of COVID-19 had resolved in June 2023. The patient was admitted to the Emergency Department in Chung Shan Medical University Hospital (Taichung, Taiwan) in June 2023, and laboratory examinations of a peripheral blood sample revealed a white blood cell count of 48,290/μl (reference range, 4,000-11,000/μl), with blasts of up to 69.2% (reference range, 0%), composed of both large myeloblast-like and small lymphoblast-like cells (Fig. 1A and B). A hemoglobin level of 8.4 g/dl (reference range, 13-17 g/dl) and a thrombocyte count of 66,000/μl (reference range, 150,000-400,000/μl) were noted at the same time.

After admission, a bone marrow aspiration and biopsy were performed on the left posterior superior iliac crest on day 2 of hospitalization. The smear revealed hypercellular marrow, with

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>90% of the nucleated cells being blasts (Fig. 1C). The initial pathological study of the biopsy specimen led to a diagnosis of acute leukemia, with the blasts staining strongly positive for myeloperoxidase (MPO), CD34 and CD79a, and focally positive for CD117. The initial flow cytometry analysis of the peripheral blood cells was performed using the EuroFlow acute leukemia orientation tube (ALOT) panel (Cytognos™; catalog no. CYT-A LOT). The flow cytometry was conducted on a BD FACSLyric flow cytometer (BD Biosciences), and data were analyzed using BD FACSuite v1.5.0.923 software (BD Biosciences). The co-expression of both myeloid and B-lymphoid markers favored a diagnosis of acute biphenotypic leukemia (Fig. 2A). Further flow cytometric analysis using the EuroFlow B-cell precursor acute lymphoblastic leukemia (BCP-ALL) panel (Cytognos) identified the following blast characteristics: $CD19^+$, terminal deoxynucleotidyl transferase $(TdT)^+$, $CD10^+$ (weak), $CD20^-$, $CD22^+$, $CD33^+$, $CD13^+$, $CD123^+$ and MPO^+ (Fig. 2B and C). The positivity of CD19, TdT, CD10 and CD22 is compatible with a B-lymphoid lineage. The positivity of CD33, CD13 and MPO is compatible with a myeloid lineage. The positivity of CD123 is compatible with both B-lymphoid and myeloid origins (6). Chromosomal analysis revealed a normal male karyotype without any recognizable abnormalities. Notably, next-generation sequencing or specific fusion gene detection was not performed for this patient during the initial diagnostic workup. Thus, the molecular risk stratification of the leukemia cannot be clearly determined accordingly. The importance of these analyses is acknowledged and they will be included in future cases to enhance the genetic characterization of similar patients.

The patient's fever subsided on day 3 of hospitalization after administration of strong broad-spectrum antibiotics [piperacillin + tazobactam, 4.5 g intravenous drip (ivd) every 8 h from days 1 to 4; cefepime, 500 mg ivd every 8 h from days 2 to 4; and fluconazole, 100 mg ivd daily from days 2 to 4]. Total leukocyte and absolute blast counts decreased to 30,220/μl (reference range, 4,000-11,000/μl) and 41.4% (reference range, 0%), respectively, after adequate intravenous hydration on day 3. Nonetheless, diffuse alveolar infiltration over the bilateral lower lung fields occurred on the same day after implantation of the port-A catheter, as shown by chest X-ray examination (Fig. 3). The patient soon developed dyspnea and respiratory distress.

Besides oral allopurinol (200 mg daily) and intravenous sodium bicarbonate (49.8 mEq every 4 h) given since day 2 of hospitalization, intravenous administration of 40 mg furosemide every 8 h was started on day 4 of hospitalization to treat the suspected pulmonary edema, despite the sparing of the bilateral upper lung fields in the initial stage of pulmonary infiltration. Pneumonia was considered a less likely cause; however, antibiotics were already in use. Pulmonary hemorrhage could not be ruled out, although there was no evidence of hemoptysis. Using frequent platelet transfusions, an attempt was made to maintain a thrombocyte count of >30,000/μl.

