Received: 13 October 2023 | Revised: 1 December 2023 | Accepted: 26 February 2024

DOI: 10.1002/mc.23715

RESEARCH ARTICLE

Molecular Carcinogenesis WILEY

BATF promotes extramedullary infiltration through TGF-β1/Smad/MMPs axis in acute myeloid leukemia

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Abstract

Acute myeloid leukemia (AML) is one of the most prevalent types of leukemia and is challenging to cure for most patients. Basic Leucine Zipper ATF-Like Transcription Factor (BATF) has been reported to participate in the development and progression of numerous tumors. However, its role in AML is largely unknown. In this study, the expression and prognostic value of BATF were examined in AML. Our results demonstrated that BATF expression was upregulated in AML patients, which was significantly correlated with poor clinical characteristics and survival. Afterward, functional experiments were performed after knocking down or overexpressing BATF by transfecting small interfering RNAs and overexpression plasmids into AML cells. Our findings revealed that BATF promoted the migratory and invasive abilities of AML cells in vitro and in vivo. Moreover, the target genes of BATF were searched from databases to explore the binding of BATF to the target gene using ChIP and luciferase assays. Notably, our observations validated that BATF is bound to the promoter region of TGF-β1, which could transcriptionally enhance the expression of TGF-β1 and activate the TGF-β1/Smad/MMPs signaling pathway. In summary, our study established the aberrantly high expression of BATF and its pro-migratory function via the TGF-β1-Smad2/3-MMP2/9 axis in AML, which provides novel insights into extramedullary infiltration of AML.

KEYWORDS

acute myeloid leukemia, BATF, extramedullary infiltration, migration, MMPs, TGF-β1

1 | INTRODUCTION

Acute myeloid leukemia (AML), the most frequently encountered form of acute leukemia in adults, is an aggressive malignancy characterized by the abnormal clonal expansion of myeloid blast cells.[1] Approximately 21,000 diagnoses and 11,000 AML-related deaths are reported annually in the United States.[2] AML treatment strategies currently encompass chemotherapy, hematopoietic stem cell transplantation, and novel targeted drugs, such as FLT3, IDH1, and BCL2 inhibitors, which have considerably prolonged the survival of AML patients.[3] However, according to the National Cancer Institute Surveillance, Epidemiology, and End Results program, the

5-year overall survival (OS) of AML patients is merely ~30.5%.[2] Besides, the majority of patients finally relapse.[4] Therefore, there is an urgent need to explore the mechanisms underlying AML development and discover new targeted therapies.

Extramedullary infiltration (EMI) has been reported in 2.5%–9.1% of patients with AML.[5] Interestingly, other studies have reported rates as high as 11%.[6] EMI of AML can involve the central nervous system (CNS), brain, skin, lymph nodes, gastrointestinal tract, mediastinum, and other tissues and organs, as well as rare sites such as the lungs, adrenal glands, and breast.[7] Notably, the presence of EMI is considered a poor prognostic indicator of AML.[8] A number of genetic molecular abnormalities, including t(8;21), inv,[9] NPM1 mutations, and CD56 expression, are hypothesized to be associated with EMI.[10–12] However, the etiology behind EMI remains elusive, presenting clinical challenges in effectively treating patients with this phenotype.

BATF was first identified in a cDNA library from human B cells infected with the Epstein-Barr virus.[13] It is located on chr14q24.3 and contains 24,539 bases. BATF family is a subgroup of basic leucine zipper (bZIP) transcription factors, comprising BATF, BATF2, and BATF3. BATF is a nuclear bZIP protein belonging to the activator protein 1(AP-1)/activating transcription factor (ATF) superfamily, which has been demonstrated to negatively regulate AP-1/ATF.[14] Meanwhile, AP-1 can govern the differentiation of lineage-specific cells in the immune system and specifically mediate the differentiation of Th17 cells, follicular T helper cells (TfH), CD8+ dendritic cells, and the class-switch recombination of B cells.[15] BATF is abundantly expressed in hematopoietic cells, especially in T cells and B cells.[16] BATF protein predominantly heterodimerizes with JUNB and also forms heterodimers with JUN and JUND.[13] Recent studies have reported that BATF expression is upregulated in multiple cancers, including non-small cell lung cancer (NSCLC), anaplastic large cell lymphoma (ALCL), colorectal cancer, and breast cancer.[9,17–19] Moreover, it has been documented to promote cancer cell proliferation, migration, and invasion both in vitro and in vivo. For example, knockdown of BATF inhibited the proliferation of A549 NSCLC cells and promoted apoptosis.[17] Additionally, BATF facilitated colon tumor progression by promoting IL-17aIL-23RIL-6CD4$^+$ T cell expression.[9] However, its function in AML remains to be elucidated.

The TGF-β signaling pathway has been established to promote tumor metastasis in several solid tumors, including hepatocellular carcinoma, breast cancer, and lung cancer.[20–22] Malignant tumor cells secrete large amounts of TGF-β1 protein, which promotes the growth and spread of cancer cells and creates an immunosuppressive tumor microenvironment that inhibits the immune system's killing of cancer cells.[23] Importantly, upregulation and activation of TGF-β1 stimulates the transformation of epithelial to mesenchymal cells and enhances the metastatic invasive ability of cancer cells.[24]

In this study, BATF expression was upregulated in AML patients. Function-loss and -gain experiments indicated that BATF promoted the migratory and invasive capabilities of cells via the TGF-β1-Smad2/3-MMP2/9 signaling axis. Our study provides a new perspective on AML development and infiltration, with BATF being a promising therapeutic target for AML.

2 | MATERIALS AND METHODS

2.1 | Public databases

The databases used in the article include cBioPortal (https://www.cbioportal.org/), UCSC Xena (https://xenabrowser.net/), Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/), LinkedOmics (http://linkedomics.org/), Cistrome DB (http://cistrome.org/db/#/), and GTRD (http://gtrd20-06.biouml.org/).

2.2 | Expression analysis of BATF in AML

The data of 173 cases of AML patients were extracted from the TCGA-LAML project. The gene expression and clinical information of AML cases in TCGA-LAML (n = 173) and GSE37642 (n = 135) were downloaded from UCSC-Xena and GEO databases. The clinical characteristics of 173 AML patients from the TCGA database are listed in Supporting Information: Table S1.

2.3 | Prognostic evaluation of BATF in AML

Kaplan–Meier (K–M) survival curves were plotted in different data sets and different subgroups to analyze the relationship between BATF expression and survival. Univariate and multivariate Cox regression analyses were used to assess the value of several clinical factors for independent assessment of patient prognosis. The nomogram with independent prognostic factors was constructed using the "rms" package. The predictive performance of the nomogram was evaluated with calibration curves.

2.4 | Patients

The diagnosis and risk assessment of AML patients were made based on the National Comprehensive Cancer Network (2022 version 1). This study included bone marrow (BM) samples of 80 adult AML patients and peripheral blood of 23 healthy individuals from the Second Affiliated Hospital of Xi'an Jiaotong University between January 1, 2018, and August 31, 2021, after providing written informed consents. Individual nucleated cells of all samples were extracted and stored at −80°C. This study was in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University (Approval No. 2015186). The clinical information of the 80 AML patients is presented in Supporting Information: Table S2.

