The epigenetic impact of suberohydroxamic acid and 5-Aza-2'-deoxycytidine on DNMT3B expression in myeloma cell lines differing in IL-6 expression

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Abstract. Gene inactivation of the cyclin-dependent kinase inhibitors p16[INK4a], p15[INK4b] and p21[WAF] is frequently mediated by promoter gene methylation, whereas histone deacetylases (HDACs) control gene expression through their ability to deacetylate proteins. The effect of suberohydroxamic acid (SBHA) and 5-Aza-2'-deoxycytidine (Decitabine) (DAC) treatments on the transcription of *CDKN2A*, *CDKN2B* and *CDKN1A* genes, and their effects on molecular biological behavior were examined in two myeloma cell lines, RPMI8226 and U266, which differ in p53-functionality and IL-6 expression. In both tested myeloma cell lines, a non-methylated state of the *CDKN2B* gene promoter region was detected with normal gene expression, and the same level of p15[INK4b] protein was detected by immunocytochemical staining. Furthermore, in myeloma cells treated with SBHA and DAC alone, the expression of both p15[INK4b] and p21[WAF] was significantly upregulated in RPMI8226 cells (p53-functional, without IL-6 expression), whereas in the U266 cell line (p53 deleted, expressing IL-6) only p21[WAF] expression was significantly increased. Moreover, the analysis revealed that treatment with DAC induced DNMT3B enhancement in U266 cells. In conclusion, in myeloma cells with IL-6 expression, significantly increased DNMT3B expression indicated the tumorigenic consequences of 5-Aza-2'deoxycytidine treatment, which requires careful use in diseases involving epigenetic dysregulation, such as multiple myeloma (MM).

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Abbreviations: AML, acute myelogenous leukemia; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; *CDKN2B* (p15[INK4b]), cyclin-dependent kinase inhibitor 2B; CML, chronic myelogenous leukemia; DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MDS, myelodysplastic syndrome; MtL, methylation level; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodine; SBHA, suberohydroxamic acid

Key words: multiple myeloma cell lines, DNA methylation, cyclin-dependent kinase inhibitor, interleukin-6

Introduction

Multiple myeloma (MM) involves crosstalk between the immune and bone systems, and as a blood cancer, homes into the bone marrow (BM) micro-environment, which is strongly tied to overexpression of interleukin-6 (IL-6) and bone loss. Like their normal counterpart, MM cells mainly proliferate and survive within the bone marrow by physiological and functional interactions with bone marrow stromal cells (BMSCs) and the surrounding BM micro environment. The later plays a central role in the pathogenesis of MM and these interactions

have shown to be important in myeloma cell survival and progression (1-3). BMSCs produce inflammatory cytokines, such as IL-6, and they regulate the expression of cell cycle inhibitors-cyclin dependent kinase inhibitors (CDK) p21^{WAF1} and p27^{Kip1}, anti-apoptotic members of the Bcl-2 family and ABC-family drug transporters in myeloma cell directly via cell receptors and adhesion molecules. Interleukins are crucial to skeletal homeostasis and some adhesion molecules, such as cadherins, facilitate the formation of multicellular structures in the bone marrow by anchoring to the actin cytoskeleton (4,5).

Epigenetic anomalies have been identified as drivers in the development of the myeloma (6). DNA methylation is an epigenetic modification of cytosine catalyzed by DNA methyltransferases (DNMT1, DNMT3a and DNMT3b). DNA methylation leads to inhibition of gene expression. Several studies have investigated whether aberrations in DNA methylation could be expressed as disease stage-specific, thus changing during disease progression. DNA hypomethylation was reported as the predominant early change during myelomagenesis that is gradually transformed to DNA hypermethylation in relapsed cases and during the disease progression (7,8). DNA methyltransferase inhibitors, such as cytidine analogs 5-azacytidine and 5-Aza-2'-deoxycytidine (Decitabine) (DAC) are currently used to revert aberrant DNA methylation patterns. The DAC is incorporated only in DNA, whereas 5-azacytidine penetrates in both DNA and RNA, and via incorporation into newly synthesized RNA, it will interfere with RNA processing, inhibiting protein synthesis. For these reasons, the 5-azacytidine is used more often as a demethylation agent for treatment of HMCLs, though the anti-myeloma activity, supported by enhanced DNA damage, cell cycle arrest, and induction of myeloma cell apoptosis was shown in both demethylation agents (9,10).

Histone deacetylases (HDACs) are enzymes responsible for removing the acetyl group from histones, leading to suppression of gene expression. This process contributes to the undesired suppression of tumor suppressor gene expression. HDAC inhibitors are known to arrest human tumor cell activity in the G1 phase of the cell cycle and activate CDK inhibitors such as p21WAF1, the *CDKN1A* gene of the CIP/KIP family, and p15^{INK4b} and p16^{INK4a} encoded by the CDKN2B and *CDKN2A* genes belonging to the INK4 family of proteins (11,12). Ng *et al* (13) reported for the first time, the high incidences of p15^{INK4b} and p16^{INK4a} alterations in MM patients, not by homozygous deletions or mutations, but solely by hypermethylation of the 5'CpG islands. However, the methylation status and transcription of these genes were subsequently studied in MM-derived cell lines *in vitro*. An aberrant methylation of p16^{INK4a} was found in six analyzed cell lines including RPMI8226 and U266 as well. Conversely, only the HS-Sultan cell line, but not RPMI8226 and U266, showed extensive methylation in the 5'upstream region of p15^{INK4b} (14). Inhibition of histone deacetylase (HDAC) activity by HDAC inhibitors on the other hand, results in the accumulation of acetylated histones leading to altered gene transcription. The HDAC inhibitor suberohydroxamic acid (SBHA), induces apoptosis, dependent on the induction of mitochondrial membrane permeability (15). The SBHA described in melanoma cell lines (15,16) is able to enhance TRAIL-induced

cell death by the up-regulation of the pro-apoptotic proteins caspase-8, caspase-3, Bim, Bid, Bak, Bax, while downregulating, at the same time, the anti-apoptotic proteins Bcl-xL, Mcl-1, and XIAP (17). The mechanism by which SBHA regulates such a diverse array of genes involved in apoptosis is not clear. Likely, SBHA targets HDACs associated with transcriptional factors that are involved in regulation apoptosis such as p53 and c-Myc.

The *PDLIM4* gene (location 5q.31) encodes PDZ and LIM protein 4 involved in bone development as was shown in a study of *PDLIM4* gene polymorphisms in the susceptibility to osteoporotic fracture (18). LIM protein RIL is an actin associated nuclear protein containing both PDZ and LIM domains. It belongs to a large family of LIM proteins (19). PDZ and LIM domain proteins (PDLIM) are involved in the regulation of a variety of biological processes, including cytoskeletal organization, and may act as adaptors between cytoskeleton and intracellular signaling components (20). The clinical and prognostic value of the PDLIM family in multiple myeloma is still unclear. The RIL, *PDLIM4* gene, which is frequently methylated in cancer, has been described as a tumor suppressor. Its methylation is associated with altered expression in various cancers, including hematological malignancies (21-23). It is known that RIL re-expression leads to suppression of cell growth, and it sensitizes cells to apoptosis (24). In this study, we examined the methylation and expression patterns of *PDLIM4* as a possible tumor suppressor gene in two human myeloma cell lines (HMCLs). RPMI8226 and U266 (U266-B1) differ in both IL-6 expression and p53-functionality. The U266 myeloma cell line has deletions of chromosome 13 and 17p, which involve the TP53 gene (25). The p53 suppressor protein is encoded by the TP53 gene located on chromosome 17p13.1, while deletion of the chromosomal region 17p13-del (17p) is associated with poor outcome in multiple myeloma (26). The second used RPMI8226 myeloma cell line has shown deletion of neither chromosome 13, nor chromosome 17p (25). We also examined the methylation and expression pattern of another tumor-suppressor gene, *CDKN2B*, which is often methylated in multiple myeloma patients (18,26). The *CDKN2B* gene belongs to INK4 family responsible for regulation of cell cycle. Aberrant methylation of this tumor suppressor gene is considered to be an important epigenetic change in molecular pathogenesis of multiple myeloma (13,26,27). Meta-analysis study of Li *et al* pointed out the effect of *CDKN2B* gene methylation on the prognosis of the disease (28). For this reason, we compared the *CDKN2B* gene expression with the expressions of other cyclin-dependent kinases genes (*CDKN2A* and *CDKN1A*). Our goal was to investigate the *in vitro* effect of SBHA and DAC alone, on tumor-suppressor and DNA methyltransferases genes transcription and the molecular biological behavior in two selected myeloma cell lines differing in p53-functionality and IL-6 expression.

