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Bone morphogenetic proteins regulate differentiation of human promyelocytic leukemia cells

Iva Topić, M.D.[1], Marina Ikić M.D.[2], Sanja Ivčević B.Sc.[3], Nataša Kovačić[4], M.D., PhD, Ana Marušić[5], M.D., PhD, Rajko Kušec, M.D., PhD[6], Danka Grčević[3], M.D., PhD

[1]Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Center Zagreb, Zagreb, Croatia
[2]Department of Clinical Immunology, Pulmology and Rheumatology, University Hospital "Sveti Duh", Zagreb, Croatia
[3]Department of Physiology and Immunology, University of Zagreb, School of Medicine, Zagreb, Croatia
[4]Department of Anatomy, University of Zagreb, School of Medicine, Zagreb, Croatia
[5]Department of Research in Biomedicine and Health, University of Split, School of Medicine, Split, Croatia
[6]Department of Hematology, University Hospital "Dubrava", University of Zagreb, School of Medicine, Zagreb, Croatia

Corresponding author:
Iva Topić, MD
Department of Otorhinolaryngology, Head and Neck Surgery
University Hospital Center Zagreb
Kispaticeva 12
HR-10000 Zagreb
Phone: +3851 2367 568
GSM: +38598 287 680
e-mail: itopic@mef.hr

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Abstract

We investigated the role of bone morphogenetic proteins (BMPs) in suppression of all-trans retinoic acid (ATRA)-mediated differentiation of leukemic promyelocytes. In NB4 and HL60 cell lines, BMPs reduced the percentage of differentiated cells, and suppressed PU.1 and C/EBPε gene expression induced by ATRA. BMP and ATRA synergized in the induction of ID genes, causing suppression of differentiation. In primary acute promyelocytic leukemia bone-marrow samples, positive correlation of PML/RARα and negative of RARα with the expression of BMP-4, BMP-6 and ID genes were found. We concluded that BMPs may have oncogenic properties and mediate ATRA resistance by a mechanism that involves ID genes.

Key words: acute promyelocytic leukemia; bone morphogenetic proteins; all-trans retinoic acid; differentiation; ID genes; cell lines; patients

Introduction

Acute promyelocytic leukemia (APL) is characterized by the accumulation of abnormal promyelocytes unable to differentiate into granulocytes and specific translocation t(15;17)(q22;q21) [1, 2]. Translocation results in the formation of PML/RARα (promyelocytic leukemia/retinoic acid receptor α) fusion gene that plays a central role in leukemogenesis of APL by blocking the function of RARα and PML proteins responsible for differentiation and apoptosis of APL cells [3]. All-trans retinoic acid (ATRA) is used for treatment of APL patients with high rate of complete remission when used alone or in combination with standard cytotoxic treatment [4]. Retinoic acid (RA) acts as morphogen through two subfamilies of nuclear receptors (RAR and RXR), ligand inducible transcription factors, in order to regulate the expression of genes containing retinoid response elements [5]. ATRA has been shown to induce terminal differentiation of APL cells by activation of RARα, and by inducing degradation of PML/RARα [2]. However, prolonged ATRA treatment often results in relapse due to the development of ATRA resistance by leukemic cells [4]. Although early studies suggested that an adaptive hypercatabolic response to pharmacological doses of ATRA is the principal mechanism of resistance, recent observations suggest that molecular disturbances in APL cells have a predominant role [6].

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor β (TGF-β) superfamily. Besides their role in bone physiology, they also regulate proliferation, differentiation, morphogenesis and apoptosis of hematopoietic cells. BMP-2 can induce hematopoietic environment after subcutaneous implantation and regulate apoptosis in various B-cell lines and primary myeloma cells [7-9]. BMP-4 has a crucial instructive role for the induction and formation of blood cell precursors [10] and induces formation of embryonic hematopoietic tissues [11]. BMP-6, produced by bone-marrow (BM) cells, participates in the regulation of proliferation, apoptosis and differentiation of B-cell lineage [12]. Dosen-Dahl et al showed that treatment with TGF-β or BMP-6 protects leukemic cells against chemotherapy-induced cell death and allow them to escape therapy [13].

BMPs are known to exert their effect through two different types of serine-threonine kinase receptors known as receptors type I (BMP receptor (BMPR)IA, BMPRIB and activin receptor

(ActR)IA) and type 2 (BMPRII, ActRIIA and ActRIIB), and intracellular SMAD (small mothers against decapentaplegic) proteins, which play a central role in BMP signaling [14]. Important direct target of SMAD pathway is the gene family of DNA binding inhibitors (ID) [15]. ID genes encode a family of proteins that block basic helix-loop-helix transcription factors to bind to DNA, thus interrupting their regulatory role in many developmental and differentiation processes. Different studies on human tumors proved their oncogenic properties in regulating cell-cycle progression, migration and invasiveness [15-19].

Our previous study has shown that BMP-2, BMP-4 and BMP-7, and their receptors were strongly expressed in patients with APL, paralleled by the expression of PML/RARα oncogene. Absence of BMP expression correlated with clearance of the tumor molecular marker [20]. In this study, we aimed to investigate molecular mechanisms by which BMPs suppress differentiation of APL cells induced by ATRA, using myeloid cell lines NB4 and HL60, and primary samples from APL patients. Since RA/RAR pathway functionally interacts with BMP/SMAD signaling [21], we proposed that BMP signal interfere with ATRA-induced differentiation of APL cells. Our findings may add to the understanding of the mechanisms of resistance to ATRA in APL patients.

Materials and methods

Patient samples and cell lines

Human myeloid leukemia cell lines NB4 and HL60 (DSMZ cell line collection, Braunschweig, Germany) were grown in RPMI 1640 with 10% fetal calf serum (FCS) (Gibco, Invitrogen Ltd., Grand Island, NY, USA), 2 mM glutamax and 100 U/ml penicillin/streptomycin, in a 5% CO_2 at 37ºC. APL cell line NB4 carries the specific t(15;17) translocation, whereas HL60, a myeloblastic leukemia cell line, lacks t(15;17) translocation but differentiates along granulocytic pathway upon ATRA treatment. To induce differentiation, cells (0.3×10^6/mL in 25 cm^2 flasks) were treated with ATRA (0.5 μM for NB4 and 2 μM for HL60). Recombinant human (rh)BMP-2, rhBMP-4 or rhBMP-6 (all from R&D Systems, Abingdon, UK), and soluble BMP antagonist NOGGIN (NOG) (PeproTech, London, UK) were added as indicated in each experiment.