2.5 | Cell culture

Human embryonic kidney cells HEK-293T (cat. no. CRL-3216), human bone marrow stromal cells HS-5 (cat. no. CRL-3611), and human AML cell lines THP-1 (cat. no. TIB-202), U-937 (cat. no. CRL-1593.2), and HL-60 (cat. no. CCL-240) were obtained from the American Type Culture Collection (ATCC) cell bank. Human AML cell lines NB4 (cat. no. CL-0676) and KG-1 (cat. no. CL-0132) were obtained from Procell Life Science & Technology Co., Ltd. THP-1, U-937, NB-4, KG-1, and HL-60 cells were cultured in RPMI-1640 medium (cat. no. L210KJ, BasalMedia), while HEK-293T and HS-5 cells were cultured in DMEM medium (cat. no. L110KJ, BasalMedia) with 10% fetal bovine serum (cat. no. FBS-E500, NEWZERUM) and 100 U/mL penicillin/streptomycin (cat. no. 15140122, Thermo Fisher Scientific). All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

2.6 | Regents

TGF-β receptor inhibitor GW788388 (10 μM, cat. no. S2750, Selleckchem) and recombinant human TGF-β1 (10 ng/mL, cat. no. 100-21, PeproTech) were dissolved according to the manufacturer's instructions.

The antibodies included BATF (1:1000, cat. no. 8638S, CST), TGF-β1 (1:4000, cat. no. 21898-1-AP, Proteintech), Smad2 (1:3000, cat. no. 12570-1-AP, Proteintech), Smad3 (1:4000, cat. no. 66516-1-Ig, Proteintech), p-Smad2 (1:1000, cat. no. bs-3419R, Bioss), p-Samd3 (1:1000, cat. no. bs-3425R, Bioss), MMP2 (1:1000, cat. no. bs-4605R, Bioss), MMP9 (1:1000, cat. no. bs-41146R, Bioss), GAPDH (1:4000, cat. no. 60004-1-Ig, Proteintech), HRP-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibody (1:10,000, cat. no. SA00001, Proteintech), and CD45 (1:2000, cat. no. 20103-1-AP, Proteintech).

2.7 | RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was extracted by Trizol reagent (cat. no. P118-05, GenStara) following the manufacturer's instructions. RNA purity and concentration were determined using NanoDrop ND-1000 (Thermo Fisher Scientific). RNA was stored at −80°C. cDNA templates were synthesized by reverse transcription using a HiFi-Script cDNA Synthesis Kit (cat. no. CW2569, CWBIO) with random primers. The reaction conditions were 15 min at 42°C and 5 min at 85°C.

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using UltraSYBR Mixture (cat. no. CW0957, CWBIO). Comparative quantification was determined using the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method. The RT-qPCR reaction conditions were set as follows: 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. GAPDH was used as the internal quantitative reference. All primer sequences are synthesized by Tsingke Biotechnology Co., Ltd and the sequences were as follows:

BATF: Forward primer: GTTCTGTTTCTCCAGGTCC
Reverse primer: GAAGAATCGCATCGCTGC
TGF-β1: Forward primer: CTGCAAGTGGACATCAACGG
Reverse primer: AAGTTGGCATGGTAGCCCTT
MMP2: Forward primer: TACAGGATCATTGGCTACACACC
Reverse primer: GGTCACATCGCTCCAGACT
MMP9: Forward primer: AGACCTGGGCAGATTCCAAAC
Reverse primer: CGGCAAGTCTTCCGAGTAGT
GAPDH: Forward primer: CAAGGTCATCCATGACAACTTTG
Reverse primer: GTCCACCACCCTGTTGCTGTAG

2.8 | Transfection

Small interfering RNAs (siRNAs) were chemically synthesized by Ribobio. The siRNA sense sequences were as follows:

si-BATF#1: 5′-GCTCTACGCAAGGAGATCA-3′
si-BATF#2: 5′-CGCATTCCACCAACCTCAT-3′

Transient transfection of si-BATF into THP-1 and U-937 cells was performed by RFect^{SP} siRNA Transfection Reagent for suspension cell (cat. no. 11026, Baidai Biotechnology) according to the manufacturer's instructions.

Overexpression plasmid of BATF and negative control plasmid were obtained from GeneChem. CV702 plasmid was used to establish the BATF overexpression vector. LipoFiter™ Liposomal Transfection Reagent (cat. no. HB-TRLF-1000, HANBIO) was used to transfect plasmids into AML cell lines.

AML cells were infected with sh-BATF lentivirus and the control lentivirus (GeneChem). Stably transfected AML cells were selected with 2 μg/mL puromycin dihydrochloride (cat. no. 1299MG025, BioFroxx).

2.9 | Western blot

Cells were collected, washed three times with PBS, and lysed by adding RIPA lysis solution (cat. no. PE01, PIONEER) premixed with protease inhibitor PMSF for 15 min, followed by centrifugation at 4°C, 12,000*g* for 15 min, and the supernatant was aspirated. After quantitatively determining the protein concentration by BCA (cat. no. SB-WB013, ShareBio), the protein was heated at 100°C for 10 min for sufficient deformation and stored at −20°C. The cells were then extracted from the cells, and the supernatant was removed from the cells. Prepare 10% or 12.5% SDS-PAGE gel for electrophoretic separation. After the protein was transferred to the PVDF membrane (cat. no. 03010040001, MilliporeSigma), 5% skimmed milk was closed at room temperature on shaker for 1 h. The primary antibody was diluted and shaken overnight at 4°C. The membrane was washed with TBST 3 times for 15 min each time, and the secondary antibody was added and incubated at room temperature for 1 h. The membrane was washed with TBST three times for 15 min each time,

and blots were visualized with ECL Western blot analysis Substrate (cat. no. SB-WB012, ShareBio).

2.10 | Enzyme-linked immunosorbent assay

The concentration of bioactive TGF-β1 in cell culture media was tested by TGF-β1 Quantikine ELISA Kit (cat. no. VAL127, R&D System). Collect the cell culture, centrifuge at 300*g* for 5 min at room temperature, and collect the supernatant into a new ep tube. All reagents were placed at room temperature before use. After sample activation, different concentrations of standards and experimental samples were added to the corresponding wells. Seal the reaction wells with a plate-sealing membrane and incubate for 2 h at room temperature. After washing the plate, add 100 μL of configured detection antibody, seal the reaction wells with a plate sealing membrane, and incubate at room temperature for 2 h. After washing the plate, add 100 μL of diluted Streptavidin-HRP B working solution, seal the reaction wells with a plate sealing membrane, and incubate for 20 min at room temperature. After washing the plate, add 100 μL of color developer and incubate for 20 min at room temperature. Add 50 μL of termination solution and measure the absorbance value at 450 nm using an enzyme meter within 30 min. The standard curve was plotted using ELISACalc software, and the corresponding sample concentration was read on the standard curve according to the detected results and multiplied by the activation factor 1.4.

2.11 | Transwell assay

1×10^5 AML cells were cultured in the upper chamber with 200 μL RPMI-1640 medium. 600 μL RPMI-1640 medium with 20% FBS was added in the lower chamber. For the invasion assay, a matrigel coating (cat. no. 356234, BD Systems) was used to precoat the upper chamber at 37°C for more than 2 h. After 24 h, cells in the lower chamber were counted. The migration rate and invasion rate were calculated by cell numbers in the experimental group/cell numbers in the NC group.