Materials and methods

Cell lines and cell culture. Cell lines RPMI8226 and U266 represent different IL-6 expression profile, IL-6 is expressed in U266, but not in RPMI8226 (7,8). Further, according to Keats Lab (www.keatslab.org), the U266 cell line has E419X

Table I. Characteristics of RPMI8226 and U266 myeloma cell lines (www.keatslab.org).

HMCL	Translocation	TP53 status	p53 expression	IL-6 dependency	(Refs.)
RPMI8226	t(14,16)	E285K	+	-	(30,57)
U266 (U266B1)	t(11,14)	A161T L36M	-	+	(30,58) (36)

Table II. Primer design for MSP-PCR.

Gene	Forward PCR primer	Reverse PCR primer
CDKN2B (U)	5'-TGAGGATTTTGTGATGTGTTT-3'	5'-CATACAATAACCAAACAACCAATCA-3'
CDKN2B (M)	5'-TGAGGATTTCGCGACGCGTTC-3'	5'-CGTACAATAACCGAACGACCGATCG-3'
PDLIM4 (U)	5'-GATGGGTTGTAGGTGTGTTAGTTG-3'	5'-CTTTTAAAATCACTTTTAAA-3'
PDLIM4 (M)	5'-GATGGGTCGTAGGTGTGTTAGTC-3'	5'-CTTTAAAATCGCTTTTAAAAACGAT-3'

missense mutation on the RB1 gene and A161T missense mutation on the TP53 gene, while the RPMI8226 cell line has E285K missense mutation on the TP53 gene (Table I). Both human multiple myeloma cell lines were purchased from ATCC (CCL155™). The cell line RPMI8226 (CCK155TM) was maintained in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics Penicillin-Streptomycin, 1% L-Glutamine and 1% 100 mM Sodium Pyruvate, while the human multiple myeloma cell line U266 (U266B1) was cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 15% fetal bovine serum (FBS), 1% antibiotics Penicillin-Streptomycin, 1% L-glutamine and 1% 100 mM sodium pyruvate. All cells were maintained at 37˚C and 5% CO_2 atmosphere.

Cell viability assay (MTT assay). Cell lines were treated with 0.5 and 5 μM DAC (Sigma-Aldrich) and 10 and 50 μM SBHA (Sigma-Aldrich) for 24 and 48 h. The RPM-I8226 cells were seeded on 96-well plates at 5,000 cells per well, and the U266 cells at 5,500 cells per well. The cell viability assay was performed in triplicate using 3-(4,5-dimethylthi-azol-2-yl)-2,5-diphenyltetrazolium bromide agent (MTT). Viable cells were detected by measuring absorbance on a spectrophotometer at a wavelength of 570 nm. The percentage of viable cells was calculated as follows: average absorbance of treated cells/average absorbance of control cells x100.

Cell cycle analysis (FACS). For fluorescence-activated cell sorting (FACS), cells were treated for 24 h (10, 50 μM SBHA, 0.5, 5 μM DAC), harvested, and fixed with 96% ice-cold ethanol overnight at -20˚C. After washing with ice cold PBS + 2% FBS, cells were incubated with 0.2 mg/ml RNAse A + PBS at RT for 30 min. Then, 200 μl of propidium iodide (PI) was added and analyzed by BD FACSVerse flow cytometry (BD Biosciences). The results were analyzed using BD FACSuite (BD Biosciences) software. The percentage of living unaffected cells was calculated to 100%, then the percentage of live treated cells was calculated as follows: x = (% of live treated cells determined by FACSuite software x100%)/% of live untreated control cells determined using FACSuite software.

Methyl-specific PCR (MSP-PCR). Genomic DNA was isolated by the Wizard® Genomic DNA Purification kit (Promega). Extracted DNA (~400 ng) was modified with sodium bisulfite using the EpiTect® Bisulfite kit according to the manufacturer's instructions (Qiagen). MethPrimer, based on Primer3 (https://www.urogene.org/methprimer/), was used for designing MSP-PCR primers specific for unmethylated (U) and methylated (M) DNA sequences (Table II). PCR products were analyzed by 2% agarose gel electrophoresis (1 h, 55 V). The percentage of methylation was subsequently quantified by methyl-specific sequencing (pyrosequencing).

Methyl-specific sequencing (Pyrosequncing). Isolated and modified DNA was used to determine the percentage of both *PDLIM4* and *CDKN2B* genes promoters methylation. DNA was isolated from both human myeloma cell lines (RPMI8226, U266) after 48 h treatment with 10, 50 μM SBHA, 0.5 and 5 μM DAC. The CpG islands identification and PCR primers design for the following pyrosequencing reaction were designed using PyroMark Assay Design SW 2.0 (Qiagen) (Table III). 1 μl of bisulfite treated DNA was added in a 25-μl PCR reaction mixture containing 12.5 μl 1X PyroMark PCR Master Mix (Qiagen), 1 μl 25 mM $MgCl_2$, 2.5 μl 1x CoralLoad Concentrate (Qiagen), 0.2 μl forward primer and 0.2 μl biotinylated reverse primer. For HotStartTaq Polymerase activation, the PCR reaction mixture was initially denatured at 95˚C for 15 min followed by 45 cycles of denaturation at 94˚C for 30 sec, annealing at 56˚C for 30 sec, elongation at 72˚C for 30 sec, and the final extension at 72˚C for an additional 10 min after the last cycle. The resulting biotinylated PCR product was immobilized on Streptavidin Sepharose® HP (GE Healthcare), precipitated with 70% ethanol, passed through a denaturation step and then a washing step using the PyroMark Q96 Vacuum Workstation (Qiagen). The amplicons were transferred to each well of the PyroMark Q96 plate containing 40 μl of 0.4 μM sequencing primer diluted in annealing buffer (Qiagen).

Table III. Primer design for pyrosequencing analysis.

Gene	Forward PCR primer	Reverse PCR primer	Sequencing primer	CpGs
PDLIM4 Promotor (216 bp)	5'-GGGTTTATGAGGA GGTATTTGAGTTG-3'	5'-biotin-ACACCCCC ACTCAACTCTC-3'	5'-TGTAGATAGTTGG GTTTGG-3'	9
CDKN2B Promotor (223 bp)	5'-AGGAGTTGAGGG TAGTGGT-3'	5'-biotin-TCCCCACC CCCTTAAACT-3'	5'-GGATATTTAGAGA GTAGTGTAGTTA-3'	13

Control samples (bisulfite modified unmethylated and methylated DNA; Qiagen) were part of a set of analyzed samples. The pyrosequencing analysis was performed according to the PyroMark CpG Software 1.0.11 (Qiagen). The methylation value was quantified in terms of the methylation level (MtL) as the average percentage of cytosines methylated per CpG: MtL (%) = ($\sum$% methylated cytosines)/No. of CpGs analyzed).

Reverse transcription-quantitative PCR (RT-qPCR). Myeloma cells were seeded in 6-well plates at a concentration of 1×10^5 cells/well, and after SBHA and DAC treatments they were harvested after 48 h. Total RNA from both cell lines was isolated with a High Pure RNA Isolation kit (Roche), and reverse transcription of 1,000 ng of total RNA was made by Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). TRI Reagent® BD (Molecular Research Centre, Inc.) was used for isolation of total RNA from unsorted BM aspirate cells. The real-time PCR was carried out using Taq-Man probes with Xceed qPCR Probe Mix (Institute of Applied Biotechnologies) using LightCycler® 480 System (Roche). The cDNA expression of *CDKN2A* (Hs00923894_m1), *CDKN2B* (Hs00793225_m1), *CDKN1A* (Hs00355782_m1), *PDLIM4* (Hs00896441_m1), *DNMT1* (Hs01041237_g1), *DNMT3A* (Hs01027162_m1), and *DNMT3B* (Hs00171876_m1) genes was normalized to the expression of endogenous housekeeping control GAPDH (Hs01041237_g1). All used probes were provided by Thermo Fisher Scientific. Untreated cells were used as the calibrator (control) for the $2^{-\Delta\Delta Cq}$ quantification approach, and commercially accessible human BM total RNA (Takara Bio USA, Inc.) for $2^{-\Delta\Delta Cq}$ quantification approach of cDNA prepared from RNA of unsorted BM patient cells. The experiments were done in triplicates, repeated in three independent measurements.