After obtaining approval from the institutional Ethics Committee and informed consent from participants, we analyzed BM and peripheral blood (PBL) samples from 5 APL patients collected as a part of routine clinical assessment, at diagnosis and during the clinical follow-up. BM specimens were obtained by sterile punction of the iliac crest. PBL was drawn by standard venipuncture, followed by mononuclear cell separation using Histopaque (Sigma, St. Louis, MO, USA; density 1.077 g/ml). Diagnosis was established by morphological characterization, cytochemistry, and cytogenetic- and immuno-phenotyping according to the criteria of French-American-British classification for AML-M3 [22]. Presence of PML/RARα fusion gene as a tumor molecular marker was confirmed by polymerase chain reaction (PCR) analysis. Patients were treated with the combination of oral ATRA (45 mg/m^2 per day), divided into 2 doses, and idarubicin (12 mg/m^2 on days 2, 4, 6 and 8) as described previously [20].

May-Grünwald Giemsa staining

Cytospin preparations for standard May-Grünwald Giemsa staining were made after ATRA treatment, as indicated in each experiment, to detect morphological changes associated with granulocyte differentiation (pale cytoplasm and mulitlobulated segmented nucleus) [23].

Flow-cytometric analysis

Myeloid leukemia cell lines NB4 and HL60 were analyzed by flow cytometry using a FACS Calibur instrument and Cell-Quest software (BD Biosciences, San Jose, CA, USA). Cells were analyzed for the phenotypic evidence of differentiation (expression of granulocyte surface markers CD11b and CD11c) by standard protocols [24]. Viability and apoptosis were evaluated by annexin V/propidium iodide (PI) staining (BD Biosciences) according to the manufacturer's instructions. Cells were classified on dot-plots as dead (PI^+), apoptotic (PI^-/annexin V^+) or viable (double negative). For cell-cycle analyses, cells were resuspended in DNA staining solution (0.01 M Tris, 10 mM NaCl, 700 U/L RNAse, 7.5×10^{-5} M PI, 0.01% Nonidet P-40). Cell-cycle distribution was presented as histograms (G_0/G_1, S, G_2/M phases) [8].

Gene expression analysis

Total RNA was extracted (TriPure; Roche Mannheim, Germany) from primary APL BM and PBL samples, or NB4 and HL60 cells, reversely transcribed to cDNA (MuLV Reverse Transcriptase; Applied Biosystems, Foster City, CA) and amplified by quantitative (q)PCR using specific TaqMan assays (Supplementary table 1) in an ABI Prism 7000 Sequence Detection system (Applied Biosystems). Each reaction was performed in triplicate in a 25 μL reaction volume as previously described [8]. The relative quantities of unknown samples for the each gene were interpolated from the six-point serial dilution standard curve of the calibrator sample (BM cells or cell lines). To equalize samples according to the amount of input cDNA, the relative quantity of the target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control.

Statistics

All experiments were repeated at least three times. Gene expression triplicates in treated cell lines were expressed as mean ± standard deviation (SD) and compared using analysis of variance with Student-Newman-Keuls *posthoc* test. Gene expressions in primary samples were correlated to clinical data using rank correlation and Spearman's coefficient of rank correlation rho (ρ) with its

95% confidence interval. Statistical analysis was performed using MedCalc software-package (Mariakerke, Belgium). For all experiments, α-level was set at 0.05.

Results

ATRA induced differentiation of NB4 and HL60 cell lines

We confirmed ATRA-induced differentiation of myeloid leukemia cell lines NB4 and HL60 by the expression of differentiation makers (CD11b/CD11c), and changes in cell proliferation and apoptosis. Differentiation was followed up to 4 days, with increase in the expression of CD11b and CD11c in both cell lines with the time of ATRA treatment (Figure 1A). Differentiated cells showed morphological changes characteristic for granulocytic differentiation, containing pale cytoplasm and multilobulated segmented nucleus (Figure 1A). At the representative time-point (day 3 of ATRA treatment for NB4 and day 4 for HL60, according to the percent of CD11b/CD11c-positive cells), we observed the suppression of cell-cycle progression (Figure 1B). Furthermore, ATRA decreased the percentage of apoptotic NB4 cells, but increased the percentage of apoptotic HL60 cells, indicating that proapoptotic effect is not associated with t(15;17) rearrangement [25]. Finally, we determined the expression of PML/RARα oncogene in NB4 cells by qPCR, which was further increased by ATRA treatment together with the expression of total RARα (Figure 1C). We also confirmed the responsiveness of cell lines to BMPs by detecting gene expression for BMP receptors (ActRIA, BMPRIA, BMPRIB, BMPRII, ActRIIA, ActRIIB), BMP-signaling related molecules (SMAD5, SMAD6, NOG, bone morphogenetic and activin membrane-bound inhibitor (BAMBI)) and BMP-target genes (ID1, ID2, cKit), with and without ATRA treatment (Figure 1C). The major difference between two analyzed cell lines, that could influence the functional response of those cells to BMP treatment, is huge induction of ID genes particularly in NB4 cells by ATRA and constitutively lower expression of cKit in HL60 cells.

BMPs suppressed differentiation of ATRA-treated NB4 and HL60 cell lines

Since our previous study on APL primary BM samples showed that treatment with ATRA was associated with the suppression of BMP expression [20], we aimed to reveal the effect of BMPs on ATRA induced differentiation of NB4 cells as an *in vitro* model for APL. HL60 cell line, not bearing the specific t(15;17)(q22;q21) translocation, was used in addition to test if the BMP effect is PML/RARα specific. Cells were treated with the combination of ATRA and BMP-2, BMP-4 or BMP-6. BMP alone did not affect the expression of differentiation markers, but in

combination with ATRA reduced the percent of cells expressing CD11b and CD11c by 40 to 60% respectively, with stronger effect in NB4 cells (Figure 2A). This effect was paralleled by expected morphological findings, i.e. more immature cell features upon combined treatment with BMP and ATRA compared to ATRA alone.

Our further aim was to evaluate BMP effect by analyzing the expression pattern of several intracellular molecules involved in proliferation, differentiation and apoptosis of APL cells. Genes for ankyrin repeat and SOCS box protein 2 (Asb2) involved in RA-induced growth inhibition and myeloid differentiation, myeloid-specific transcription factors PU.1 and CCAAT-enhancer-binding proteins ε (C/EBPε), and ubiquitin-conjugating enzyme UBE2D3 [26, 27] were up-regulated in ATRA-treated groups in both cell lines. Expression of proto-oncogene Bcl-2, tyrosine-protein kinase cKit and distal-less homeobox gene 4 (Dlx4) associated with cell proliferation and tumorigenesis [28], were decreased by ATRA treatment (Figure 2B). In general, BMPs more effectively suppressed genes associated with cell differentiation, specifically PU.1, UBE2D3 and C/EBPε in NB4 cell line, compared with very weak or negligible effect on the restoration of proliferative/antiapoptotic oncogene expression.