2.12 | Animal experiment

The animal study was approved by the Animal Ethics Committee of Xi'an Jiaotong University (No:2022-1602). Female NOD/SCID mice aged 4 weeks were purchased from the Gempharmatech Co., Ltd. The mice were housed in specific pathogen-free conditions. Each mouse was injected with 1×10^7 AML cells transfected with shBATF or NC through the tail vein. The general condition of the mice was examined daily. The body weight was measured twice a week. At the end of the observation (weight loss >20%, weakness of lower limbs, hair erection, etc.), BM samples of mice were collected. The proportion of GFP^+ cells in BM was detected through flow cytometry for detecting the infiltration of leukemia cells. An IVIS Lumina imaging system was used to monitor AML cells.

2.13 | Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP Assay Kit (cat. no. P2078, Beyotime) according to the manufacturer's instructions. THP-1 cells were cultured to a density of 1×10^8 cells and formaldehyde was added for fixation. DNA was lysed by sonication into 100–1000 bp fragments. BATF antibody and IgG antibody (cat. no. 2729S, CST) were added to the precleared chromatin and incubated overnight. Protein A+G-agarose beads were added to precipitate the chromatin fragments bound to the target protein. After inversion cross-linking, ChIP enrichments were isolated and used in the q-PCR reaction. Primers used for ChIP assay: forward 5′-CCCTTCCATCCTTCAGGTGT-3′ and reverse 5′-CCAAAGCGGGTGATCCAG-3′ and control primers for glyceraldehyde 3-phosphate dehydrogenase. The PCR products were subjected to agarose gel electrophoresis, and a gel imaging system was used to analyze the bands.

2.14 | Luciferase reporter assay

Wild-type (WT) and mutant (MUT) sequences of the TGF-β1 promoter were amplified and inserted into a pGL3-basic promoter vector (Tsingke). HEK-293T cells were cotransfected with si-NC/si-BATF or NC/BATF-OE and pGL3- TGF-β1-WT or pGL3-TGF-β1-MUT in six-well plates. At 48 h after cotransfection, luciferase activity was measured on an EnSpire Multimode Plate Reader using the Luciferase Reporter Gene Assay Kit (cat. no. 11401ES80, Yeasen) according to the manufacturer's instructions.

2.15 | Statistical analysis

The survival rate was determined using the K–M method and accessed with the log-rank test. The Student's *t* test and Mann–Whitney *U* test were used to compare the differences between the two groups. All experiments were conducted for three independent times. Statistical analysis was carried out using SPSS 26.0 (IBM, SPSS) and GraphPad Prism 8.0 (GraphPad). $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | BATF expression is upregulated in AML and is an independent risk factor for AML survival

The gene expression and clinical information of 173 AML cases were first retrieved from the TCGA database. The expression of BATF was subsequently analyzed, and the results demonstrated that BATF

expression level was significantly higher in AML patients compared with controls (Figure 1A). As anticipated, patients with poor and intermediate genetic risk exhibited higher BATF expression levels (Figure 1B). Likewise, BATF expression levels were significantly increased in patients with poor OS outcomes (Figure 1C). Next, K–M survival curves were plotted based on the TCGA-LAML (n = 173) and GSE37642 (n = 135) data sets. The curves illustrated that patients with high BATF expression levels had shorter OS than those with low

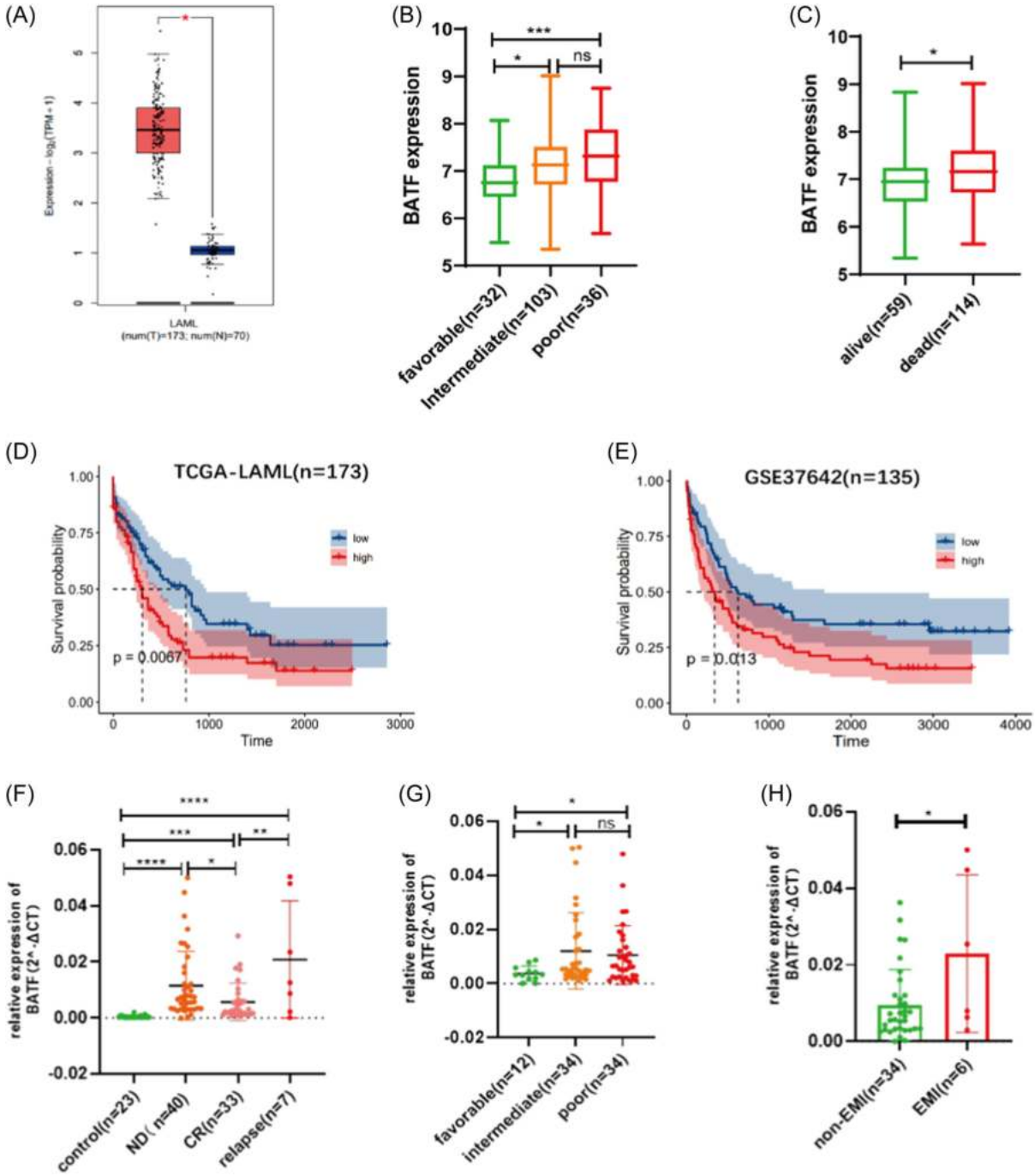

FIGURE 1 BATF was upregulated and positively associated with poor OS in AML patients. (A) BATF was upregulated in AML patients compared to controls in TCGA database. (B) BATF was overexpressed in AML patients with poor and intermediate risk compared to favorable risk AML patients. (C) BATF was highly expressed in dead patients compared to alive patients in TCGA database. (D, E) K–M curve analysis of OS between high and low BATF expression groups in TCGA-LAML data set (D, n = 173) and GSE37642 data set (E, n = 135). (F) BATF expression was significantly higher in AML patients than in the controls. (G) BATF expression was elevated in poor and intermediate genetic risk AML patients than in the favorable genetic risk AML patients. (H) BATF was elevated in EMI AML patients than in the non-EMI patients among ND-AMLs. AML, acute myeloid leukemia; EMI, extramedullary infiltration; K–M, Kaplan–Meier; ND-AML, newly diagnosed AML; OS, overall survival. Error bars are mean ± SD. *p < 0.05, **p < 0.01,***p < 0.001, ****p < 0.0001.

