DNA Methylation in ATRA-treated Leukemia Cell Lines Lacking a PML–RAR Chromosome Translocation

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Abstract. *A deficient retinoic acid signaling has been suggested to be an important cause of the clinical inefficacy of all-trans retinoic acid (ATRA) therapy in non-promyelocytic (non-PML) forms of acute myeloid leukemia (AML). The general aim of the present work was to explore novel ways to take advantage of the anti-leukemic potential of ATRA, and, specifically, to search for a synergism between ATRA and epigenetic drugs. Because previous reports have found no major influence of ATRA on DNA methylation, we investigated whether ATRA-mediated differentiation of the U937 and HL-60 AML cell lines, both lacking a PML–retinoic acid receptor (RAR) fusion product, is accompanied by early-appearing and weak changes in CpG methylation. We report that in HL-60 cells, by using a highly quantitative analysis of a set of genes found to be abnormally expressed in AML, polymerase chain reaction (PCR)-amplified p16 gene promoter molecules (each with 15 CpG sites), exhibited a CpG methylation level of 0-4% in untreated cells, which increased to 4-21% after treatment with ATRA for seven days. In contrast to HL-60 cells, U937 cells exhibited a very high CpG methylation level in p16, and ATRA did not influence the promoter methylation of this gene. In the total CCGG sites of the genome, analysed using a methylation-sensitive restriction enzyme, CpG methylation was significantly lower in ATRA-treated HL-60 ($p<0.01$) and U937 cells ($p<0.05$) than in controls. Taken together, our findings show that ATRA can influence DNA methylation, and suggest that future research should investigate whether epigenetic modulation may evoke a clinical effect of ATRA in leukemia.*

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All-trans retinoic acid (ATRA) induces clinical remission in patients with promyelocytic leukemia (PML), by causing differentiation and apoptosis of the leukemia cell population; a similar effect can be seen in many cell lines representing acute myelogeneous leukemia (AML) types harbouring no translocation engaging the retinoic acid (RA) receptor (RAR) gene. It has been suggested that a deficiency in RA signalling is an important determinator of the clinical inefficacy of ATRA therapy for patients with forms of AML other than the PML subtype (1-3), and studies on mixed lineage leukemia (MLL) forms of acute lymphocytic leukemia lines have shown that ATRA induces differentiation by mechanisms other than those found in PML (4). Several lines of evidence indicate an important role of modulation of DNA methylation for the different effects of ATRA in many solid cancer and leukemia cell lines. For example, *p16* de-methylation in hepatoma lines is required for ATRA-induced senescence (5), furthermore, epigenetic modulation can potentiate ATRA-induced differentiation in non-PML leukemia cell lines (6-8).

The overall aim of the present study was to explore novel ways to take advantage of the anti-leukemic potential of ATRA. Specifically, our work is an attempt to document whether the anti-leukemic influence of ATRA on two non-PML AML lines, U937 and HL-60, both lacking a PML–RAR fusion product, is accompanied by changes in DNA methylation. Although the expression level of many genes is clearly altered during ATRA treatment, previous reports have found no major influence of ATRA on DNA methylation (6, 9). Therefore, our analysis was designed to focus on early-appearing and weak changes (so called 'seeding') (10-13) in CpG methylation, using a highly quantitative analysis of promoter CpG islands of a set of genes found to be abnormally expressed in AML, as well as of the total CCGG sites in the genome, after up to seven days of *in vitro* exposure to ATRA.

Materials and Methods

Cells and ATRA treatment. U937 and HL-60 cells were purchased from the American type culture collection (ATCC, Manassas, VA, USA). U937 cells were cultured in RPMI–1640 supplemented with

10% fetal bovine serum, and HL-60 cells in Iscove's modified Dulbecco's media (IMDM) supplemented with 20% fetal bovine serum, at 37˚C in a humidified atmosphere with 5% CO_2. ATRA (Sigma, St Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO):ethanol (5:1) and added to a final concentration of 1 μM. Cells were washed and suspended in fresh culture medium, with ATRA or DMSO, as indicated, after the first 72 h and then every 48 h.

Cellular differentiation analysis. Expression of integrin alpha X (CD11c) and CD38 was determined by flow cytometry, using a FACS Calibur (BD Biosciencies, Franklin Lakes, NJ, USA) and FCS Express software (De Novo Software, Los Angeles, CA, USA), and anti-CD11c/phycoerythrin and anti-CD38/fluorescein (BD Biosciencies). Background autoflourescence was monitored with two-colour isotypic non-specific mouse Ig. Apoptosis was analysed using a standard FITC-labelled annexin V plus 7-aminoactinomycin D (7-AAD) protocol, with apoptosis defined by staining with annexin but not 7-AAD.