Effect of BMP-2 and Noggin on promyelocytic cell lines treated with ATRA

Since we did not find significant difference in the effectiveness between tested BMPs, we assumed that their action was mediated by the same receptor complex (possible BMPRIA in combination with BMPRII or ActRIIB, according to the expression pattern presented at Figure 1C). Therefore we used BMP-2 as a model to further study the role of BMPs in ATRA-resistance. Again we confirmed that BMP-2 suppressed NB4 and HL60 ATRA-induced differentiation, assessed by CD11b/CD11c expression, but also revealed that the effect was BMP-specific since we were able to reverse BMP-mediated suppression of differentiation by NOG pretreatment (Figure 3A).

Further on, we aimed to identify molecular cross-road between BMP-signaling pathway and induction of genes that regulate promyelocyte differentiation. ID genes are well documented direct targets of BMP signaling [29], which, at the same time, have important role in granulocyte

differentiation [30] as well as malignant transformation in several cell types [15]. ID1 and ID2 genes were induced by BMP-2 in both tested cell lines, but up-regulated by ATRA treatment only in NB4 cells. Combined treatment with BMP-2 and ATRA synergistically enhanced ID genes, particularly ID1 in both cell lines (Figure 3B). In addition, we hypothesized that more pronounced differentiation-suppressive effect of BMPs seen in NB4 cells compared with HL60 (approximately 50% suppression of CD11b expression in NB4 compared with 30% in HL60 cells, Figure 3A) could be explained by constitutively higher expression of cKit, transcriptional target of BMPs with a role in hematopoietic cell survival and proliferation [31]. Nevertheless, cKit expression was highly suppressed by ATRA treatment, and not significantly affected by BMP-2. Combined treatment was able to only weakly increase the expression compared to ATRA alone (Figure 3B).

Correlation of PML/RARa oncogene expression and BMP-related molecules in APL patients

To translate the importance of the obtained findings to clinical setting, we collected the follow-up BM and PBL samples of 5 APL patients and assessed the expression of BMP-related genes in correlation with the disease molecular marker (Figure 4). The patients were included at the time of diagnosis, with detectable expression of PML/RARα oncogene in BM and PBL. Patients showed prolonged molecular remission with ATRA treatment, except for patient #1, who died upon relapse and disease complications. In parallel, expression of total RARα was assessed, showing reciprocal expression profile compared with the fusion oncogene PML/RARα (ρ=-0.430, 95% CI -0.692 to -0.068, p=0.025).

Based on the expression pattern of BMP-related molecules, we further analyzed their correlations (specifically BMP-2, -4, -6, ID1, ID2, cKit, BAMBI, NOG, PU.1, UBE2D3, C/EBPε) with PML/RARα and RARα (Table 1). The most significant positive correlations were found for PML/RARα and BMPs as well as their transcription target ID1, whereas significant negative correlations were found for BMP antagonist BAMBI and transcription factor PU.1, with the reciprocal correlations for RARα (Table 1). Finally, expression of cKit [32] was in negative correlation with PML/RARα and positive with RARα.

Discussion

We studied mechanisms by which BMPs affect response of APL cells to ATRA treatment and found ID genes as a possible molecular link between BMP signaling and suppression of myeloid differentiation. Previous studies showed that ID genes were induced by ATRA in NB4 cells and APL patients [33], and by BMP-4 in embryonic stem cells and a range of cell lines [34], but this is the first report of the synergistic enhancement of ID genes by combined treatment with BMPs and ATRA. Addition of BMP antagonist NOG to ATRA/BMP-2 treatment suppressed ID1 and ID2 expression, which is consistent with the observed inhibition of SMAD1/5/8 phosphorylation and ID protein up-regulation upon treatment with BMP-2 and NOG [35]. Moreover, in primary APL BM samples we found positive correlation of PML/RARα and negative of RARα with ID genes as well as their upstream activators BMP-4 and BMP-6.

To better characterize the effect of BMPs on differentiation of APL cells, we used NB4 and HL60 cell lines. NB4 is RA sensitive APL cell line that harbors the specific t(15;17) translocation. HL60, on the other hand, is a myeloblastic leukemia cell line that responds to RA but lacks t(15;17) translocation, suggesting that genetic alterations distinct from translocation involving RARα might generate APL-like phenotype [36]. In NB4 cells ATRA enhanced the expression of oncogene PML/RARα and total RARα, restoring RA-modulated myeloid differentiation [2, 37]. In parallel, ATRA reduced apoptotic/dead population of NB4 cells, which may be related to APL-differentiation syndrome seen upon ATRA treatment [38]. Syndrome is caused by the accumulation of mature granulocytes and can lead to a fatal outcome in APL patients. Increase in the percentage of cells in G_0/G_1 cell-cycle phase is known to be caused by ATRA-induced over expression of ID1 and ID2 [33]. Among tested BMPs, NB4 expressed exclusively BMP-2 and HL60 BMP-4 (not shown), which act through the same receptor complexes, and both lines expressed BMP receptors, specifically BMPRIA, BMPRII and ActRIIB. BMPs and their receptors has been detected in BM in different pathological conditions [7, 8, 20], suggesting an autocrine action.

BMP signaling pathway plays an important role in regulation of hematopoietic niches and stem cell population size maintaining BM homeostasis [39] as well as differentiation and growth of leukemic cells [9, 10, 12]. Therefore we proposed that BMP signal may target genes important

for granulocyte differentiation, causing promyelocyte resistance to ATRA. Several genes were identified to be rapidly induced by ATRA, i.e. SOCS box family gene Asb2, due to binding of RARα to Asb2 promoter [26], and hematopoietic transcription factor PU.1, found to be suppressed in APL cell lines [27]. PU.1 shares some transcription targets with BMPs, such as ID2 [40], and interacts with SMAD1 to enhance macrophage expression of interleukin 1β after BMP-6 treatment [41]. ATRA induction of PU.1 in cells harboring PML/RARα is mediated by up-regulation of the C/EBP proteins, important for terminal granulocytic differentiation [27]. We observed that both PU.1 and C/EBPε were increased after treatment with ATRA, and repressed by BMPs, indicating that this could be one of the mechanisms involved in resistance to ATRA-induced differentiation [42]. C/EBP transcription factors interact with SMAD proteins leading to formation of C/EBP-SMAD complexes. Possible mechanism of C/EBP-SMAD antagonism could be the interference with C/EBP coactivator or competition for a common coactivator [43]. On the other hand BMPs could not restore the expression of proliferative genes highly suppressed by ATRA, including oncogene Bcl-2 and homebox gene Dlx4 [28, 44, 45]. Nevertheless, expression of cyclin D1 degradation enzyme UBE2D3, important for ATRA-mediated cell growth arrest [46], was down regulated by addition of BMPs, possible causing an increase in the proportion of cells in the active cell-cycle phases (not shown).