BATF expression levels (Figure 1D,E). Afterward, subgroup survival analysis determined that in the white blood cell (WBC) ≤ 10×10^9/L, abnormal cytogenetics, FLT3-negative, NPMc-negative, and IDH1-negative subgroups, patients with high BATF expression levels had a poorer OS than those with low BATF expression levels (Supporting Information: Figure S1A–J). Following this, univariate and multivariate Cox regression analyses were conducted, which exposed that old age, upregulated BATF expression, poor genetic risk, and high WBC count were independent risk factors for a poor prognosis in AML patients (Table 1). To assess the quantitative prediction of these factors on the prognosis of AML patients, a nomogram incorporating age, BATF expression, genetic risk, and BM blast cells was constructed (Supporting Information: Figure S1K). The calibration curve depicted that the predicted OS of the nomogram was comparable to the actual ratio of 1-year and 2-year OS (Supporting Information: Figure S1L).

Thereafter, 80 samples of AML patients and 23 healthy controls were collected, respectively. QRT-PCR determined that BATF expression levels were higher in AML patients compared with healthy controls, while newly diagnosed AML (ND-AML) and relapsed AML patients displayed a significantly higher BATF expression level than the CR patients (Figure 1F). Patients with intermediate and poor risk exhibited a higher BATF expression level than patients with favorable risk (Figure 1G). However, BATF expression was similar between patients of different ages, genders, BM blast count, and WBC count (Supporting Information: Figure S2A–D). Interestingly, among 40 ND-AML patients, the expression level of BATF was significantly higher in 6 AML patients with EMI than in 34 AML patients without EMI (Figure 1H). Similarly, BATF was highly expressed in the M4 and M5 subtypes in the TCGA-LAML database, which are speculated to be more invasive and often exhibiting the EMI than other FAB subtypes (Supporting Information: Figure S2E). Noteworthily, despite the absence of a significant difference, the expression level of BATF was numerically higher in the EMI group than in the non-EMI group in the GSE116618 data set (Supporting Information: Figure S2F). In short, our findings collectively suggested that BATF is highly expressed in AML and significantly associated with poor risk stratification and EMI. Consequently, we postulate that BATF can be used as an independent risk factor to assess the prognosis of AML patients.

3.2 | Altered BATF expression affects the migratory and invasive abilities of AML cells in vitro

Next, the role of BATF was assessed in AML tumorigenesis. To begin, the expression level of BATF was detected in AML cell lines, uncovering that BATF was significantly overexpressed in AML cell lines (Supporting Information: Figure S2G). Then, BATF-specific siRNAs were transfected into AML cell lines to silence BATF expression. Thus, the BATF expression level was significantly reduced after transfecting siRNAs into AML cells (Figure 2A). Interestingly, the proportion of migrating cells was significantly lower in the BATF knockdown group compared to the NC group (Figure 2B, Supporting Information: Figure S3A). Additionally, matrigel-coated Transwell assays delineated that silencing the expression of BATF significantly inhibited the invasive ability of AML cells (Figure 2C, Supporting Information: Figure S3B). However, cell proliferation, apoptosis, and cell cycle assays showed no significant difference between si-NC and si-BATF groups (Supporting Information: Figure S4A–D).

To further validate the role of BATF, BATF-overexpressing plasmids were transfected into AML cells to upregulate BATF expression. The results showed that BATF was significantly

TABLE 1 Univariate and multivariate Cox regression analyses for OS of AML patients in TCGA database.

Variables	Univariate analysis			Multivariate analysis		
	HR[a]	95% CI[b]	*P* value	HR	95% CI	*P* value
BATF expression	1.651	1.138–2.396	**0.008**	1.66	1.134–2.430	**0.009**
Age	2.654	1.819–3.874	**<0.0001**	2.574	1.720–3.851	**<0.0001**
Genetic risk	1.834	1.355–2.481	**<0.0001**	1.557	1.127–2.150	**0.007**
WBC count	1.004	1.000–1.007	**0.034**	1.005	1.001–1.009	**0.006**
Gender			0.69			
Peripheral blood blasts			0.223			
Bone marrow blasts			0.212			
FLT3 mutation			0.216			
IDH1 mutation			0.447			
RAS mutation			0.941			
NPMc mutation			0.823			

Abbreviations: AML, acute myeloid leukemia; OS, overall survival; WBC, white blood cell.

[a]HR: hazed ratio.

[b]CI: credibility interval.