Despite continuous treatment with furosemide, the pulmonary infiltration worsened on day 5 of hospitalization (Fig. 3). However, with persistent furosemide therapy at the same dose as before, the white patches in the lung began to resolve on day 8 of hospitalization (Fig. 3), and the lung fields became clear on day 11 of hospitalization (Fig. 3).

Despite the pulmonary problem preventing the prescription of induction chemotherapy, it was noted that the patient's peripheral leukocyte counts and the percentage of blasts concurrently decreased at a rapid speed during the period of furosemide treatment without the use of any chemotherapeutic agents (Fig. 3). Nevertheless, the peripheral blood white cell counts and blast percentage did not decrease further and remained at a stable level (leukocytes, 1,610-2,020/μl; blasts, 6.9-19%) from day 10 to day 17 of hospitalization (Fig. 3).

A second bone marrow examination was performed on day 16 of hospitalization. The smear showed that the marrow space was still filled with blasts (Fig. 1D). However, the size of the blasts appeared to be smaller and the cytoplasm was less abundant than that detected at the previous examination. While awaiting the pathological study results of the second bone marrow biopsy, the patient, who now had a provisional diagnosis of acute myeloid leukemia (AML) based on traditional morphology and immunohistochemical staining, began treatment with a standard AML induction regimen comprised of cytarabine (200 mg/m^2 daily for 7 days) and idarubicin (12 mg/m^2 daily for 3 days), on day 19 of hospitalization for one cycle (Fig. 3). The second bone marrow biopsy result, however, led to a diagnosis of acute lymphoblastic leukemia (ALL) corresponding to a pro-B CD10-positive type, with >90% blasts staining positive for CD34, CD10, CD79a and TdT upon immunohistochemical analysis. By contrast, only 5% of blasts stained positive for MPO upon immunohistochemical analysis.

After the peripheral hematological profile of the patient achieved satisfactory recovery, with absolute neutrophil counts of >500/μl, a third bone marrow biopsy on day 29 of hospitalization revealed that the residual lymphoblasts still occupied ~20% of the nucleated marrow cells. Therefore, the patient was treated with the rituximab-hyper-CVAD protocol (cycle 1: Rituximab, IV 375 mg/m^2 on days 1 and 8; cyclophosphamide, IV 300 mg/m^2 every 12 h x 6 doses on days 1-3; mesna, IV 300 mg/m^2 on days 1-3; doxorubicin, IV 50 mg/m^2 on day 4; vincristine, IV 2 mg on days 1 and 8; dexamethasone, IV 40 mg on days 1-4 and days 11-14; and intrathecal methotrexate, 12 mg on days 2 and 8. Cycle 2: Rituximab, IV 375 mg/m^2 on days 1 and 8; methotrexate, IV 750 mg/m^2 on day 1; cytarabine, IV 2 g/m^2/dose every 12 h x 4 doses on days 2 and 3; methylprednisolone, 50 mg every 12 h x 6 doses on days 1-3; and intrathecal cytarabine, 100 mg on days 2 and 8) for ALL on day 58 of hospitalization. The patient failed to achieve complete remission after cycles 1 and 2 of rituximab-hyper-CVAD treatment and was subsequently transferred to another tertiary hospital for rescue therapy with inotuzumab ozogamicin (IV 0.8 mg/m^2 on day 1, 0.5 mg/m^2 on days 8 and 15, then rest for 7 days, for a total of 2 cycles). The patient proceeded to receive an allogeneic hematopoietic stem cell transplantation, with his father as the donor, upon achieving complete remission. The patient is currently followed up on a weekly basis at the Outpatient Clinic due to acute graft-vs.-host disease. The prognosis is poor in general for this patient; nevertheless, they are currently free from leukemia.