Immunocytochemical staining. Immunocytochemical staining method was used for detection of target p15INK4b and PDZ and LIM protein 4. After 48-h treatment (10, 50 μM SBHA, 0.5, 5 μM DAC) untreated control (DMSO) and treated cells (RPMI8226 and U266 cell lines) were spread on a microscope slide and fixed by mixture of acetone: methanol (1:1). After antigen recovery in citrate buffer (pH 6.0) for 15 min in 120℃, a block of endogenous peroxidase (5% H_2O_2) was performed for 15 min, followed by a block of non-specific background staining with Protein Block (Dako) for 10 min. For washing between these steps were used Tris buffer (pH 7.6). The cells were incubated over night with primary antibodies (Elabscience), and protein detection was performed using the EnVision™ Detection System Peroxidase/DAB, Rabbit/Mouse (Dako). Nuclei of cells were stained with hematoxylin and samples were dehydrated and coverslipped. Results were evaluated by ImageJ software by measuring staining intensity of microscopic photos, taken from at least five different field of vision. The results were evaluated as reciprocal staining intensity: reciprocal staining intensity = 255-measured staining intensity (29) and then converted to percentages as follows: (reciprocal staining intensity of treated cells/reciprocal staining intensity of control untreated cells) x100.

Statistical analysis. The significance of results was determined using unpaired Student's t-test with Bonferroni correction. All statistical analyses were performed using SPSS software version 20 (IBM Corp.) and P<0.05 was considered to indicate a statistically significant difference.

Results

The SBHA anti-proliferative effect in myeloma cells. The MTT assay and FACS analysis were used as an assessment of the anti-proliferative effects of both, histone deacetylase inhibitor SBHA and demethylation agent DAC. The RPMI8226 cells were more potent in regard to cell death induction than the U266 cell line, as evidenced by treatment with both tested compounds (Figs. 1-3). As depicted in Fig. 2, the U266 cells terminate the cell cycle predominantly at the G1 checkpoint and the proportion of dead cells in Sub-G1 population increased in both agents gradually, in a dose-dependent manner compared to the untreated U266 cell culture. Fig. 3 shows the differences in the response of both myeloma lines to used agents, with a more pronounced effect after 48 h of treatment in RPMI8226 cell line. The increase in sub-G1 fraction in RPMI8226 cells treated with 50 μM SBHA (29.8%) (Fig. 1) contrasts with the G1 accumulation observed after treatment of U266 cells with 50 μM SBHA (8.4%) (Fig. 2), which indicates a difference in cell cycle regulation between these cell lines. For this reason, the cyclin-dependent kinase inhibitors p15[INK4b], p16[INK4a], p21[WAF1] cDNA expression was analyzed (Fig. 4).

Different CDKs and DNMTs expressions in myeloma cell lines treated with SBHA and DAC. The significant (P<0.05) increased expression of *CDKN2B* gene was detected in RPMI8226 cells after treatment of both 0.5 and 5 μM DAC concentrations (Fig. 4A; Table IV), while 10 and 50 μM SBHA treatments resulted to significant (P<0.0001) increase
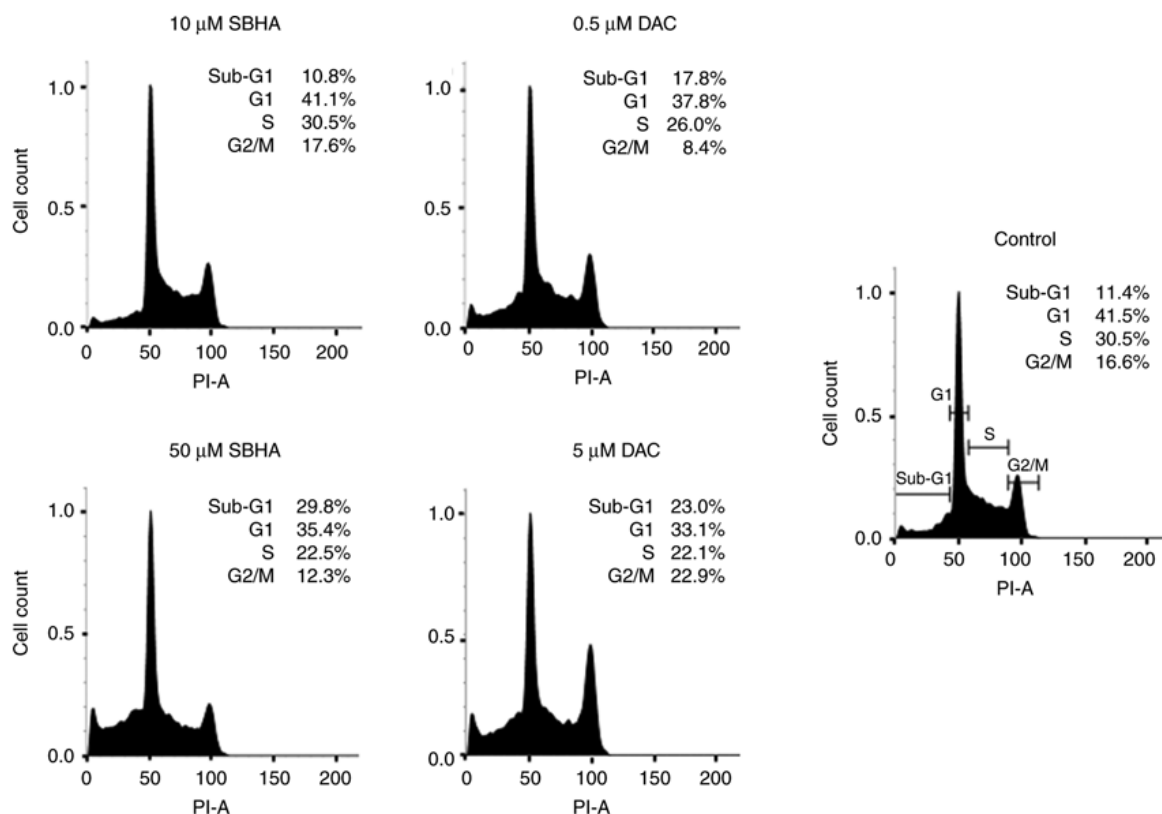
Figure 1. Cell cycle analysis of RPMI 8226 cells after 24-h of 10 and 50 μM SBHA, 0.5 and 5 μM DAC treatments. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; PI, propidium iodide; SBHA, suberohydroxamic acid.
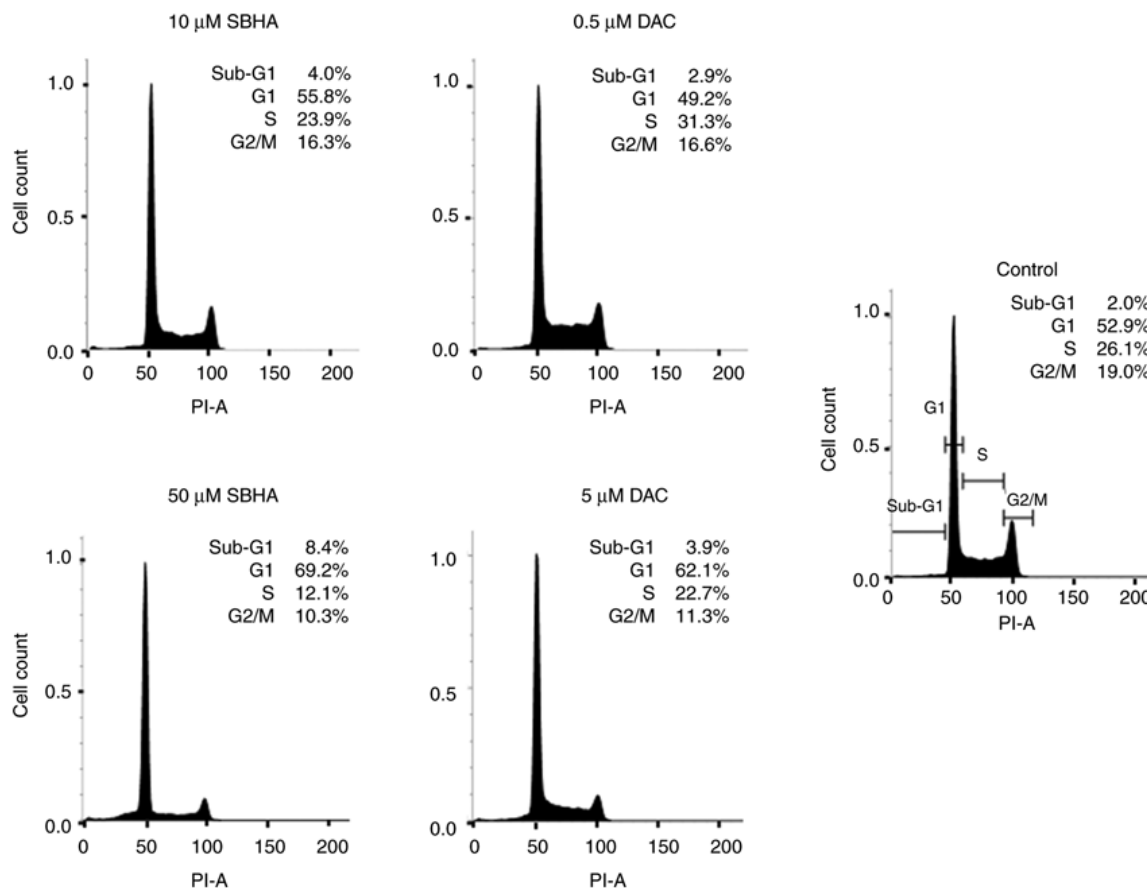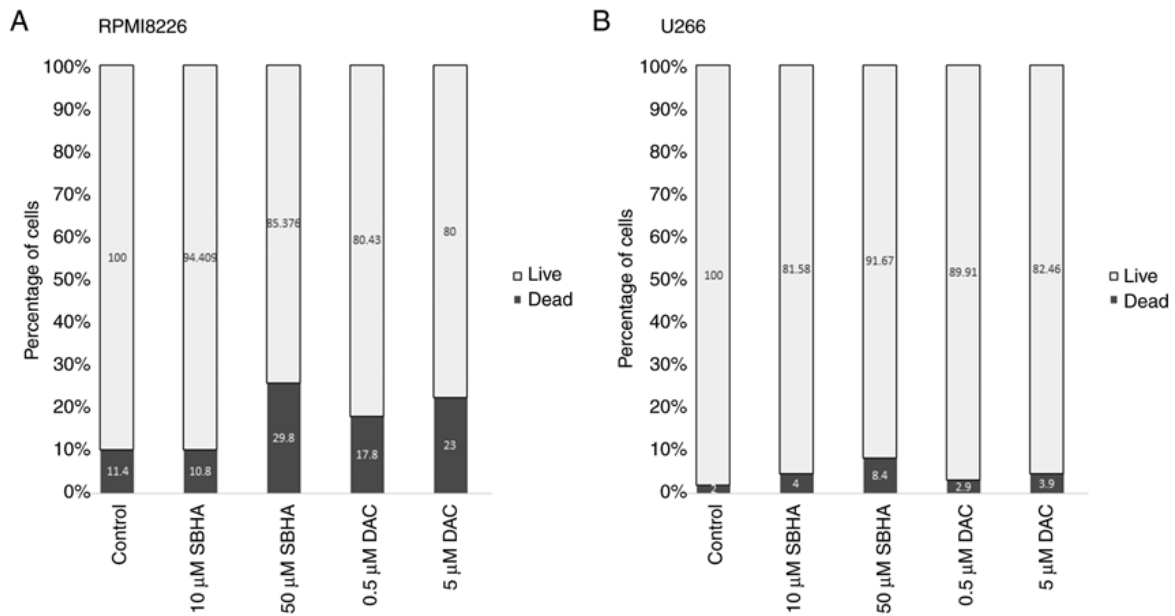