Since it has been shown that SMAD3 interacts with RARα in a TGF-β dependent manner [47], we proposed that simultaneous activation by ATRA and BMPs causes those two signals to interfere perpetuating immature APL phenotype. BMP signal during ATRA treatment synergizes in the induction of ID genes, which may lead to inhibition of differentiation and propose BMPs as oncogenic proteins. Despite the role in granulocyte differentiation, ID genes promote cell proliferation and malignant transformation in different cell types including hematopoietic cells, particularly myeloid [48]. cKit has also been involved in RA as well as BMP action [49, 50]. It has an important role in proliferation, differentiation and survival of normal hematopoietic and leukemic cells [49, 50]. We found negative correlation of cKit with oncogene PML/RARα and positive with RARα in APL BM samples, indicating its role in differentiation of APL cells upon ATRA treatment rather than proto-oncogenic property [32].

Finally, we confirmed the importance of BMPs and ID genes in the pathogenesis of APL by observing positive correlation between their expressions with the fusion oncogene PML/RARα in BM of APL patients. Molecular remission and enhanced expression of total RARα, able to restore RA-mediated differentiation, was paralleled by the suppression of those genes, suggesting that BMPs play important role in resistance to ATRA often seen in APL patients. It has been shown that RA promotes degradation of phosphorylated SMAD1 reducing BMP signal [21], but in our study ATRA decreased expression of BMP antagonists NOG and BAMBI, and inhibitory SMAD6, which would have the opposite effect. This suggests BMP antagonists as possible complementary therapeutic strategy for ATRA-resistant patients.

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Figure legends:

Figure 1. Effect of all-trans retinoic acid (ATRA) treatment on differentiation of NB4 and HL60 cell lines. A) Cell lines were treated with ATRA (NB4 0.5 μM, left panel; HL60 2 μM, right panel) and analyzed at several time-points (day 1 to day 4) for the morphological changes characteristic for granulocyte differentiation (standard May-Grünwald Giemsa staining, magnification 1000×, under immersion oil) and cell surface phenotype markers (CD11b/CD11c by flow cytometry) compared to ATRA-non treated control (ctrl). B) Effect of ATRA on the surface phenotype (expression of CD11b/CD11c), cell cycle distribution (G_0/G_1, S, G_2/M phases) and apoptosis (percentage of dead/apoptotic cells by Annexin V/PI staining) was further analyzed at the representative time-point (day 3 of ATRA tretment for NB4, left panel; day 4 for HL60, right panel) by flow cytometry. C) Expression of acute promyelocytic leukemia (APL) molecular markers (promyelocytic leukemia/retinoic acid receptor α (PML/RARα) fusion oncogene and total RARα receptor), bone morphogenetic protein (BMP) receptors (activin receptor (ActR)IA, BMP receptor (BMPR)IA, BMPRIB, BMPRII, ActRIIA, ActRIIB), and BMP-related molecules (inhibitors of DNA binding (ID)1, ID2, tyrosine-protein kinase cKit, small mothers against decapentaplegic (SMAD)5, SMAD6, NOGGIN (NOG), and bone morphogenetic and activin membrane-bound inhibitor (BAMBI)) in NB4 and HL60 cell lines (day 2 of ATRA tretment for NB4 and day 3 for HL60). RNA was extracted from leukemia cell lines NB4 and HL60 and analyzed by quantitative PCR. Expression of target genes was presented as RNA relative quantity and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control. Data are representative of three similar experiments.