As discussed later, a retrospective immunohistochemistry study of the patient's bone marrow biopsy specimens was performed after the patient was transferred to another hospital for investigating the possible underlying mechanism

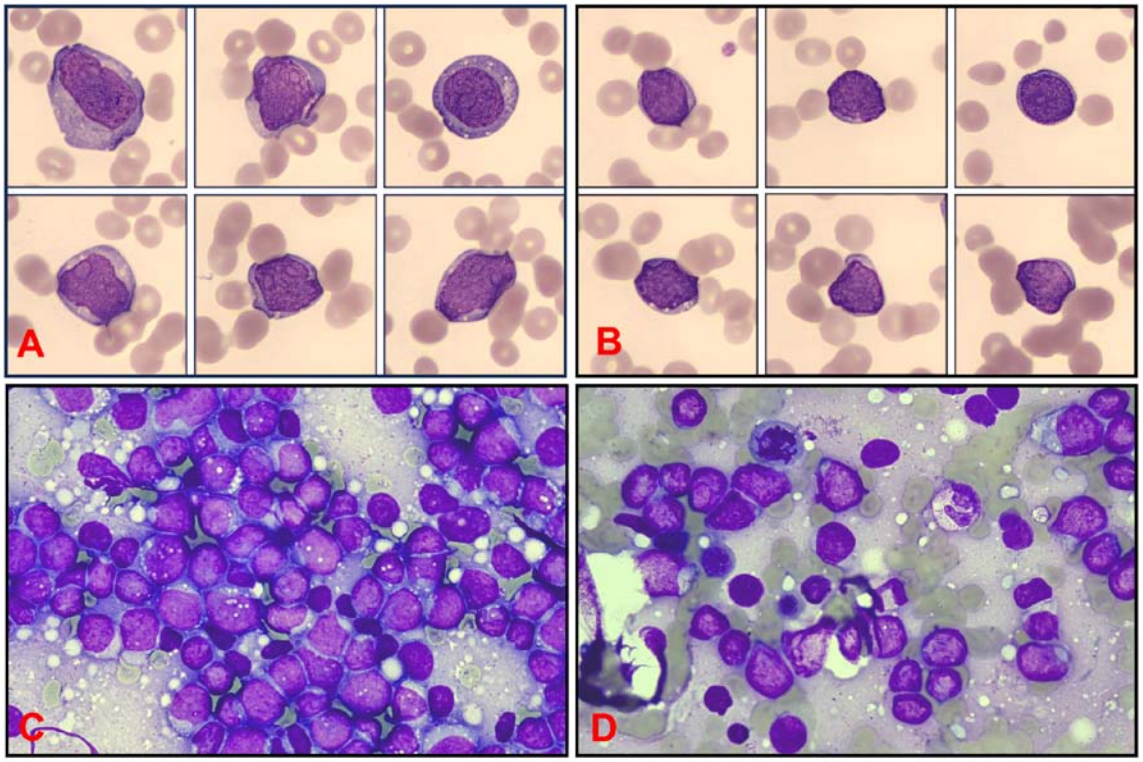

Figure 1. Leukemia cells in the peripheral blood (Wright-Giemsa stain; x1,000 magnification) and bone marrow (Liu's stain; x1,000 magnification). (A) Myeloblast-like cells and (B) lymphoblast-like cells in the peripheral blood at presentation on day 2. (C) Bone marrow smear full of blasts at presentation on day 2. (D) Bone marrow smear with residual blasts after furosemide treatment on day 16.