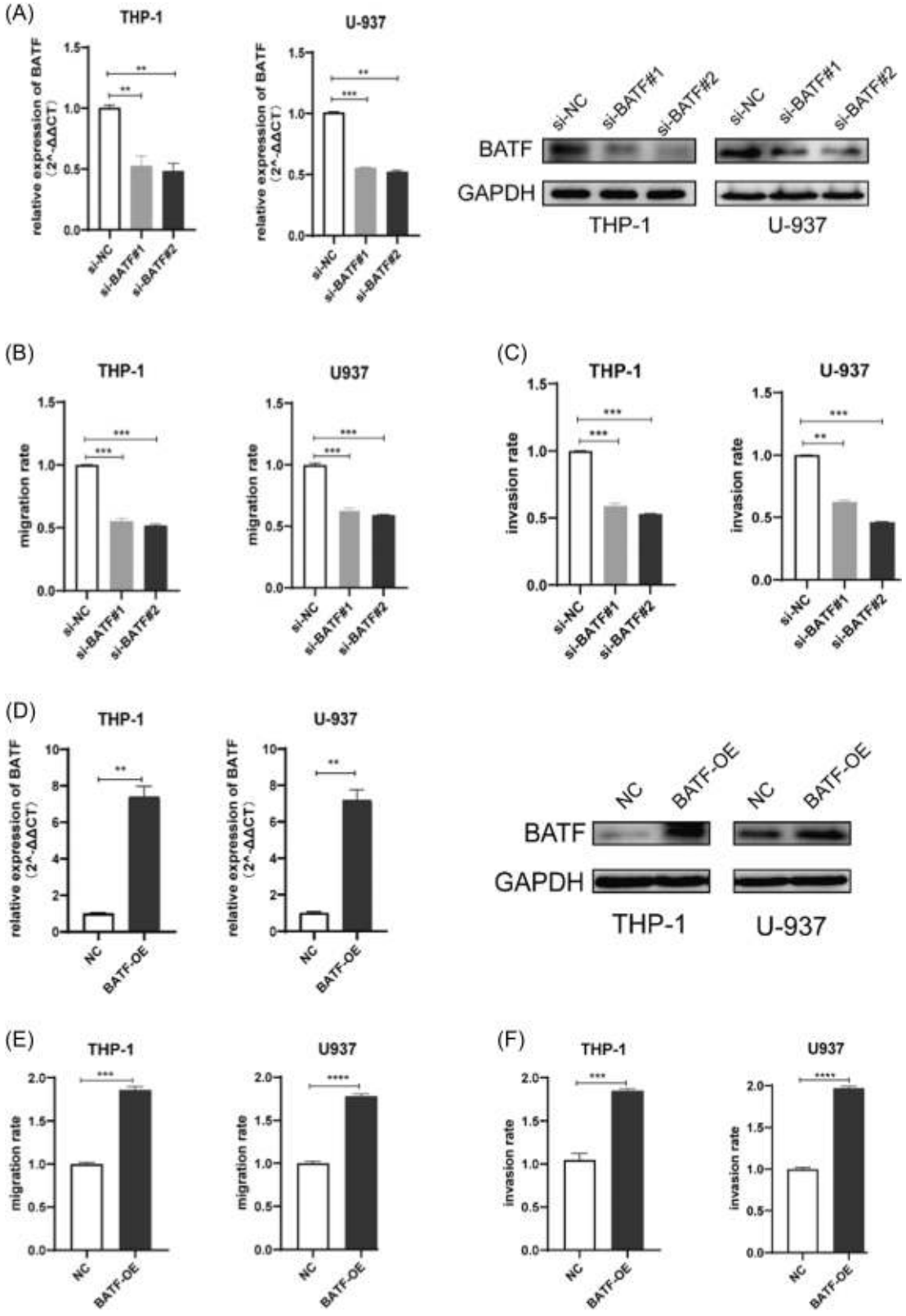

FIGURE 2 Knockdown/overexpression of BATF inhibited/promoted AML cell migration and invasion in vitro. (A) Decreased expression of BATF after transfecting siRNA was confirmed in AML cell lines THP-1 and U-937 by qRT-PCR and Western blot analysis. (B, C) Transwell assay showed that silencing BATF inhibited cell migration (B) and cell invasion (C) in THP-1 and U-937 cells. (D) Increased expression of BATF after transfecting plasmid was confirmed in AML cell lines by qRT-PCR and Western blot analysis. (E, F) Transwell assays showed that overexpressing BATF promoted cell migration (E) and cell invasion (F) in THP-1 and U-937 cells. Error bars are mean ± SD. $n = 3$ independent experiments. AML, acute myeloid leukemia; RT-qPCR, real-time quantitative polymerase chain reaction. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

upregulated at the mRNA and protein levels following plasmid transfection (Figure 2D). Meanwhile, Transwell assays determined that BATF overexpression significantly enhanced the migratory and invasive properties of AML cells (Figure 2E,F, Supporting Information: Figure S3C,D). Taken together, these results signified that BATF could promote the migratory and invasive abilities of AML cells.

3.3 | Knockdown of BATF inhibits AML infiltration in vivo

Furthermore, lentiviral vectors of shBATF and shNC were transfected into THP-1 cells to construct the stable BATF knockdown AML cell line and control group. Then, NOD/SCID mice were injected with AML cells transfected with shNC or shBATF through the tail vein. Following the observation period, the mice were euthanized by decortication after anesthesia, and BM samples of mice were collected. The number of GFP^+ cells in BM was significantly higher in NC mice than in shBATF mice (Figure 3A). At the same time, NC mice exhibited lesser weight and mild splenomegaly compared with the shBATF group (Supporting Information: Figure S5A,B). To evaluate survival, 10 mice in the NC group and 9 mice in the shBATF group were observed after cell injection until death. The K–M curve presented that shBATF mice survived longer than NC mice (Figure 3B). Bioluminescent imaging results highlighted a significant inhibition in AML cell infiltration in shBATF mice on both Days 15 and 30 (Figure 3C). Consistently, CD45 + IHC unveiled a lesser degree of AML infiltration in the liver, spleen, kidney, and CNS of shBATF mice (Supporting Information: Figure S5C,D).

3.4 | BATF transcriptionally regulates the expression of TGF-β1

BATF belongs to the AP-1/ATF superfamily of transcription factors that can bind DNA target sequences and thereupon participate in target gene regulation.[25] Thus, genes positively associated with BATF in AML were analyzed, and intersections with target genes of BATF in the ChIP database were selected for the ensuing analyses (Supporting Information: Figure S6A). Among these target genes, TGF-β1 was significantly positively correlated with BATF expression (Supporting Information: Fiure S6B, $r = 0.47$). Meanwhile, survival analysis showed that AML patients with high TGF-β1 expression exhibited shorter OS than those with low TGF-β1 expression (Figure 4A). To validate the correlation between BATF and TGF-β1, the expression level of TGF-β1 was detected after altering BATF expression in AML cell lines. The results demonstrated that the expression level of TGF-β1 was significantly reduced after knocking down BATF expression (Figure 4B). Conversely, TGF-β1 expression was significantly elevated upon upregulating BATF expression (Figure 4C). In addition, the expression level of TGF-β1 in cell culture medium was significantly decreased after BATF knockdown (Figure 4D).

Besides, a putative promoter containing BATF binding sites was identified at −2000 to +99 bp of the TGF-β1 gene using JASPAR online database (Supporting Information: Figure S6C,D). Then, a ChIP assay was conducted, which revealed that BATF could bind the TGF-β1 promoter (Figure 4E). To further corroborate the interaction between BATF and TGF-β1, luciferase reporter plasmids carrying TGF-β1-WT and TGF-β1-MUT sequences were synthesized (Supporting Information: Figure S6D). The luciferase assay determined that cotransfection with pGAL3-TGF-β1-WT resulted in BATF knockdown inhibiting luciferase activity, whereas luciferase activity was higher following BATF overexpression compared with the NC group. However, modulating BATF expression did not significantly alter pGAL3-TGF-β1-MUT fluorescence intensity (Figure 4F). In short, these results conjointly indicated that BATF bound the promoter region of TGF-β1 and transcriptionally activated its expression.

3.5 | BATF activates the TGF-β1/Smad/MMPs signaling pathway

In the canonical TGF-β signaling pathway, activated TGF-β1 phosphorylates Smad2/3 and then forms a heterotrimeric complex with SMAD4, which translocates to the nucleus and activates downstream genes.[26] To examine the effect of BATF on the canonical TGF-β signaling pathway, the expression level of Smad2/3 and p-Smad2/3 was detected. Notably, BATF knockdown reduced the expression level of p-Smad2 and p-Smad3 but exerted a marginal effect on the expression of total Smad2 and Smad3 (Figure 5A). In contrast, BATF overexpression increased the expression level of p-Smad2 and p-Smad3 (Figure 5B).