Figure 2. Cell cycle analysis of U266 cells after 24-h of 10 and 50 μM SBHA, 0.5 and 5 μM DAC treatments. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; PI, propidium iodide; SBHA, suberohydroxamic acid.

Figure 3. Effects of 10 and 50 μM SBHA, and 0.5 and 5 μM DAC on the live (MTT assay)/dead (FACS analysis, Sub-G1 phase, Figs. 1 and 2) percentage cell ratios after incubation with compounds for 24 h in (A) RPMI8226 and (B) U266 cells. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; SBHA, suberohydroxamic acid.
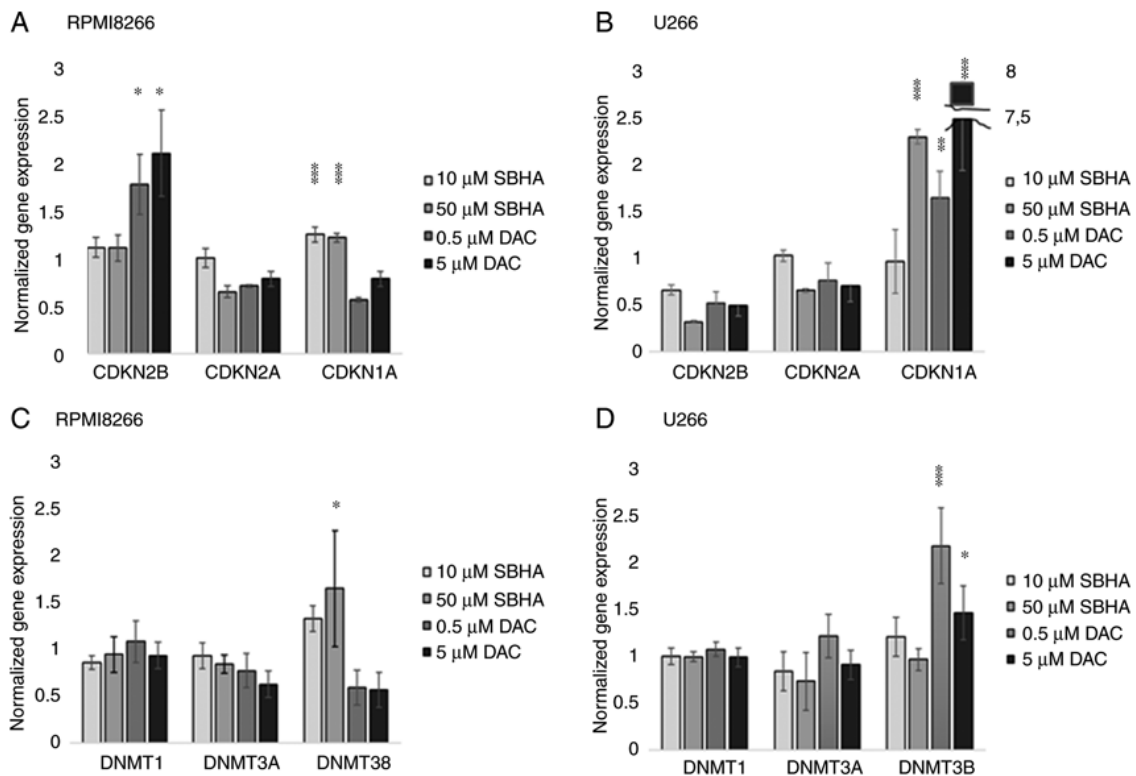

Figure 4. The cDNA expression profile of p15^{INK4b}, p16^{INK4a}, p21^{WAF1} in (A) RPMI8226 and (B) U266 after 48-h treatment with SBHA (10 and 50 μM) and DAC (0.5 and 5 μM). The cDNA expression profile of DNMT1, DNMT3A and DNMT3B in (C) RPMI8226 and (D) U266 cell lines treated with SBHA (10 and 50 μM) and DAC (0.5 and 5 μM) for 48 h. The significance was determined using unpaired Student's t-test with Bonferroni correction. *P<0.05 vs. Control (DMSO), **P<0.01 vs. Control (DMSO) and ***P<0.001 vs. Control (DMSO). The groups were normalized to the control (DMSO) group, which was set at 1. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MtL, methylation level; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodide; SBHA, suberohydroxamic acid.

the *CDKN1A* expression in U266 cells (Fig. 4B). A similar significant increase in CDKN1A expression was detected in U266 cells treated with both used DAC concentrations, and 10 μM SBHA as well (Fig. 4B). Conversely, the *CDKN2A* gene expression was not significantly changed, although this gene is methylated in both of these myeloma cell lines (25,28).

Table IV. Expression profile of CDK genes in RPMI8226 and U266 cell lines after 48 h treatment with SBHA (10 and 50 μM) and DAC (0.5 and 5 μM).

Cell treatments	CDKN2B	CDKN2A	CDKN1A
RPMI8226			
10 μM SBHA	1.123±0.10	1.010±0.10	1.260±0.08[c]
50 μM SBHA	1.121±0.14	0.656±0.06	1.227±0.04[c]
0.5 μM DAC	1.790±0.32[a]	0.715±0.01	0.569±0.03
5 μM DAC	2.122±0.46[a]	0.791±0.08	0.791±0.08
U266			
10 μM SBHA	0.662±0.05	1.031±0.06	0.970±0.34
50 μM SBHA	0.318±0.01	0.657±0.02	2.750±1.19
0.5 μM DAC	0.522±0.12	0.764±0.18	1.655±0.28[b]
5 μM DAC	0.494±0.11	0.707±0.17	7.811±0.55[c]

The significance was determined using unpaired Student's t-test with Bonferroni correction. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$. All data are expressed as mean + SD. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MtL, methylation level; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodide; SBHA, suberohydroxamic acid.

Table V. Expression profile of DNMTs genes in U266 and RPMI8226 cell lines after 48 h treatment with SBHA (10 and 50 μM) and DAC (0.5 and 5 μM).