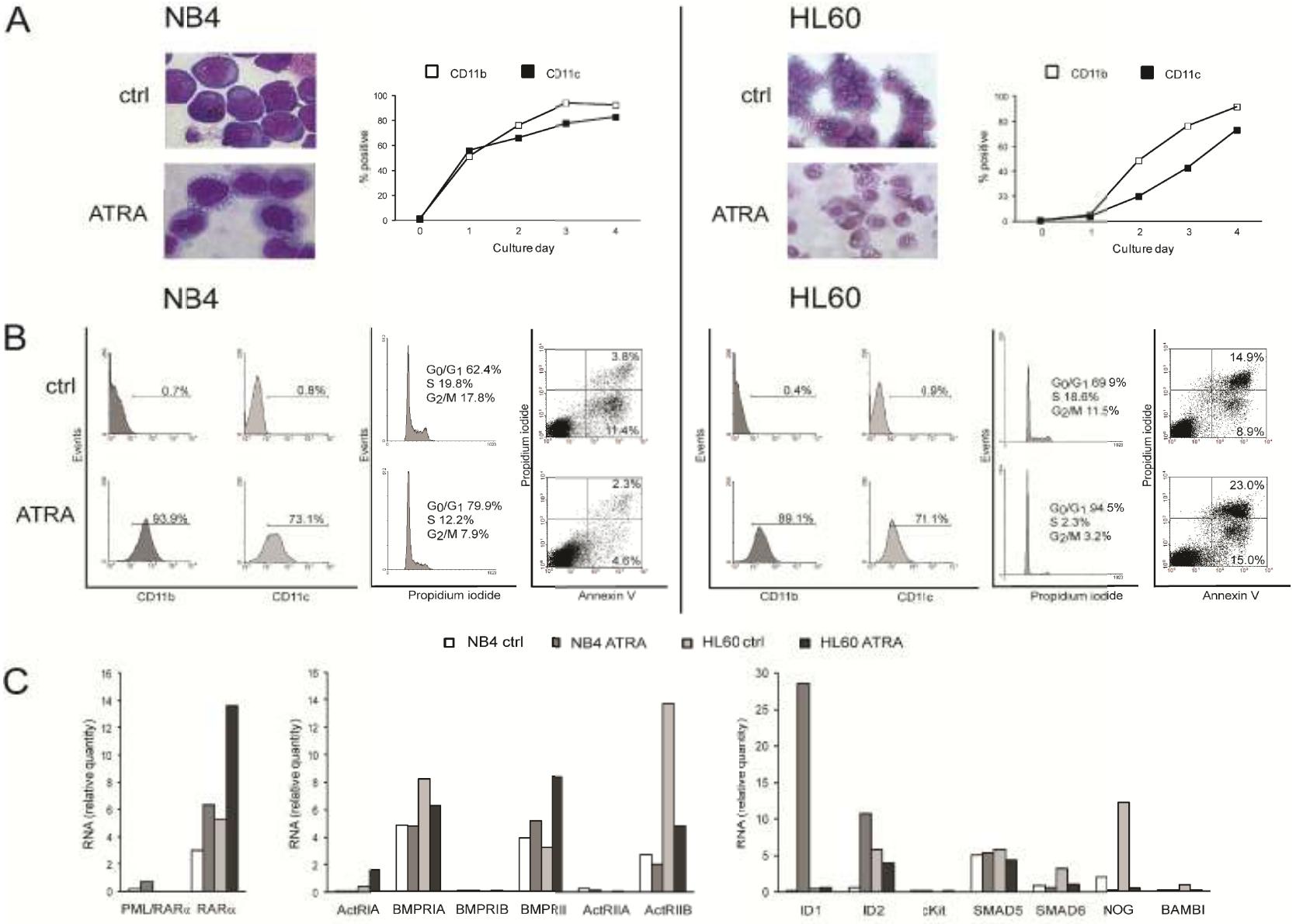

A
NB4
HL60
ctrl
ATRA
CD11b
CD11c
% positive
Culture day
B
NB4
HL60
ctrl
ATRA
0.7%
0.8%
93.9%
73.1%
G0/G1 62.4%
S 19.8%
G2/M 17.8%
G0/G1 79.9%
S 12.2%
G2/M 7.9%
3.8%
11.4%
2.3%
4.6%
0.4%
0.9%
89.1%
71.1%
G0/G1 69.9%
S 18.8%
G2/M 11.5%
G0/G1 94.5%
S 2.3%
G2/M 3.2%
14.9%
8.9%
23.0%
15.0%
Events
Propidium iodide
Annexin V
CD11b
CD11c
C
NB4 ctrl
NB4 ATRA
HL60 ctrl
HL60 ATRA
RNA (relative quantity)
PML/RARα
RARα
ActRIA
BMPRIA
BMPRIB
BMPRII
ActRIIA
ActRIIB
ID1
ID2
cKit
SMAD5
SMAD6
NOG
BAMBI

Figure 2. Effect of bone morphogenetic protein (BMP)-2, BMP-4 and BMP-6 on all-trans retinoic acid (ATRA)-induced differentiation of NB4 and HL60 cell lines. Cell lines were treated by ATRA (NB4 0.5 μM, left panel; HL60 2 μM, right panel) and BMP-2, BMP-4 or BMP-6 (25 ng/mL for all), and assessed at day 2 of ATRA treatment for NB4 and day 3 for HL60. A) Cells were analyzed for morphological changes of differentiation (standard May-Grünwald Giemsa staining, magnification 1000×, under immersion oil) and cell surface phenotype (expression of CD11b/CD11c by flow cytometry) compared to non-treated cells (ctrl). B) Expression of genes affected by ATRA-induced differentiation (ankyrin repeat and SOCS box protein 2 (Asb2), transcription factor PU.1, CCAAT-enhancer-binding-proteins (C/EBP)ε and C/EBPα, ubiquitin-conjugating enzyme (UBE)2D3), tyrosine-protein kinase cKit, apoptotic regulator B-cell lymphoma (Bcl)-2, and distal-less homebox (Dlx)4) in NB4 and HL60 cell line. RNA was extracted from myeloid leukemia cell lines NB4 and HL60 after treatment with ATRA and BMPs, and analyzed by quantitative PCR. Expression of target genes was presented as RNA relative quantity and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control. Data are representative of three similar experiments.