To verify the effect of the TGF-β/Smad pathway on the migratory and invasive abilities of AML cells, AML cells were cocultured with the TGF-β receptor inhibitor GW788388, which inhibited the protein expression of p-Smad2 and p-Smad3 in AML cells (Figure 5C). Transwell assays showed that blocking the TGF-β signaling pathway in vitro suppressed the migratory and invasive properties of AML cells (Figure 5D,E, Supporting Information: Figure S3E,F). At the same time, Western blot results suggested that stimulation of recombinant human TGF-β1 could rescue the downregulation of p-Smad2 and p-Smad3 expression promoted by BATF downregulation (Figure 5F). Rescue assay further demonstrated that recombinant human TGF-β1 stimulation reversed the attenuation of cell migration and invasion caused by BATF downregulation (Figure 5G, Supporting Information: Figure S3G,H).

Moreover, the expression levels of MMP2 and MMP9 were found to be downregulated after BATF knockdown (Figure 5A, Supporting Information: Figure S7A). On the other hand, MMP2 and MMP9 expression were upregulated following BATF overexpression (Figure 5B, Supporting Information: Figure S7B). Importantly, the expression levels of MMP2 and MMP9 were lower following treatment with a TGF-β receptor inhibitor GW788388 (Figure 5C, Supporting Information: Figure S7C).

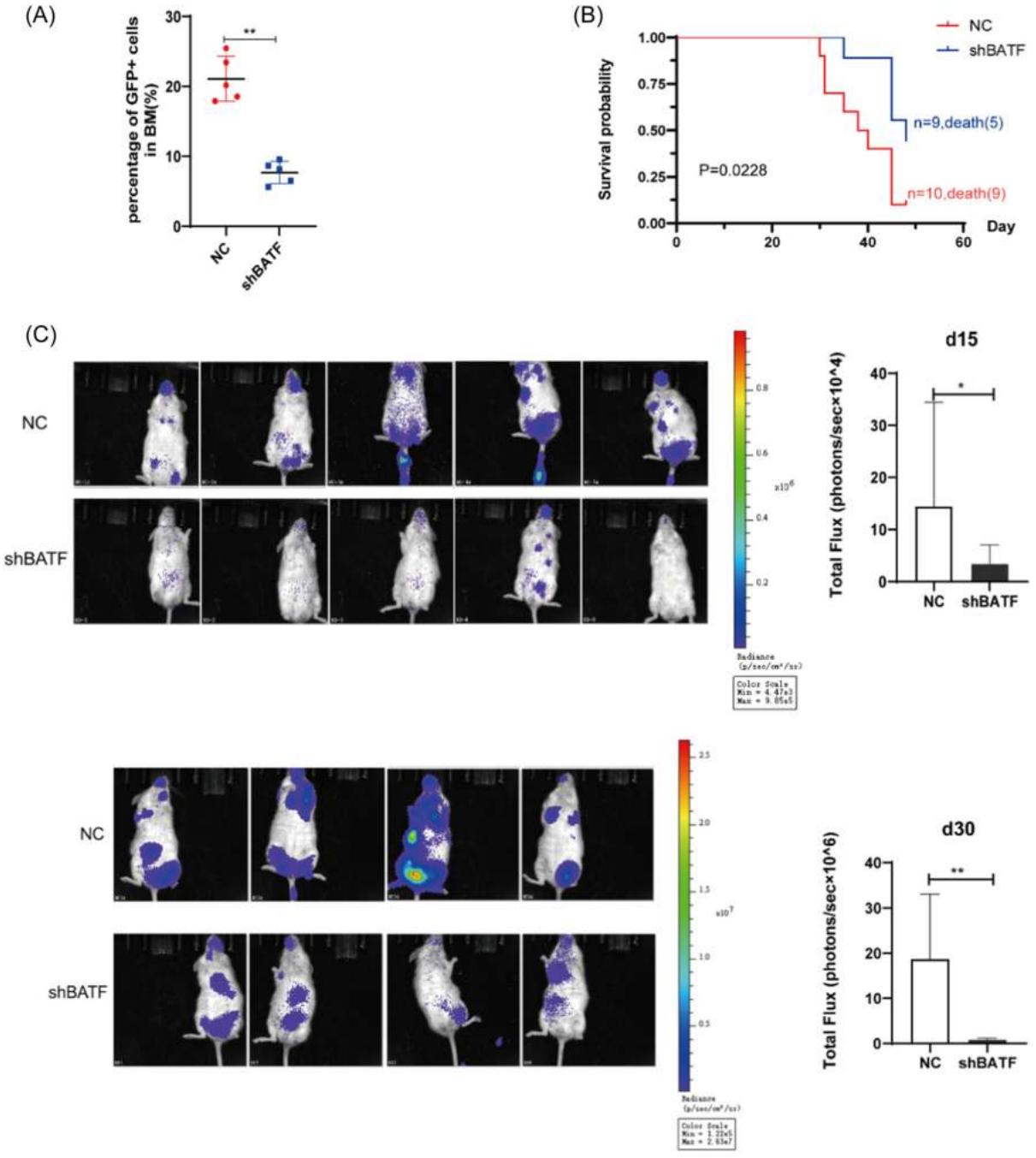

FIGURE 3 Knockdown of BATF inhibited AML infiltration in vivo. (A) The BM GFP+ cell percentage was significantly higher in NC mice than shBATF mice detected by flow cytometry. (B) NC mice showed shorter survival time than the shBATF mice. (C) The bioluminescent imaging showed that the infiltration of AML cells was significantly inhibited in the shBATF mice at d15 and d30 after vein infection of AML cells. AML, acute myeloid leukemia. $^{*}p < 0.05$, $^{**}p < 0.01$.

In addition, the expression level of MMP2 and MMP9 was also rescued following the introduction of recombinant human TGF-β1 (Figure 5F, Supporting Information: Figure S7D). In short, BATF promoted leukemia cell invasion by elevating MMP2 and MMP9 expression via the TGF-β/Smad signaling pathway. Figure 6 illustrates a model outlining the mechanism by which BATF promotes leukemia cell migration by mediating the TGF-β1/Smad/MMPs signaling axis.

4 | DISCUSSION

In the bone marrow and other hematopoietic tissues, leukemia cells proliferate and accumulate, suppressing normal hematopoiesis and infiltrating other organs and tissues, finally developing into an EMI of AML. However, the mechanisms by which EMI occurs are intricate and underexplored. Herein, a new mechanism involving the BATF/TGF-β1/Smad/MMPs signaling pathway that