Cell treatments	DNMT1	DNMT3A	DNMT3B
RPMI8226			
10 μM SBHA	0.858±0.07	0.929±0.14	1.324±0.14
50 μM SBHA	0.943±0.19	0.842±0.10	1.865±0.66[a]
0.5 μM DAC	1.080±0.22	0.772±0.18	0.591±0.19
5 μM DAC	0.932±0.14	0.624±0.14	0.567±0.19
U266			
10 μM SBHA	1.005±0.09	0.843±0.21	1.216±0.21
50 μM SBHA	0.999±0.05	0.736±0.31	0.968±0.12
0.5 μM DAC	1.080±0.07	1.222±0.23	2.192±0.4[b]
5 μM DAC	0.992±0.10	0.912±0.16	1.469±0.29[a]

The values of DNMTs expression of the treated cells were normalized to the expression values of the unaffected/control cells. The significance was determined using unpaired Student's t-test with Bonferroni correction. [a]$P<0.05$, [b]$P<0.001$. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MtL, methylation level; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodide; SBHA, suberohydroxamic acid.

An expression analysis of DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) showed significantly increased relative *DNMT3B* ($P<0.001$) gene expression after 0.5 μM ($P<0.001$) and 5 μM DAC ($P=0.05$) treatments in U266 cell line (Fig. 4D). On the contrary, in RPMI8226 cells, the significantly increase *DNMT3B* gene expression was determined after their treatment with 50 μM SBHA ($P<0.05$) (Fig. 4C; Table V).

Expression profile of the unmethylated CDKN2B and the methylated PDLIM4. To determinate the methylation state of promoter sequences, gene methylation status was performed by MSP-PCR with primers specific for unmethylated (U) and methylated (M) promoter sequences. The unmethylated *CDKN2B* gene promoter sequence was detected in both untreated myeloma cell lines, RPMI8226 (3%) and U266 (3%) (Figs. 5 and 6), a methylation was therefore not determined after epigenetic treatment. Moreover, the 62% average promoter *PDLIM4* gene methylation in RPMI8226 (Figs. 5A and 7B), and 59% in U266 (Figs. 6A and 8B), contrasted with the unmethylated state of the *CDKN2B* gene promoter (3%) (Figs. 5B and 6B). The unmethylated *CDKN2B* promoter analyzed region in both myeloma cell lines was detected whereas the *PDLIM4* analyzed promoter region was detected as methylated in control cells (DMSO) (Figs. 7 and 8). These results were confirmed quantitatively by the pyrosequencing, when 13 CpGs of the *CDKN2B* and 9 CpGs of the *PDLIM4* were analyzed. In RPMI82226, but not U266, cells, 0.5 μM DAC demethylation treatment caused significant reduction of *PDLIM4* promoter MtL to 44% in comparison to untreated cells (P-value 0.004; $P<0.05$) (Figs. 9 and 10). Although the methylation reduction was not significantly confirmed by elevated protein level using immunochemistry staining

detection (Fig. 11), U266 cells treated with 10 μM SBHA and 5 μM DAC showed increased normalized *PDLIM4*/RIL expression compared with that in control (DMSO) cells, detected by both RT-PCR and ICC staining methods, thus both at the cDNA and protein level (Fig. 12).

Discussion

Two human myeloma cell lines, differing in p53-functionality and IL-6 expression, RPMI8226 and U266, were used in the study (30,31). The IL-6 has been shown to regulate DNA methylation by inducing expression of FLI-1, a transcription factor of DNMT1 (32). Furthermore, IL-6 signal enhances DNMT1, which promotes the methylation with subsequent deactivation of p53 enabling cells to escape cell cycle checkpoint (33). Moreover, although the molecular mechanism by which DAC induces cancer cell death is not fully understood, the cytotoxic effect of DAC may be mediated primarily through Dnmt3a and Dnmt3b, as described in null mutant embryonic stem (ES) cells (34).

Human myeloma cell lines are widely used for their representation of primary myeloma cells because they cover patient diversity (35,36). However, the HMCLs harbor the 14q32 abnormality, which occurs early at the MGUS stage, and display frequent mutations in NRAS and KRAS genes (37,38). Tessoulin *et al* performed whole-exon sequencing of 33 HMCLs, and recurrent bi-allelic losses were found in genes involved in cell cycle regulation (*RB1, CDKN2C*), the NF-κB pathway (TRAF, BIRC2), and the p53 pathway (*TP53, CDKN2A*) (36). The U266 cell line, used in our study, contained 378 missense mutations including two ones with TP53 gene (Table I), and 231 mutations leading to different
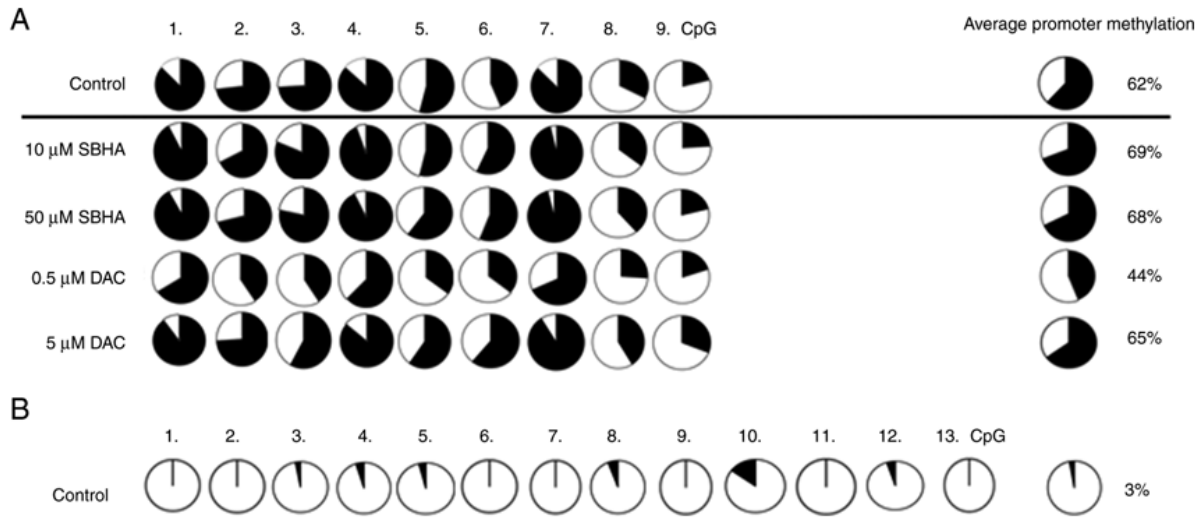
Figure 5. (A) Comparison of methylation status at 9 CpGs of the *PDLIM4* gene promoter sequence (216 bp) in control cells and after 48-h of RPMI8226 treatments (10, 10 µM SBHA, 0.5, 5 µM DAC) and (B) 13 CpGs of the *CDKN2B* gene promoter sequence (233 bp) in control cells performed by bisulfite pyrosequencing. Black areas in the pie charts represent % of methylated, white areas % of unmethylated cytosine residues. Pyromarks of sequences obtained from RPMI8226 cell line. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MtL, methylation level; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodide; SBHA, suberohydroxamic acid.
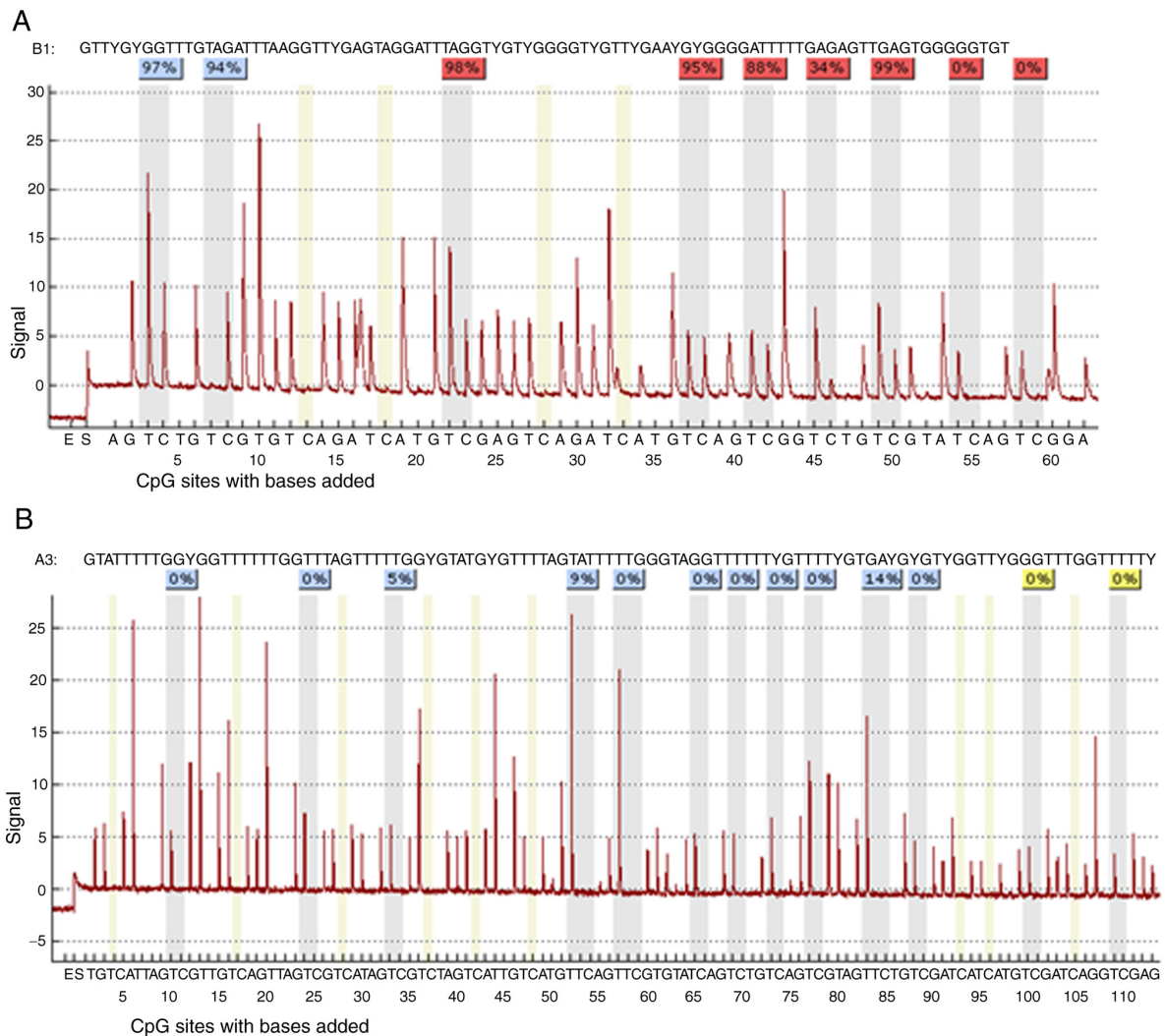