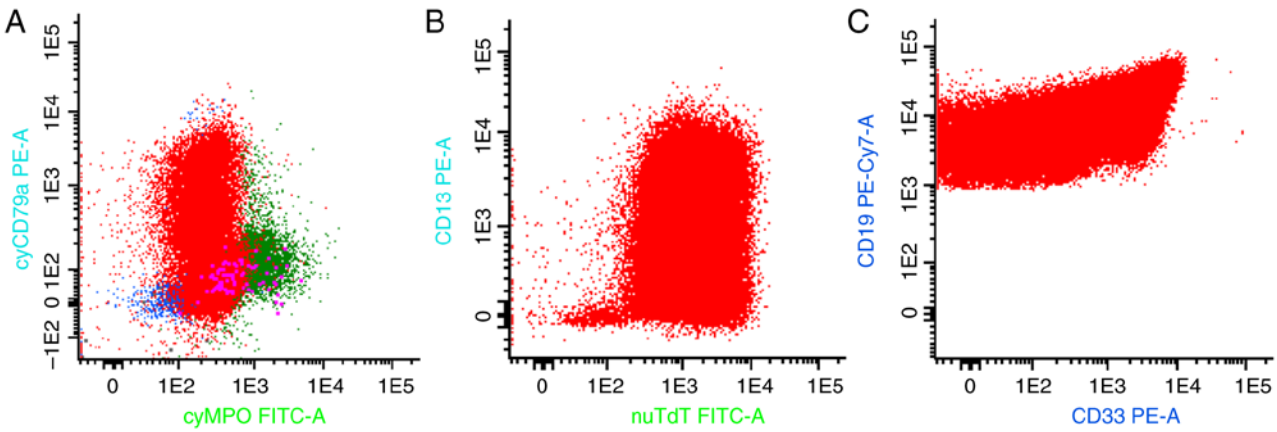

Figure 2. Key results of the flow cytometry analysis of the peripheral blood pathological cells (red dots) leading to the diagnosis of acute biphenotypic leukemia using the EuroFlow acute leukemia orientation tube and B-cell precursor acute lymphoblastic leukemia panels. Leukemia cells were positive for both myeloid and B-lymphoid markers in (A) MPO and CD79a, (B) CD13 and TdT, and (C) CD33 and CD19. Green dots represent granulocytes, blue dots represent lymphocytes and pink dots represent monocytes. MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase; cy, cytoplasm; nu, nucleus; PE-A, phycoerythrin area; FITC-A, fluorescein isothiocyanate area; PE-Cy7-A, phycoerythrin-cyanine7 area.

by which furosemide might have eradicated myeloblasts. The targets of interest were TNF-α and its receptors. The research procedure is listed later. Notably and with compatibility with our assumption, the expression of TNF-α and its two types of receptor was much decreased in the bone marrow after furosemide treatment. This indicates that the growth of the patient's myeloblasts may be dependent on certain autocrine and/or paracrine TNF-α feedback loops and that furosemide had opportunely interrupted the loops. More detailed arguments will be presented in the subsequent discussion.

Immunohistochemical (IHC) staining for MPO, CD79a, CD117, CD34, TNF-α, TNF receptor (TNFR)1 and TNFR2. The bone marrow samples taken on a series of three separate dates as aforementioned were fixed in 10% neutral-buffered formalin at room temperature (22-25˚C) for 24 h, and embedded in paraffin. Subsequently, 4-μm sections were prepared for hematoxylin and eosin staining, and IHC analysis. For IHC staining, sections were deparaffinized in xylene, rehydrated through a graded series of ethanol, and permeabilized using 0.1% Triton X-100. Heat-induced epitope retrieval was then performed in citrate