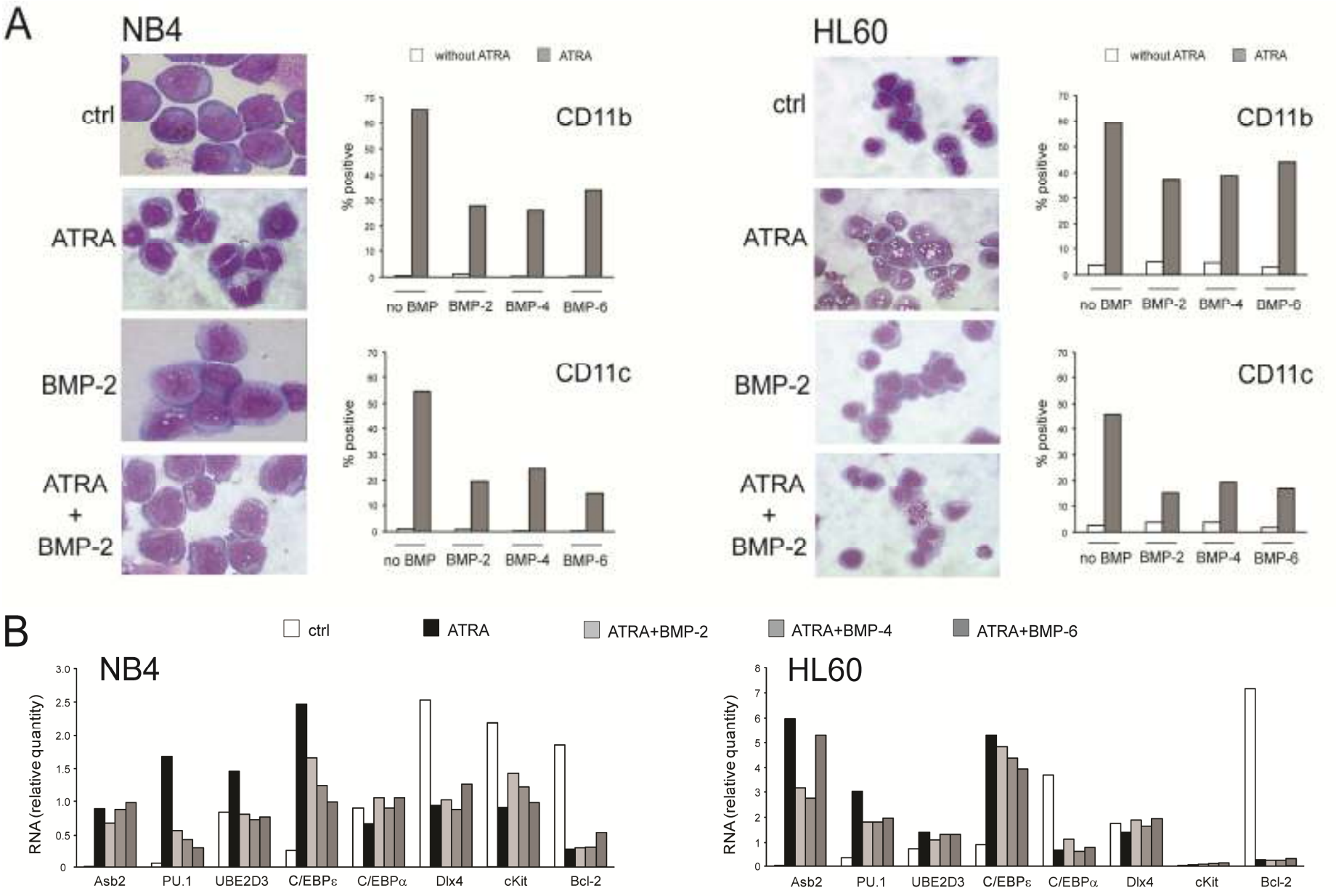
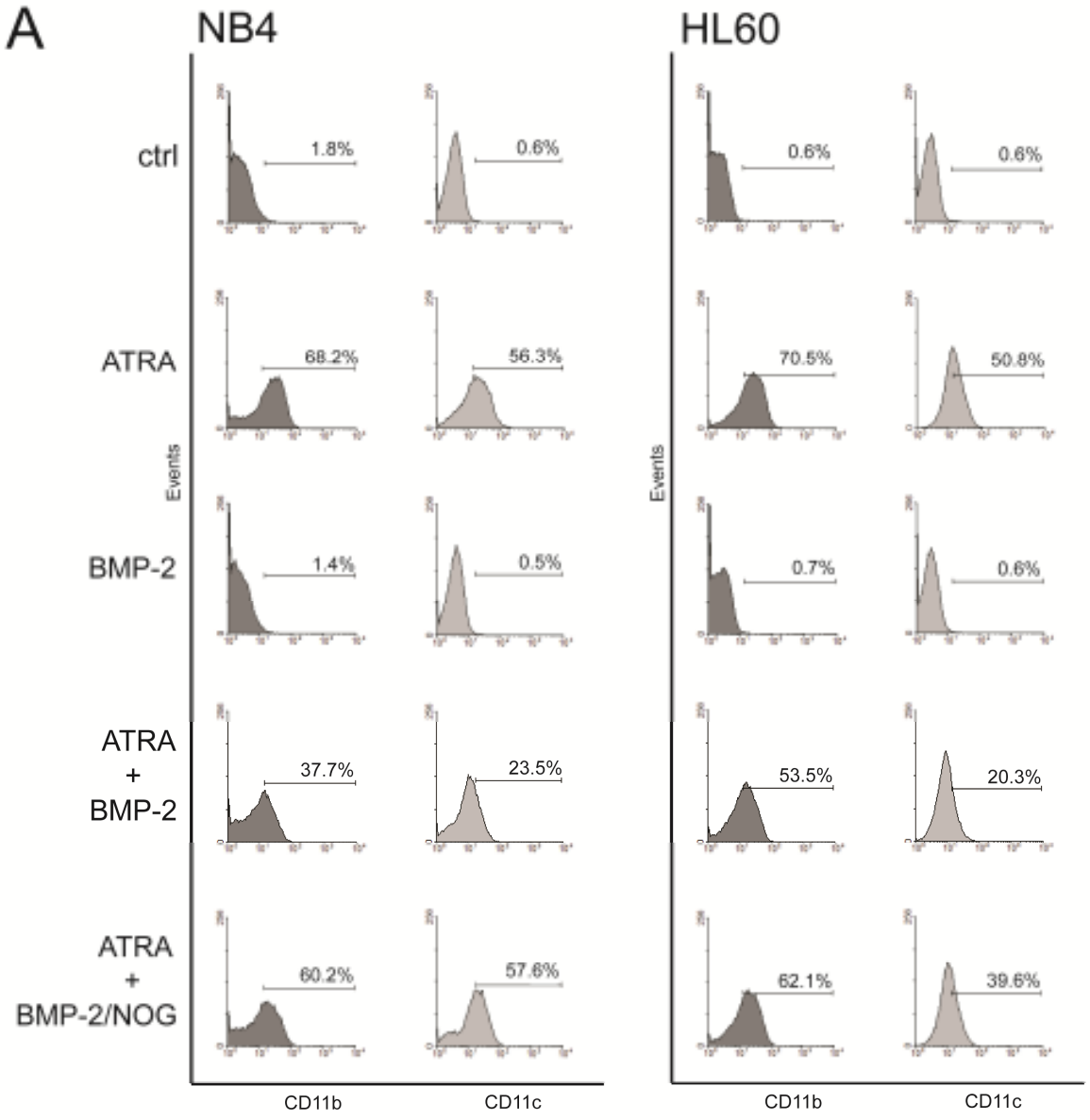

A
NB4
ctrl
ATRA
BMP-2
ATRA + BMP-2
without ATRA
ATRA
CD11b
% positive
no BMP
BMP-2
BMP-4
BMP-6
CD11c
HL60
B
ctrl
ATRA
ATRA+BMP-2
ATRA+BMP-4
ATRA+BMP-6
RNA (relative quantity)
Asb2
PU.1
UBE2D3
C/EBPε
C/EBPα
Dlx4
cKit
Bcl-2

Figure 3. Effect of bone morphogenetic protein (BMP)-2 and NOGGIN (NOG) on NB4 and HL60 cells treated with all-trans retinoic acid (ATRA). Cell lines were treated with different combinations of ATRA (NB4 0.5 μM, left panel; HL60 2 μM, right panel), BMP-2 (25 ng/mL) and NOG (100 ng/mL). A) Cells were analyzed for cell surface phenotype markers (CD11b/CD11c) by flow cytometry, at day 1 of ATRA treatment for NB4 and day 2 for HL60, in non-treated (ctrl) and treated samples (ATRA, BMP-2, ATRA+BMP-2 and ATRA+BMP-2/NOG). Data are representative of three similar experiments. B) Expression of inhibitors of DNA binding (ID)1, ID2 in NB4 and HL60 cell lines and tyrosine-protein kinase cKit in NB4 cell line. RNA was extracted from leukemia cell lines NB4 and HL60 after 12 hours or 24 hours of treatment, respectively, and analyzed by quantitative PCR. Expression of target genes was presented as RNA relative quantity and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control. Data were presented as triplicate mean ± standard deviation (SD) and analyzed using analysis of variance with Student-Newman-Keuls *posthoc* test; $p \leq 0.05$ were considered statistically significant. *, indicates statistically significant difference compared to BMP-2 or ATRA alone; **, indicates statistically significant difference compared to combined ATRA/BMP-2 treatment.