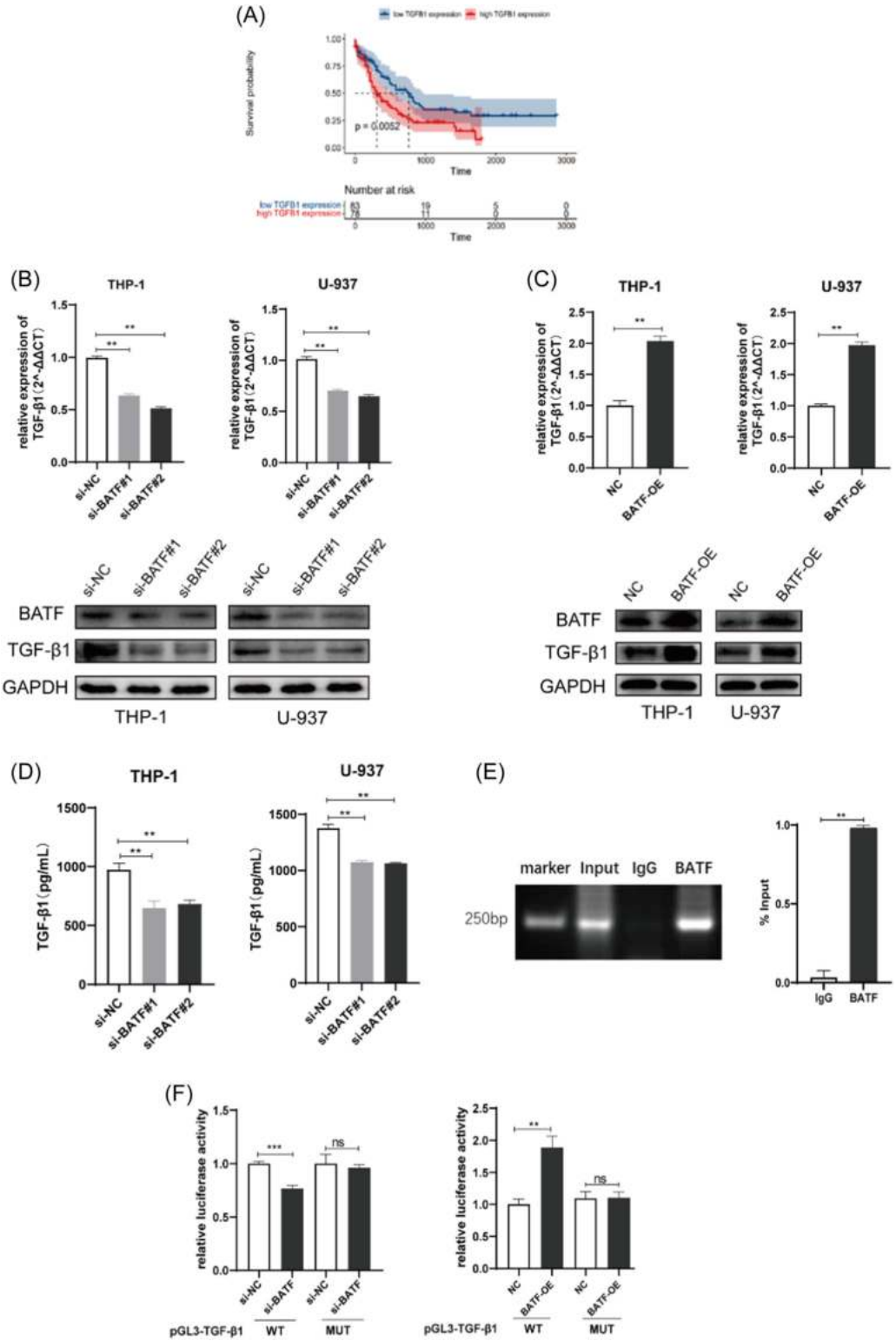

FIGURE 4 BATF transcriptionally regulated the expression of TGF-β1. (A) High TGF-β1 expression was significantly related to poor survival of AML patients in TCGA data set. (B) TGF-β1 expression was significantly decreased after knocking down BATF in AML cell lines by transfecting siRNA. (C) TGF-β1 expressions were significantly increased after overexpressing BATF in AML cell lines by transfecting plasmid. (D) TGF-β1 secretion levels were downregulated in the culture medium after knocking down BATF in AML cells. (E) ChIP analysis of interaction between BATF and the TGF-β1 promoter. (F) Luciferase assays showed that BATF activated TGF-β1 transcription in HEK-293T cells. AML, acute myeloid leukemia; ChIP, chromatin immunoprecipitation; ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