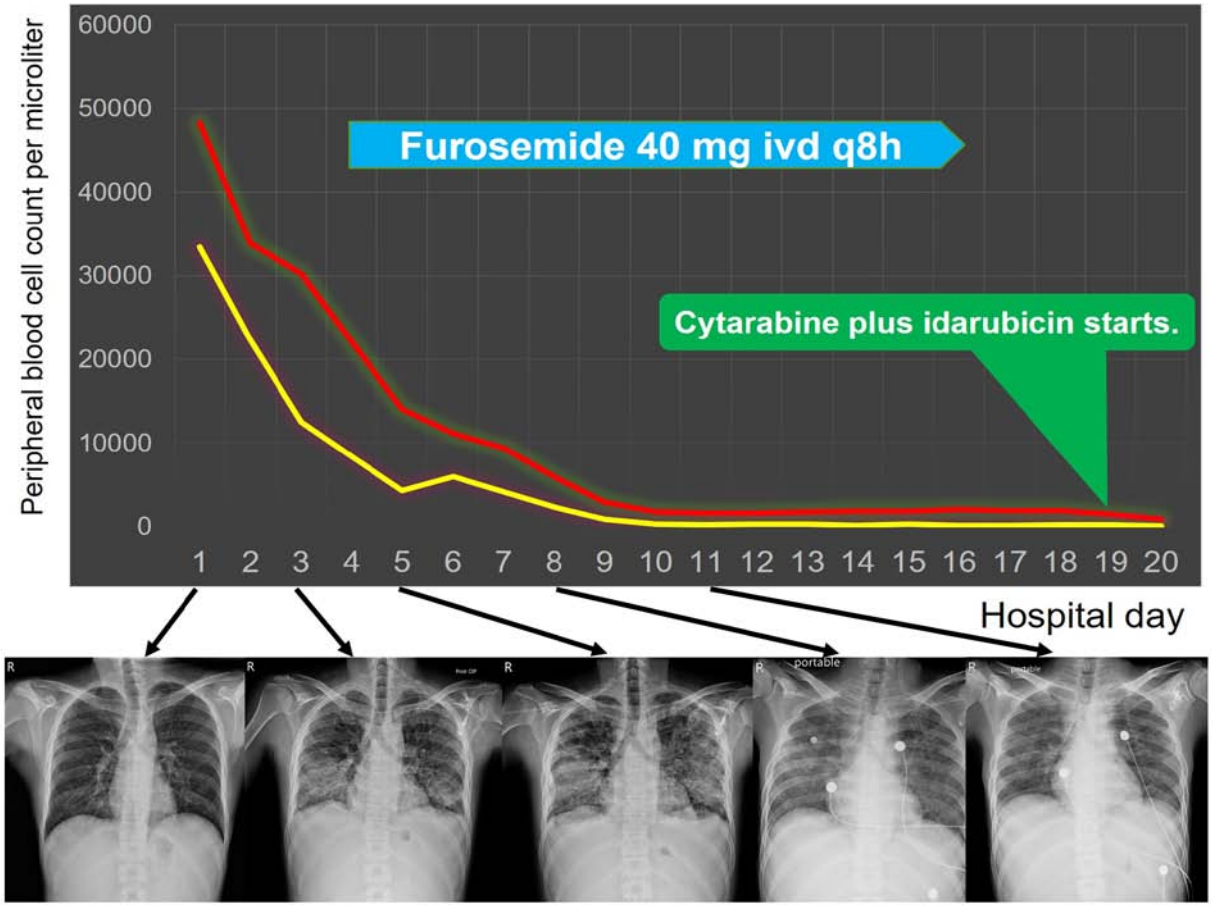

Figure 3. Peripheral blood total white cell counts (red) and absolute blast counts (yellow) during the clinical course. Furosemide treatment was prescribed from day 4 to day 16. Induction chemotherapy with cytarabine and idarubicin started on day 19. A series of chest X-ray films with day post-admission (indicated by black arrows) is presented at the bottom of the figure. q8h, every 8 h; ivd, intravenous drip.