A
NB4
HL60
ctrl
ATRA
BMP-2
ATRA + BMP-2
ATRA + BMP-2/NOG
Events
1.8%
0.6%
0.6%
0.6%
68.2%
56.3%
70.5%
50.8%
1.4%
0.5%
0.7%
0.6%
37.7%
23.5%
53.5%
20.3%
60.2%
57.6%
62.1%
39.6%
CD11b
CD11c
CD11b
CD11c

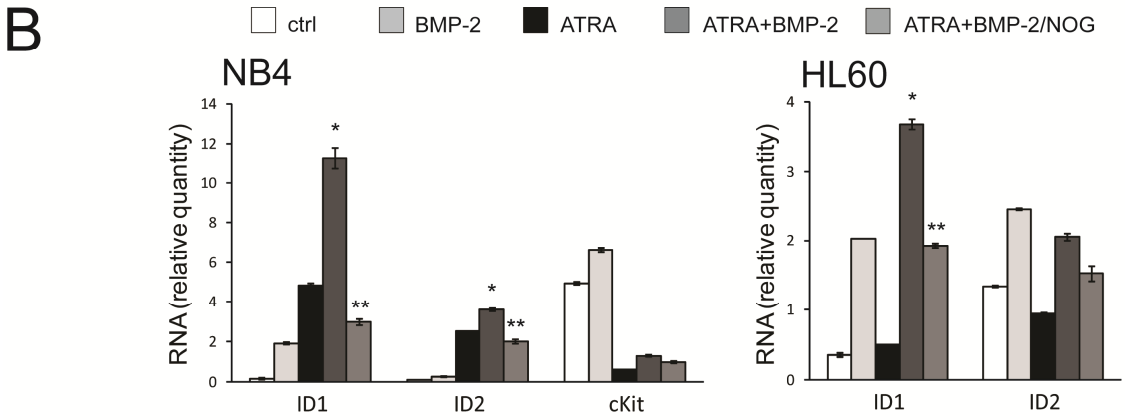

B
ctrl
BMP-2
ATRA
ATRA+BMP-2
ATRA+BMP-2/NOG
NB4
HL60
RNA (relative quantity)
ID1
ID2
cKit
ID1
ID2

Figure 4. Five patients with the diagnosis of acute promyelocytic leukemia before and after the combined cytostatic/all-trans retinoic acid (ATRA) treatment. Bone-marrow (BM) and peripheral blood (PBL) samples were collected from patients at the time of diagnosis (the first presented time-point in all patients) and at clinical follow-up time-points during ATRA treatment (7 time points for patient #1, 5 for #2, 2 for #3, 6 for #4 and 3 for #5). Diagnosis was established according to French-American-British classification for AML-M3. A) Expression pattern of bone morphogenetic protein (BMP)-2, BMP-4 and BMP-6, and BMP-related genes (bone morphogenetic and activin membrane-bound inhibitor (BAMBI), BMP antagonist NOGGIN (NOG), inhibitors of DNA binding (ID)1, ID2, tyrosine-protein kinase cKit, transcription factor PU.1, ubiquitin-conjugating enzyme (UBE)2D3, CCAAT-enhancer-binding-proteins (C/EBP)ε) was analyzed in BM samples by quantitative (q)PCR. B) The expression of fusion oncogene promyelocytic leukemia/retinoic acid receptor α (PML/RARα) and total RARα was verified in BM and PBL at corresponding time-points by qPCR. Expression of target genes was presented as RNA relative quantity and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control. nd, not determined.