Figure 6. The sequence analyzed is the (A) *PDLIM4* and (B) *CDKN2B* genes promoters. The percentages at the top of the figure indicate the level of methylation of a particular CpG site, the resulting number for a sample is then calculated by software as the average methylation of all CpG sites of the sequence under investigation. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MtL, methylation level; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodide; SBHA, suberohydroxamic acid.
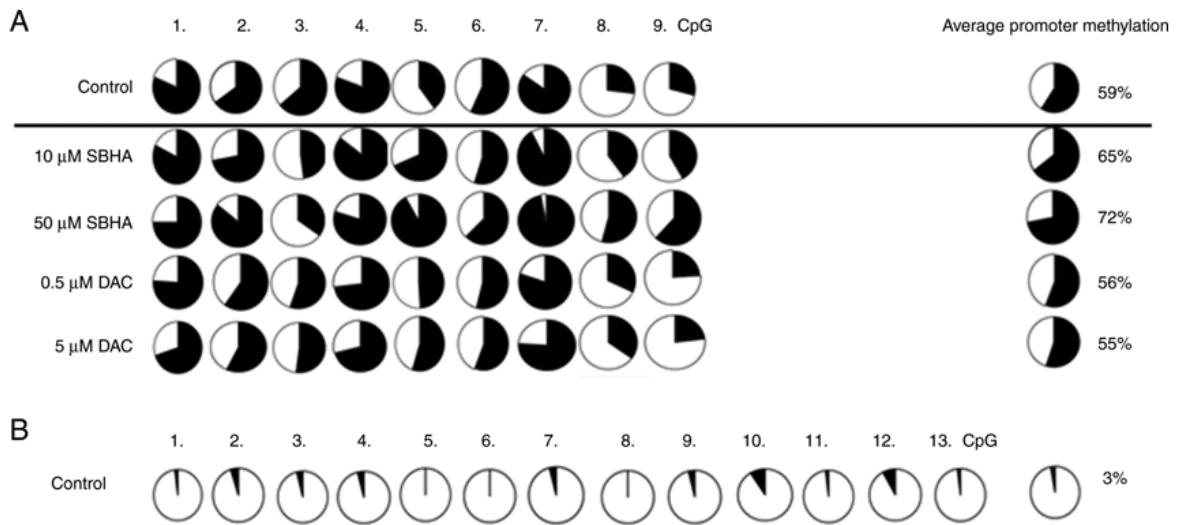
Figure 7. (A) Comparison of the methylation status at 9 CpGs of the *PDLIM4* gene promoter sequence (216 bp) in control cells and after 48-h U266 treatments (10, 10 μM SBHA, 0.5, 5 μM DAC) and (B) 13 CpGs of the *CDKN2B* gene promoter sequence (233 bp) in control cells (B) performed by bisulfite pyrosequencing. Black areas in the pie charts represent % of methylated, white areas % of unmethylated cytosine residues. Pyromarks of sequences obtained from U266 cell line. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MtL, methylation level; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodide; SBHA, suberohydroxamic acid.
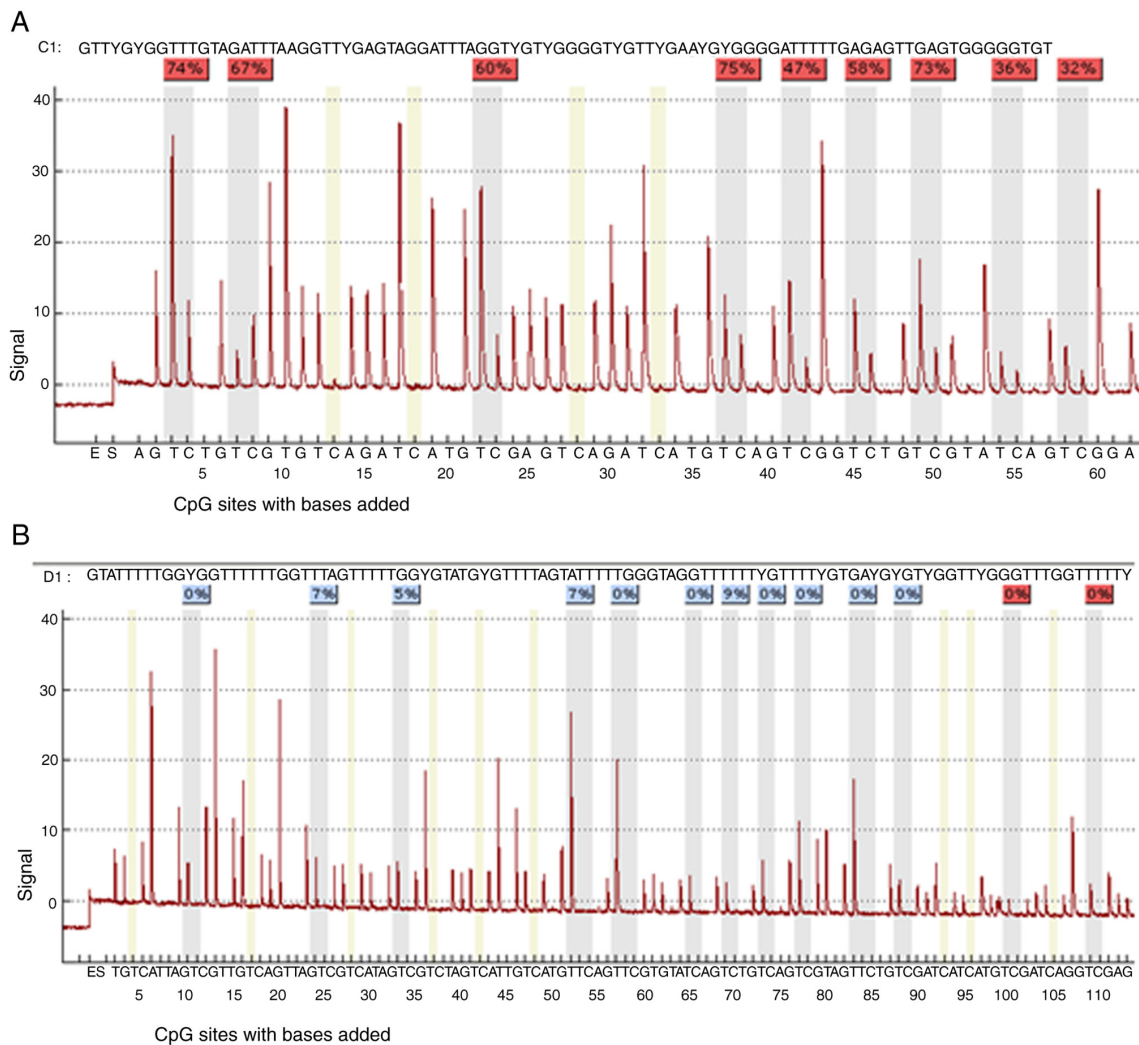