buffer (pH 6.0) using a microwave. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol and the sections were incubated overnight at 4˚C with the following primary antibodies: Anti-TNF-α (cat. no. bs-2081R; Bioss), anti-TNFR1 (cat. no. ab19139; Abcam) and anti-TNFR2 (cat. no. GB111013; Wuhan Servicebio Technology Co., Ltd.). After washing, the sections were treated with a secondary antibody provided in the IHC stain kit (cat. no. TAHC04D; BioTnA) for 30 min. The signal was developed using 3,3'-diaminobenzidine and counterstained with hematoxylin (incubated for 10 sec at 25-28˚C). Staining was quantified using ImageJ software (Version 1.49; National Institutes of Health), with pixel intensity measured and significance determined. Images were captured at x400 magnification under a whole slide digital scanner (MoticEasyScan Pro; Motic Asia). For each sample, at least 5 random fields were selected. The staining intensity (measured in arbitrary units, AU pixels) were automatically detected and analyzed using ImageJ software. The average intensity from these fields were used to evaluate the expression of TNF-α, TNFR1 and TNFR2. Additionally, IHC staining for MPO (Polyclone, DAKO Omnis; Agilent Technologies, Inc.), CD79α (SP18, Thermo Fisher Scientific, Inc.), CD117 (EP10; Roche Tissue Diagnostics) and CD34 (QBEnd/10; Biocare Medical LLC) was performed using the Ventana Benchmark Ultra IHC system (Roc Roche Tissue Diagnostics) at Chung Shan Medical University Hospital (CSMUH). The automated staining procedure followed the manufacturer's protocols for deparaffinization, antigen retrieval, antibody incubation and detection. The results were interpreted by pathologists at CSMUH, ensuring consistency and accuracy in evaluating the expression of these markers. Quantification of IHC staining was determined in pixel intensity and presented as mean ± SEM. Statistical analysis was conducted using repeated measures ANOVA in GraphPad Prism (version 10.2.0; Dotmatics) to compare the means of different groups. Independent IHC quantitative results were obtained by randomly capturing pixel intensity from the indicated samples (n=5). Post hoc analysis was performed using a paired test with Bonferroni's correction to assess statistical significance between groups. $P<0.05$ was used to indicate a statistically significant difference.

Discussion

During the initial disease course, it was recognized that congestive heart failure with pulmonary edema would not happen so easily in a young man, that pulmonary hemorrhage would not disappear so rapidly and that there were no fever or other septic signs that supported the existence of pneumonia. Despite the lack of a lung biopsy and the fact that the diagnosis remained an assumption, it was determined that the initial pulmonary change detected in the patient described in the present case report was actually due to leukemic infiltration,