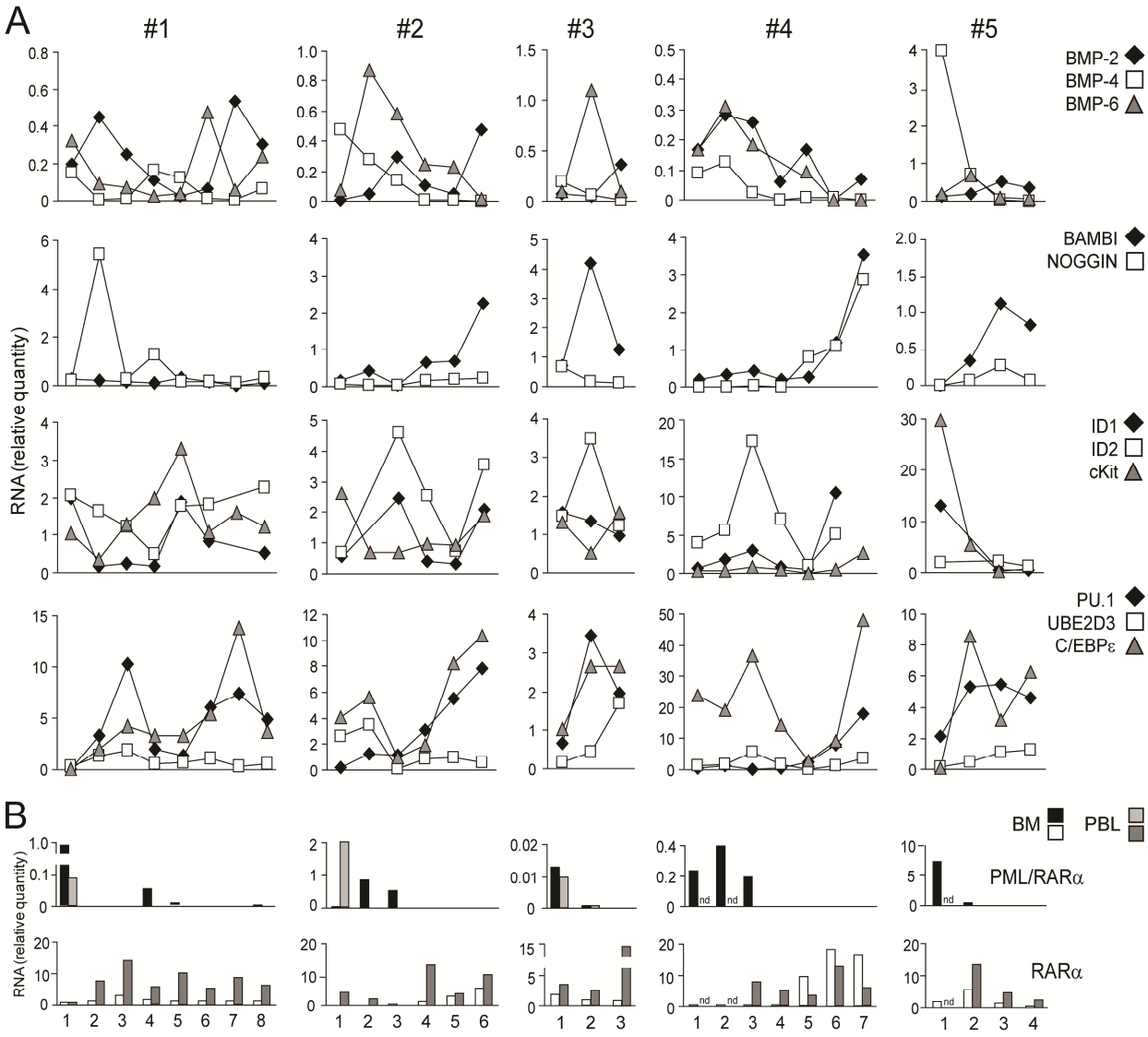

A
#1
#2
#3
#4
#5
BMP-2
BMP-4
BMP-6
BAMBI
NOGGIN
ID1
ID2
cKit
PU.1
UBE2D3
C/EBPε
RNA (relative quantity)
B
BM
PBL
PML/RARα
RARα
RNA (relative quantity)
nd

Table 1. Correlation of the expression of BMPs and BMP-related genes with the disease molecular marker PML/RARα and total RARα receptor

PML/RARα vs*	Spearmans rho (95% CI), p**	RARα vs	Spearmans rho (95% CI), p
BMP-2	-0.17 (-0.51 to 0.22), p=0.385	BMP-2	0.01 (-0.36 to 0.38), p=0.952
BMP-4	0.82 (0.65 to 0.91), **p<0.001**	BMP-4	-0.22 (-0.55 to 0.17), p=0.252
BMP-6	0.51 (0.16 to 0.74), **p=0.010**	BMP-6	-0.48 (-0.72 to -0.12), **p=0.015**
ID1	0.46 (0.06 to 0.73), **p=0.029**	ID1	-0.10 (-0.48 to 0.32), p=0.642
ID2	0.29 (-0.13 to 0.62), p=0.160	ID2	-0.34 (-0.65 to 0.08), p=0.106
cKit	-0.49 (-0.73 to -0.15), **p=0.011**	cKit	0.43 (0.07 to 0.69), **p=0.025**
BAMBI	-0.55 (-0.77 to -0.23), **p=0.004**	BAMBI	0.69 (0.42 to 0.84), **p<0.001**
NOGGIN	0.16 (0.22 to 0.51), p=0.398	NOGGIN	0.07 (-0.31 to 0.43), p=0.716
PU.1	-0.68 (-0.84 to -0.41), **p<0.001**	PU.1	0.68 (0.42 to 0.84), **p<0.001**
UBE2D3	-0.14 (-0.49 to 0.25), p=0.474	UBE2D3	-0.33 (-0.63 to 0.05), p=0.088
C/EBPε	-0.14 (-0.48 to 0.25), p=0.481	C/EBPε	0.01 (-0.37 to 0.38), p=0.975

* Correlations between gene expression of fusion promyelocytic leukemia/retinoic acid receptor α (PML/RARα) or total RARα receptor with bone morphogenetic proteins (BMP) and BMP-related genes (inhibitors of DNA binding (ID)1, ID2, cKit, NOGGIN, bone morphogenetic and activin membrane-bound inhibitor (BAMBI), transcription factor PU.1, CCAAT-enhancer- binding-proteins (C/EBP)ε and ubiquitin-conjugating enzyme (UBE)2D3) in bone-marrow samples of acute promyelocytic leukemia patients analyzed by quantitative PCR.

** Gene expression values were analyzed using rank correlation and Spearman's coefficient of rank correlation rho (ρ) with its 95% confidence interval (CI). Significant correlations ($p \leq 0.05$) are presented in bold.

Supplementary table 1. TaqMan assays used for quantitative PCR analysis

Gene*	Gene symbol	Assay ID
PML/RARα	*PML-RARA fusion*	Hs03024794_ft
RARα	*RARA*	Hs00940446_m1
BMP-2	*BMP2*	Hs00154192_m1
BMP-4	*BMP4*	Hs00370078_m1
BMP-6	*BMP6*	Hs00233470_m1
ActRIA	*ACVR1*	Hs00153836_m1
BMPRIA	*BMPR1A*	Hs01034910_g1
BMPRIB	*BMPR1B*	Hs00176144_m1
BMPRII	*BMPR2*	Hs00176148_m1
ActRIIA	*ACVR2A*	Hs00155658_m1
ActRIIB	*ACVR2B*	Hs00609603_m1
ID1	*ID1*	Hs03676575_s1
ID2	*ID2*	Hs00747379_m1
cKit	*KIT*	Hs00174029_m1
SMAD5	*SMAD5*	Hs01557453_mH
SMAD6	*SMAD6*	Hs00178579_m1
NOGGIN	*NOG*	Hs00271352_s1
BAMBI	*BAMBI*	Hs00180818_m1
Asb2	*ASB2*	Hs00387867_m1
PU.1	*SPI1*	Hs00231368_m1
UBE2D3	*UBE2D3*	Hs00701790_s1
C/EBPε	*CEBPE*	Hs00357657_m1
C/EBPα	*CEBPA*	Hs00269972_s1
Dlx4	*DLX4*	Hs00231080_m1
Bcl-2	*BCL2*	Hs00153350_m1
GAPDH	*GAPDH*	Hs99999905_m1

* Assays used for quantitative PCR analysis were commercially available and used in accord to the manufacturer recommendations (Applied Biosystems).

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Authors' contributions: IT participated in study design, collected samples, performed the experiments, and drafted the manuscript. MI and SI assisted in experiments, and helped in data analysis and interpretation. NK and AM helped in experiment design, data interpretation, and manuscripts preparation. RK participated in study design, collected clinical samples and data, and helped in manuscripts preparation. DG conceived and designed the study, participated in experiments, data analysis and interpretation, and prepared the final draft of the manuscript. All authors critically revised the manuscript and approved the final version.

Conflict of interest: The authors declare no potential conflicts of interest.