Figure 8. The sequence analyzed is the (A) *PDLIM4* and (B) *CDKN2B* genes promoter. The percentages at the top of the figures indicate the level of methylation of a particular CpG site, the resulting number for a sample is then calculated by software as the average methylation of all CpG sites of the sequence under investigation. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MtL, methylation level; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodide; SBHA, suberohydroxamic acid.
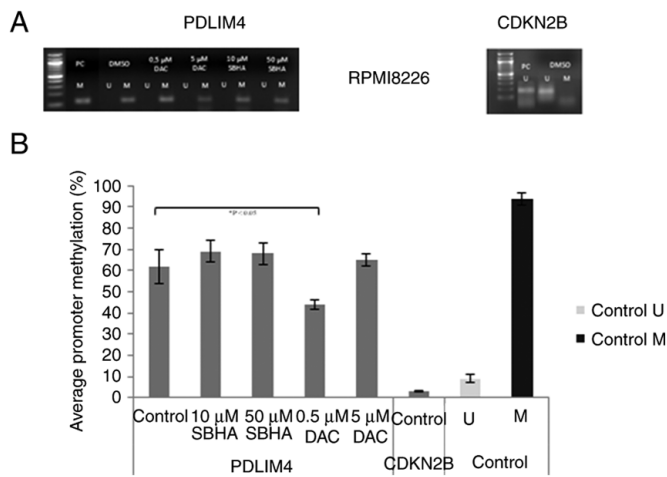
Figure 9. (A) Methylation status of *PDLIM4* and *CDKN2B* genes after 48-h treatment of the RPMI8226 cell line detected by MSP-PCR. (B) Average *PDLIM4* gene promoter methylation in treated RPMI8226 cells in comparison to average *CDKN2B* gene promotor methylation, and to unmethylated (U) (9%) and methylated (M) (94%) control DNA detected by pyrosequencing. The significance was determined using unpaired Student's t-test and Bonferroni correction. $^{*}P<0.05$ vs. PDLIM4 Control (DMSO). DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MtL, methylation level; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodide; SBHA, suberohydroxamic acid.
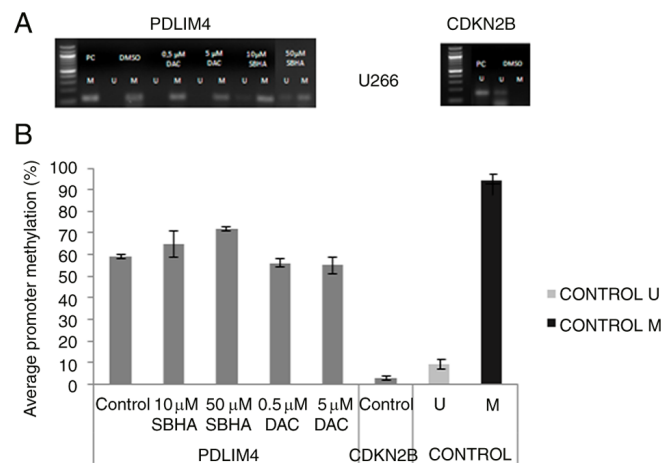

Figure 10. (A) Methylation status of *PDLIM4* and *CDKN2B* genes after 48-h treatment of the U266 cell line detected by MSP-PCR. (B) Average *PDLIM4* gene promoter methylation in treated U266 cells in comparison to average *CDKN2B* gene promotor methylation, and to unmethylated (U) (9%) and methylated (M) (94%) control DNA detected by pyrosequencing. The significance was determined using unpaired Student's t-test and Bonferroni correction. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; MtL, methylation level; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide agent; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodide; SBHA, suberohydroxamic acid.

gene silencing. In addition to mutually exclusive and associated mutations/deletions in genes involved in the MAPK and p53 pathways, were identified in epigenetic regulator/modifier genes, such as histone methyltransferases and DNA methylation modifiers (35,39). Above these findings, up to 53% of MM patients may present with mutated histone acetylation-, DNA methylation-, and chromatin remodeling-related genes (40).

Since the identification of IL-6 was used as a main growth factor for myeloma cells in the past, there is a large cohort of HMCLs, culturing primary myeloma cells from patients with extramedullary proliferation with IL-6 (41). Subsequent evaluation on a limited number of HMCLs lacking IL-6 type HMCLs showed that HMCLs did not reflect their genetic diversity and chromosomal abnormalities (42,43). As Moreaux *et al* described, TP53 gene abnormalities were found in 65% of HMCLs culturing without adding exogenous IL-6, while HMCLs depending on the addition of IL-6, had a trend to have less TP53 abnormalities than HMCL without IL-6, 58% vs. 81% (37). Thus, HMCLs are heterogenous in term of how they were obtained, IL-6-dependence, phenotype, and gene abnormalities. In our study, the significantly increased DNMT3B expression in the DAC treated U266 cells (Fig. 4D) was determined in an IL-6-expressing U266 cell line, while the expression of the DNMT1 and DNMT3A was not affected (Fig. 4). Although this finding has not yet been published, and we may assume that the increased DNMT3B expression is due to the interaction between DAC and IL-6, it is important to mention the non-negligible effect of the large number of missense/nonsense mutations detected in the U266 IL-6-expressing cell line. Further, epigenetic silencing via DNA methylation is an alternative mechanism that can result in silencing of genes. On the contrary, acetylation of histones is associated to gene activation (44).

In the RPMI8226 cell line, the 0.5 μM 5-Aza-2'-deoxycytidine treatment caused a reduced level of the *PDLIM4* gene promoter methylation enabling the increase of gene transcription (Figs. 5 and 6), whereas in IL-6-expressing U266 cells, we found no demethylation changes after either DAC or SBHA agents (Figs. 7 and 8). It is very likely that the DAC impact leading to an increased DNMT3B expression in the IL-6-expressing cell line may be due to a repressive transcriptional mechanism including interaction between DNMT3B and IL-6. However, although the mechanisms of transcriptional repression are not fully understood yet, they probably involve interactions of methylated DNA with regulatory proteins, such as binding of methylated DNA binding proteins (MBPs). In addition, the methylated cytosines in the promoter region can bind methyl-CpG binding protein 2 (MeCP2), which form complexes with corepressors that include HDACs (45).