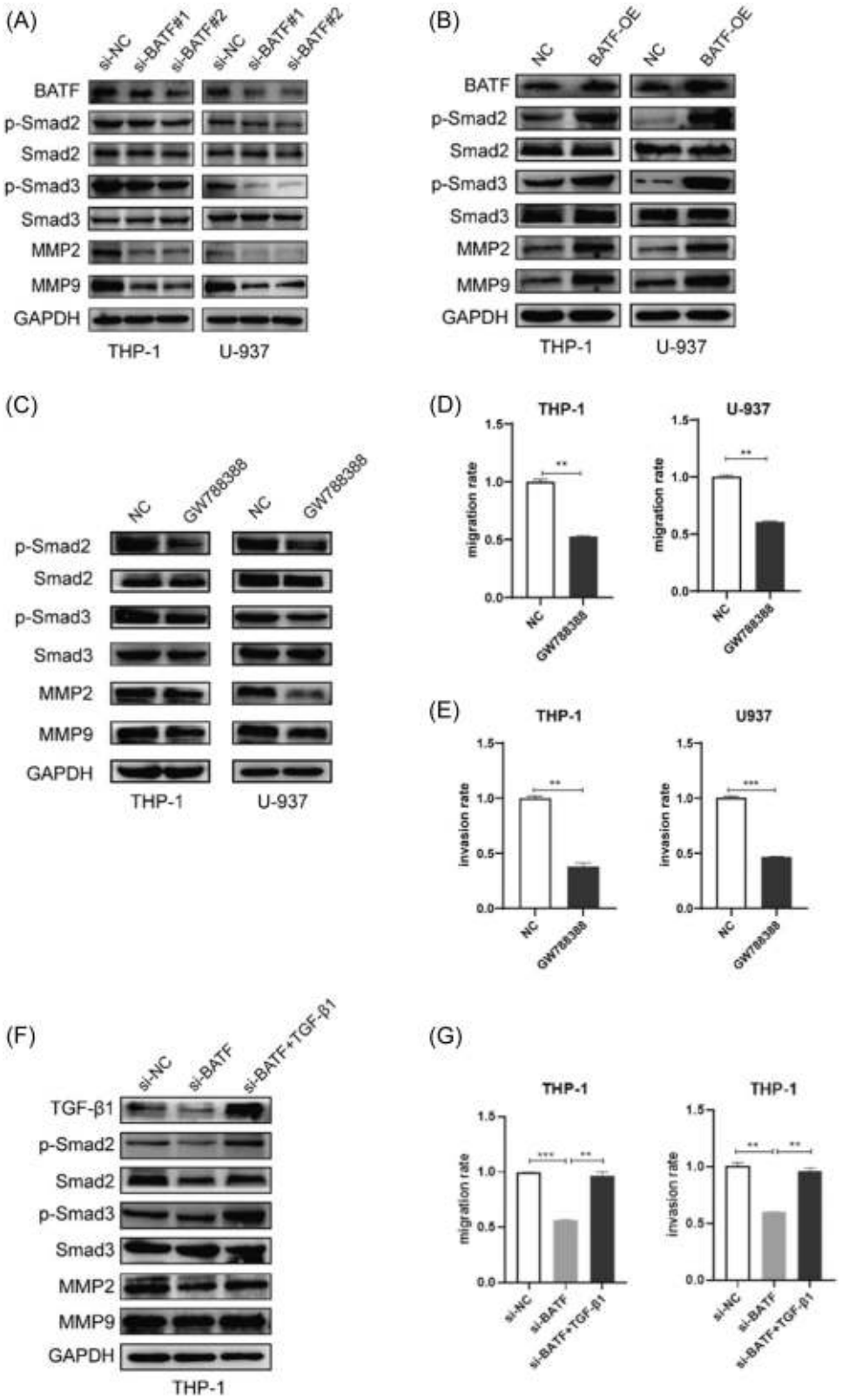

FIGURE 5 BATF activated TGF-β1/Smad/MMPs signaling pathway. (A) Western blot analysis showed that p-Smad3, p-Smad2, MMP2, and MMP9 were decreased in BATF-silenced AML cells. (B) Western blot analysis showed that p-Smad3, p-Smad2, MMP2, and MMP9 were increased in BATF-overexpressed AML cells. (C) TGF-β receptor inhibitor GW788388 downregulated the protein levels of p-Smad2, p-Smad3, MMP2, and MMP9. (D) Transwell assay showed that GW788388 inhibited cell migration in AML cells. (E) Transwell assay showed that GW788388 inhibited cell invasion in AML cells. (F) Western blot analysis showed relative levels of TGF-β1-Smad2/3-MMP2/9 pathway proteins in BATF-silencing THP-1 cells with or without TGF-β1. (G) Recombinant human TGF-β1 stimulation could rescue the inhibition of si-BATF on AML cell migration and invasion in THP-1 cells. AML, acute myeloid leukemia. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

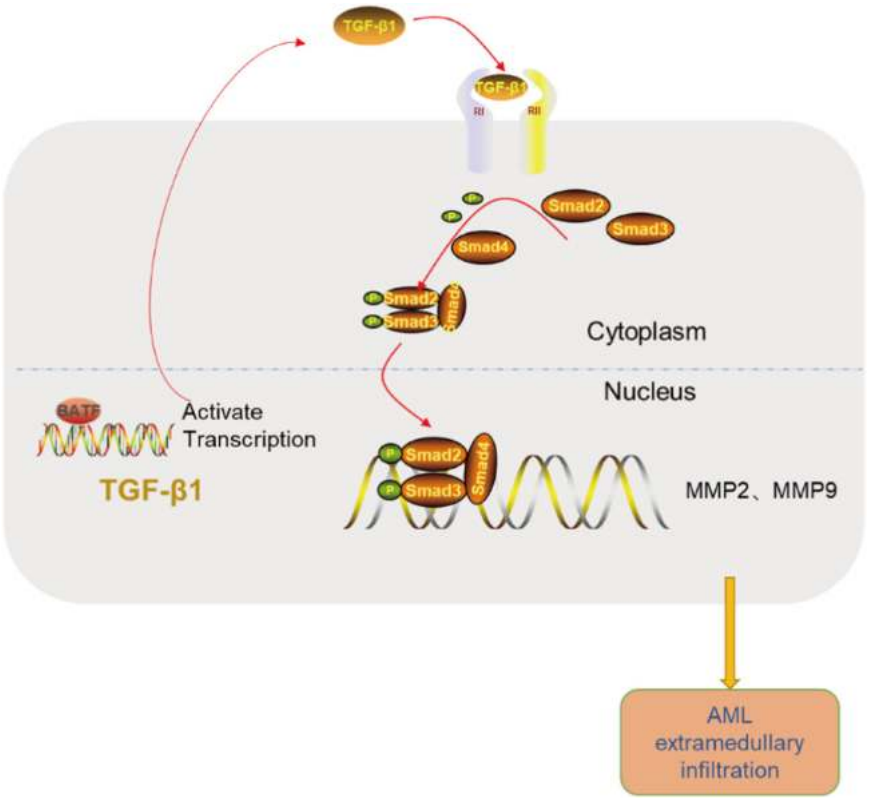

FIGURE 6 A model proposing the mechanism by which BATF promotes leukemia cell migration through TGF-β1/Smad/MMPs signaling.

accounts for the occurrence of infiltration in AML was discovered.

BATF is a crucial transcription factor implicated in several biological processes, including immune regulation, cancer genesis and development, and immune inflammation.[13,27–29] A growing number of studies have established its oncogenic role in multiple cancers.

In the present study, BATF was upregulated in AML BM samples and cell lines. AML patients with poor risk exhibited higher BATF expression levels. AML patients with EMI displayed a higher BATF expression level compared to non-EMI patients. Moreover, high BATF expression was shown to be an independent and adverse factor for the OS of AML patients. Function assays were conducted, which found that BATF could trigger AML cell infiltration both in vitro and in vivo. Simultaneously, ChIP experiments and luciferase reporter assays validated that BATF can bind to the promoter region of TGF-β1, then activate TGF-β1 transcription and regulate the TGF-β1-Smad2/3-MMP2/9 axis. Rescue experiments confirmed that supplementation with recombinant human TGF-β1 could rescue the diminished migratory and invasive capacities of cells following the knockdown of BATF.

Extramedullary tumor occurrence has been reported in various hematological neoplasms. The incidence of EMI in hematologic tumors has been essentially underestimated due to the paucity of large-sample prospective studies. EMI may involve several systemic organs, including skin and soft tissues, liver, kidneys, lymph nodes, CNS, breast, pleura, pericardium, and so on.[30] Genetics and the microenvironment collaborate to cause extramedullary disease. Various genetic changes endow tumor cells with key phenotypic characteristics that enable proliferative self-renewal, adhesion, angiogenesis, migration, and invasion. In the BM microenvironment, various cellular components, such as osteoclasts, endothelial cells, stromal cells, and immune cells, as well as cell surface adhesion molecules, chemokines, and stromal molecules, act synergistically to promote the development of EMI. In AML, patients with abnormal karyotypes, such as t(8;21), inv (16), and 11q23 translocations, are more prone to suffer from extramedullary disease.[31] In addition, the MMP family has been theorized to contribute to cellular invasion.[32–34] To date, there are no specific treatment options for extramedullary disease. Surgical resection and radiotherapy may be implemented for localized masses, while chemotherapy regimens remain focused on systemic disease.[5] Patients who develop EMI generally have a poor prognosis, and their response to standard therapy is less favorable than those without EMI.[35] Herein, TGF-β receptor inhibitors alleviated AML cell migration and invasion, suggesting that TGF-β1 might be a candidate therapeutic target to overcome AML infiltration.

5 | CONCLUSION

In summary, our study revealed that BATF is overexpressed in AML and correlated with poor survival. Mechanistically, BATF promotes the migratory and invasive capabilities of AML cells by promoting TGF-β1 transcription and activating the TGF-β1/Smad/MMPs signaling pathway.

AUTHOR CONTRIBUTIONS

Ru Zhang designed the study. Ru Zhang, Jiyu Miao, and Meng Zhai performed the in vitro experiments. Ru Zhang drafted the manuscript. Ru Zhang and Fangmei Li carried out the statistical analyses. Ru Zhang and Lingjuan Huang contributed to the animal experiments. Ting Wang, Xuezhu Xu, Rui Yang, Ruoyu Yang, and Yiwen Wang collected clinical samples. Rui Liu, Jianli Wang, and Aili He revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data sets used and/or analyzed during this study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Zhang R, Miao J, Zhai M, et al. BATF promotes extramedullary infiltration through TGF-β1/Smad/MMPs axis in acute myeloid leukemia. *Mol Carcinogen*. 2024;63:1146-1159. doi:10.1002/mc.23715