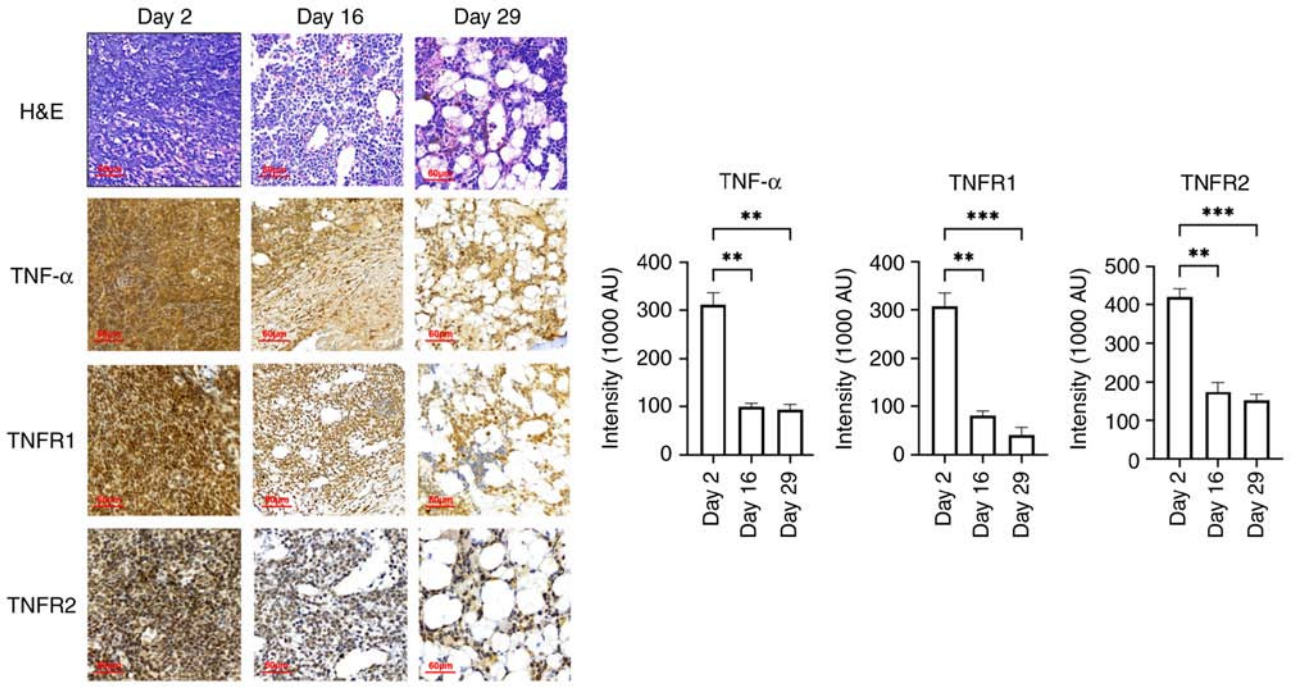

Figure 4. Expression of TNF-α and its receptors in bone marrow during the clinical course. Bone marrow biopsy specimens stained with H&E, and IHC studies for expression of TNF-α, TNFR1 and TNFR2 on day 2, 16 and 29 of hospitalization (scale bar, 60 μm). Quantification of IHC staining was determined in pixel intensity using ImageJ software (n=5; mean ± SEM). **P<0.01 and ***P<0.001. H&E, hematoxylin and eosin; IHC, immunohistochemical; TNF-α, tumor necrosis factor-α; TNFR, TNF receptor.

but that it was mistakenly treated as pulmonary edema with furosemide. Notably, all myeloblasts in the lung, peripheral blood and bone marrow were eliminated under furosemide treatment without the administration of any chemotherapy agents, including hydroxyurea and steroids. From the time of admission, the patient received orally administered allopurinol and an intravenous sodium bicarbonate infusion for the prevention of tumor lysis syndrome. Nevertheless, to the best of our knowledge, no literature has supported the anti-leukemia effects of these two drugs thus far. Although lymphoblast-like leukemia cells remained unaffected by furosemide, the mechanism by which furosemide eliminated the myeloblasts was unclear.

It has been recognized that dysregulation of pro- and anti-inflammatory cytokine networks may markedly influence the progression of AML, with TNF-α considered one of the main pro-leukemogenic stimulators (7). TNF-α mediates biological effects by acting on two different receptors, TNFR1 and TNFR2, while production of TNF-α relies on releasing its secretory form through proteolytic cleavage of the transmembrane form in macrophages, natural killer cells, T-lymphocytes and neutrophils (8). It was previously discovered that TNF-α could act synergistically with granulocyte/macrophage colony-stimulating factor to promote the proliferation of clonogenic cells of AML (9). Importantly, the leukemia-initiating cells of AML have been found to exhibit constitutive NF-κB activity, which is maintained via autocrine TNF-α secretion, forming an NF-κB/TNF-α positive feedback loop (10). In a clinical study of patients with AML, the serum levels of TNF-α were shown to be significantly higher at diagnosis compared with those in control samples, and were considerably reduced after induction chemotherapy, highlighting the autocrine-facilitating roles of TNF-α in AML (11).

As a well-known diuretic and antihypertensive agent, furosemide used at a high dose with hypertonic saline solution has been shown to be capable of lowering serum TNF-α levels in patients with congestive heart failure (12) or refractory ascites due to liver cirrhosis (13). Additionally, pharmacological doses of furosemide have been found to reduce TNF-α production by cultured peripheral blood mononuclear cells (PBMCs) and placenta samples both from women with preeclampsia and those with a normal pregnancy (14,15). Furthermore, it has been reported that furosemide not only inhibits TNF-α production by cultured PBMCs but also exhibits cytotoxic effects on the cells (16).