Bone remodeling is a balance between bone resorption and bone apposition controlled by two cell types, osteoclasts, and osteoblasts (46). Osteoblasts are responsible for bone apposition whereas osteoclasts are specialized for bone resorption. Osteoclastic precursors differentiate into mature osteoclasts after interaction with osteoblastic/stromal cells. The cell-cell interactions are necessary as well as the production of soluble factors by osteoblasts. Thus, inflammatory cytokines, such as IL-6, can modulate skeletal homeostasis and osteoclast differentiation. IL-6-type cytokines utilize the transducing receptor β-subunit gp130 as a part of a multimeric receptor complex (47). Ligand-induced oligomerization of receptor subunits activates Janus protein-tyrosine kinases (JAKs), which allows activation of the signal transducer and activator of transcription (STATs, predominantly STAT3) (46). Other signaling cascades known to be activated by IL-6 are phosphoinositide-3-kinase (PI3K)/AKT, and PKCδ (48,49). However, the activation of STAT3 is necessary for osteoblast
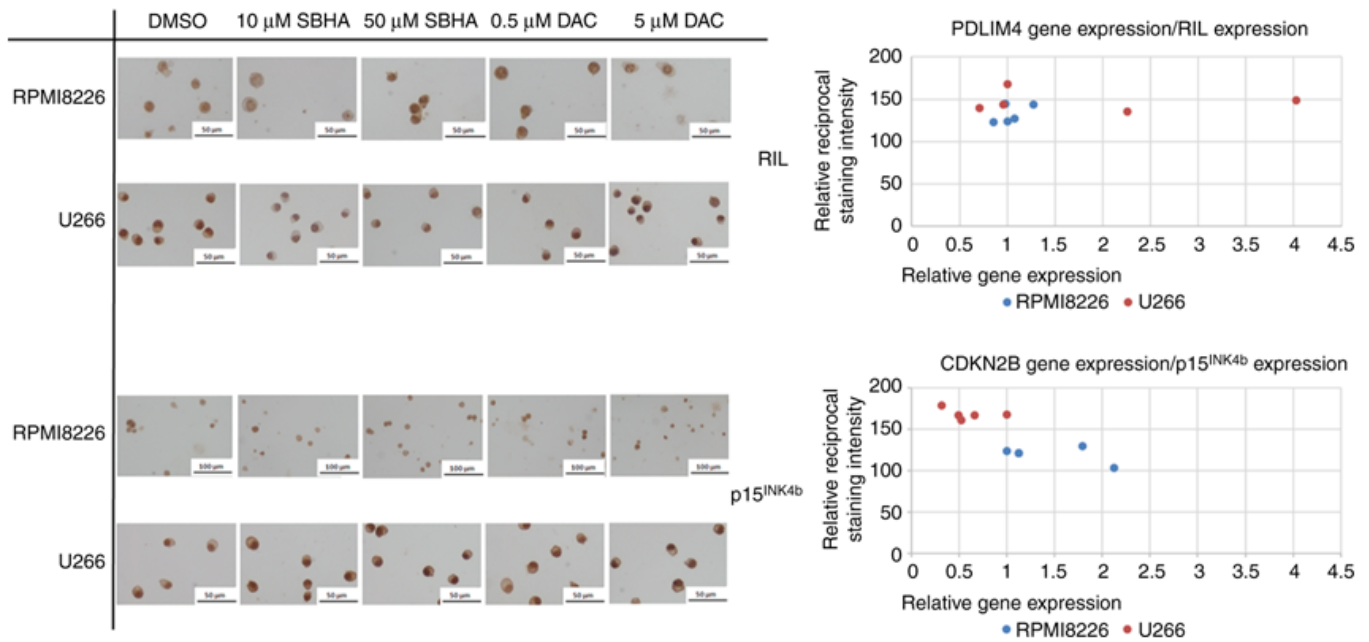
Figure 11. ICC staining of RIL and p15^{INK4b} in RPMI8226 and U266 cell lines at control cells (DMSO) and after 48 h of 10 and 50 μM SBHA, 0.5 and 5 μM DAC treatments. Reciprocal staining intensity was re-calculated to the relative reciprocal staining intensity, where the intensity of staining in control cells is considered to be 100%. Post-treatment staining intensity values were normalized to control untreated cells. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; ICC, Immunocytochemical staining; *PDLIM4* (RIL), PDZ and LIM domain 4; PI, propidium iodide; SBHA, suberohydroxamic acid.
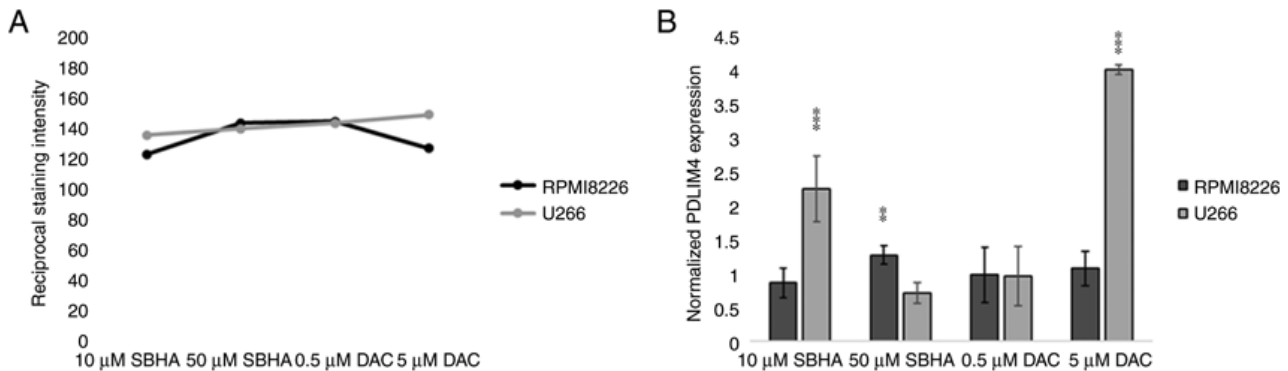

Figure 12. Comparison results of the PDLIM4/RIL relative expression obtained after (A) ICC staining and (B) RT-qPCR between RPMI8226 and U266 cell lines after 48 h of 10 and 50 μM SBHA, 0.5 and 5 μM DAC treatments. The significance in *PDLIM4* gene expression (B) was determined using unpaired Student's t-test with Bonferroni correction. $^{**}P<0.01$ vs. Control (DMSO) and $^{***}P<0.001$ vs. Control (DMSO). The groups were normalized to the control (DMSO) group, which was set at 1. DAC, 5-Aza-2'-deoxycytidine (Decitabine); DMSO, dimethyl sulfoxide; ICC, Immunocytochemical staining; *PDLIM4* (RIL), PDZ and LIM domain 4; SBHA, suberohydroxamic acid.

differentiation and bone formation induced by IL-6 (50). IL-6 can reduce proliferation of various osteoblastic cells through activation of STAT3 and enhanced expression of p21^{WAF1} (51). Then, IL-6-type cytokines can then protect osteoblastic cells from apoptosis induced by serum depletion or tumor necrosis factor α (TNFα) (52). Therefore, in our study, increased p21^{WAF1} expression detected as result of the action of both SBHA and DAC agents may not be the desirable therapeutic outcome in IL-6-expressing U266 myeloma cells.

DAC has a significant therapeutic value for treatment of patients with myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and is clinically used as a DNA methylation inhibitor for the treatment of MDS and AML (53). In MM, phase 1 trial of DAC has been performed to study its biological and clinical effectiveness as monotherapy or combined with lenalidomide or dexamethasone (54). However, its therapeutic efficacy has not been well established. In AML cells, for example, the DAC treatment resulted in the induction of p21^{WAF1}, which correlated with the arrest of AML cells in the G1 cell cycle checkpoint (55). Our analyzes reveal that the DAC treatment induced the DNA methyltransferase 3B enhancement in IL-6-expressing U266 cells.

In the current study, the cell cycle and viability of RPMI8226 and U266 cell lines were analyzed, and the increase in sub-G1 phase was found to tend to increase in the RPMI8226 cells following SBHA or DAC treatment (Fig. 1). An increase in sub-G1 and distinctly in G1 arrest were found to have a

tendency to increase in U266 cells following SBHA treatment (Fig. 2). Although, these findings do not correspond to U266 cell viability by SBHA or DAC treatment, a significant reduction in the RPMI8226 cell viability following 48 h DAC treatment may be due to a later onset of DAC (Fig. 3).

Moreover, the enhanced level of p21^{WAF1} was detected after U266 cell treatment with DAC, while in RPMI8226 cells, the DAC caused a significant increase in p15^{INK4B} expression. As was reported in several studies (56-58), the p21^{WAF1} expression is regulated through both p53-dependent and p53-independent mechanism. We found that the 50 μM SBHA treatment caused a significant increase in p21^{WAF1} expression in the p53-deleted U266 cell line (Fig. 4). This finding is in accordance with the previously described SBHA inhibiting effect of terminating the cell cycle predominantly at the G1 checkpoint (11,12), which we observed in p53-deleted U266 cells (Fig. 2), but no in RPMI8226 cells with functional p53 (Fig. 1). Moreover, SBHA was able to induce Notch1 intracellular domain levels, coupled with increase in p53 and p21^{WAF1}, and may have anti-tumor functions via regulating Notch1/p53 (59). Owing to the *in vitro* detected cyclin-dependent kinase inhibitor 2B (*CDKN2B*) (p15^{INK4B}) unmethylated promoter regions in both cell lines (Figs. 5A, B, 7A, B, 9A and 10A) the unmethylated *CDKN2B* gene may be included in the SBHA-activated cell cycle inhibiting signaling pathway in a p53-dependent manner as was detected in the RPMI8226 cell line (Fig. 4A). However, unlike studied myeloma cell lines, in MM patients *in vivo*, the methylated state of the *CDKN2B* gene has been confirmed methylated in several studies (27,60,61). On the other hand, the results of this study correspond to our previous results, indicating a low level of *CDKN2B* gene methylation and higher level of *PDLIM4* gene methylation in patients with MM (62). Additionally, the frequency of p16^{INK4a} (*CDKN2A*) hypermethylation increases with the progression of MM (18,63,64). Hence, during MM development, the methylation status of the *CDKN2B* gene can vary in individual stages of the disease, which may be further accompanied by increased frequency of *CDKN2B* gene methylation.

In conclusion, the DAC treatment induces the DNMT3B enhancement in IL-6-expressing U266 cells, which indicates the controversial role of DAC treatment as a demethylation agent in multiple myeloma patients. From the obtained data we assume that analysing of the IL-6 pathway in multiple myeloma may be a promising therapeutic target of multiple myeloma since the effect of blocking IL-6 may act at least in part through regulation of cell cycle gene expression. Moreover, in IL-6-expressing cells, the increased expression of cyclin dependent kinase inhibitor p21^{WAF} may not be indicative of the desired multiple myeloma cell apoptosis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

KST, PL and JM designed the study, and wrote and revised the manuscript; PL, DWD, LJ, IF and DP performed the experiments; KC, JG and MH analysed the dataset. All authors read and approved the final manuscript. KST and JM confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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