In the present study, it was therefore proposed that most myeloblasts at presentation were eradicated by furosemide through the inhibition of TNF-α production in leukemia cells, interrupting an autocrine and/or paracrine cytokine cycle, and consequently leading to cytotoxicity in the patient during the first 4 weeks of hospitalization. To test this hypothesis, IHC analysis was performed to assess the expression of TNF-α, TNFR1 and TNFR2 in bone marrow biopsy specimens obtained on days 2, 16 and 29 of hospitalization.

The results of IHC analysis are shown in Fig. 4. Leukemic cells were strongly stained for TNF-α, TNFR1 and TNFR2 at presentation on day 2. As hypothesized, after furosemide treatment, the expression of TNF-α, TNFR1 and TNFR2 was significantly decreased in the residual cells on days 16 and 29, which is consistent with the speculation that myeloblasts dependent on autocrine and/or paracrine stimulation by TNF-α were eliminated by furosemide.

Notably, it has previously been demonstrated in laboratory experiments that anti-TNF-α antibody treatment can

induce apoptosis of leukemia cells and that the effects may be enhanced when combined with an NF-κB inhibitor (17). During the experiment, this previous study found that the expression of Akt could be induced by either anti-TNF-α antibody or the NF-κB inhibitor, while a combination of both anti-TNF-α antibody and NF-κB inhibitor resulted in a further increase in the expression of Akt, indicating a possible role of Akt in the apoptotic process of leukemia cells treated with these two agents. Due to marked inhibitory effects on normal hematopoietic cells, the clinical use of NF-κB inhibitors has some limitations to be overcome. Therefore, attacking transmembrane TNF-α (tmTNF-α) on the surface of leukemia cells has been raised as a potential therapeutic modality for acute leukemia. A previous study discovered that an anti-tmTNF-α antibody mediated its AML-eradicating effects by affecting the expression of important genes involved in cell cycle progression, including proapoptotic genes Apaf1, Bad, Bax, Bcl-2, Fas and TRAIL, in addition to complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity (18).

Based on its anti-inflammatory efficacy through inhibition of the secretion of pro-inflammatory TNF-α, IL-6 and nitric oxide in lipopolysaccharide-stimulated microglia cells, furosemide has also been suggested as a useful molecule in the treatment of β-amyloid-induced neuroinflammation in Alzheimer's disease (19). Furthermore, inhaled furosemide was recommended as a potential therapeutic agent for controlling cytokine storm in SARS-CoV-2 infection, after scientists disclosed its capability for reducing TNF-α and IL-6 levels in experiments on macrophages stimulated by lipopolysaccharide (20). Thus, it is our hope that furosemide can be tested clinically for the treatment of AML in the near future, perhaps concurrently with an NF-κB inhibitor. Nonetheless, since furosemide is traditionally recognized as a diuretic, it may be difficult to persuade researchers involved in the field of hematological malignancies to conduct a clinical trial on this drug in AML; however, the present case report provides evidence for this innovative idea.

In summary, the present study describes the case of a notable 'spontaneous' disappearance of the myeloblasts in a patient with acute biphenotypic leukemia, which indicates the possibility that furosemide might have therapeutic effects on AML, at least as an adjunct to standard chemotherapy and gene targeted therapy. Further laboratory studies and clinical trials are thus warranted.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YWC performed the IHC analysis of TNF-α and its receptors. WRC performed the pathological study of bone marrow biopsies. YTL and FSF took care of the patient. YTL arranged the cytogenetic and flow cytometry examinations. FSF conceptualized the idea, collected clinical data, analyzed all investigation results and wrote the article. YWC and FSF confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Chung Shan Medical University, Taichung, Taiwan (approval no. CS1-24024).

Patient consent for publication

Written informed consent was obtained from the patient for the publication of this case report and any accompanying images.

Competing interests

The authors declare that they have no competing interests.

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