Promoter DNA methylation. The DNA methylation analysis for promoters, using bisulfite-treated DNA (EZ DNA Methylation Kit; Zymo Research, Irvine, CA, USA) has been described elsewhere (13), and was modified here by using the epigenetic sequencing methylation (ESME) software permitting quantification of CpG methylation (Epigenomics AG, Berlin, Germany) (14). Primers were chosen from the literature or designed with the MethPrimer software (urogene.org). Primer sequences were: *p16*, forward: TTTTTAGAGGATTTG AGGGATAGG and reverse: CTACCTAATTCCAATTCCCCTACAAA CTTC; cyclin A1 region 1, forward: TATAGTTGGAGTTGGAGGGT, reverse: AAACAACTAACAAATACACTAAAA; cyclin A1 region 2, forward: TATAAGGATTTTAGGGATTTTTTTT, reverse: ACCCTC CAACTCCAACTATAC; RARα2, forward: GGTYGGAGTTATATAT GATGT, reverse: AATAATCCCRATATCCTCCCCTTAA; estrogen receptor alpha (ERα), forward: GTGATGTTTAAGTTAATGTTA GGGTAAGG, reverse: CCAAATAATAAAACACCTACTAACC; DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3A), forward: GGGAA GGTGTGTTTTTAAGG, reverse: ACTCAAATTCAAATCTATACAA TACC; caudal type homeobox 1 (CDX1), forward: TTGTGTGAA GTTGGT TAGAATTT, reverse: ACACATAACCCACATACATAA TAA; cyclin-dependent kinase 2 (CDK2), forward: GGATTTAAAG TAGGTATTTGGGAAGA, reverse: TCCACCTTTTAAAAATT CTCCA TAA. PCR amplicons were sequenced using these primers.

Total DNA methylation. Total cellular CCGG methylation was performed using methylation-sensitive restriction enzymes and a nick-translation assay (15). DNA was digested with *Hpa*II and *Msp*I, respectively, recognizing unmethylated CCGG and all CCGG sites, respectively, followed by a ^{3}H-dCTP *in vitro* DNA synthesis incorporation assay (Amersham Bioscience, Buckinghamshire, UK) with all determinations in triplicate.

Statistical analysis. The non-parametric Mann–Whitney rank test was used (16).

Results

The present study was designed to determine whether the capacity of ATRA, at concentrations used for treatment of patients with leukemia, to cause differentiation of leukemia cells is accompanied by changes in DNA methylation, including early or weak signs of cytosine methylation. Therefore, before DNA methylation was determined, we performed control experiments to verify the anticipated differentiating influence of ATRA on our leukemia cell lines.

Verification of ATRA-induced differentiation. We found an increase in the frequency of cells undergoing early apoptosis upon ATRA treatment, as defined by flow cytometric analysis of expression of annexin V coupled with a lack of 7-AAD staining. The apoptosis rate of U937 cells increased from 1.5% to 4.5% after treatment with ATRA for 48 h, and correspondingly increased from 0.7% to 8% by ATRA in HL-60 cells (Figure 1). Acquisition of the cell surface markers CD38 and CD11c after treatment with ATRA for 48 h and 168 h, respectively, indicative of monocyte differentiation, was documented by flow cytometry for both U937 and HL-60 cells (Figure 2).

Total DNA CCGG methylation. The level of DNA methylation was first analysed in the total CCGG sites of the genome, using a methyl-sensitive restriction enzyme assay (12). Total cellular DNA was digested with *Hpa*II and *Msp*I, recognizing methylated CCGG (*i.e.* $CC_{meth}GG$) and all CCGG sites, respectively, followed by an *in vitro* DNA synthesis ^{3}H-dCTP incorporation assay. The baseline level of proliferating cells with no exposure to ATRA was 82.6% methylated CCGG sites for U937 cells, *i.e.* similar to that in mitogen-stimulated T-lymphocytes obtained from a healthy blood donor (79.5%), but higher than that observed for HL-60 cells (70.7%) (Figure 3). A clearly lower percentage of DNA methylation (53.5%), was found, as expected, using a cell line established from a laryngeal solid cancer tumour (Hep-2). U937 cells exposed to ATRA for 72-96 h (no analysis of a longer incubation time was carried out, and the results of three 72-h experiments and four of 96-h were pooled) had less methylated CCGG (74.6%), as compared with untreated U937 cells (82.6%) ($p<0.05$). For HL-60 cells, ATRA had no significant effect on CCGG methylation after 72 h of treatment. However, after 168 h of ATRA exposure, there was a decrease from 70.7% in controls to 57.5% in ATRA-treated HL-60 cells ($p<0.01$). Thus, our total DNA methylation results suggest that ATRA induces de-methylation at CCGG sites.

Promoter DNA methylation. Secondly, the DNA methylation level in promoter sequences was determined. The set of genes analysed was selected to include genes with a CpG-rich promoter, thus, with a potential to be regulated by DNA methylation, with some of the genes functioning in cell cycle regulation (*p16*, cyclin-dependent kinase-2 (CDK2) and cyclin A1) or cellular differentiation (CDX1), to be directly involved in retinoic acid signalling (RARα) or in DNA methylation (DNMT3A), or to be known to be altered in many cases of leukemia (ERα). Furthermore, CDX1 was

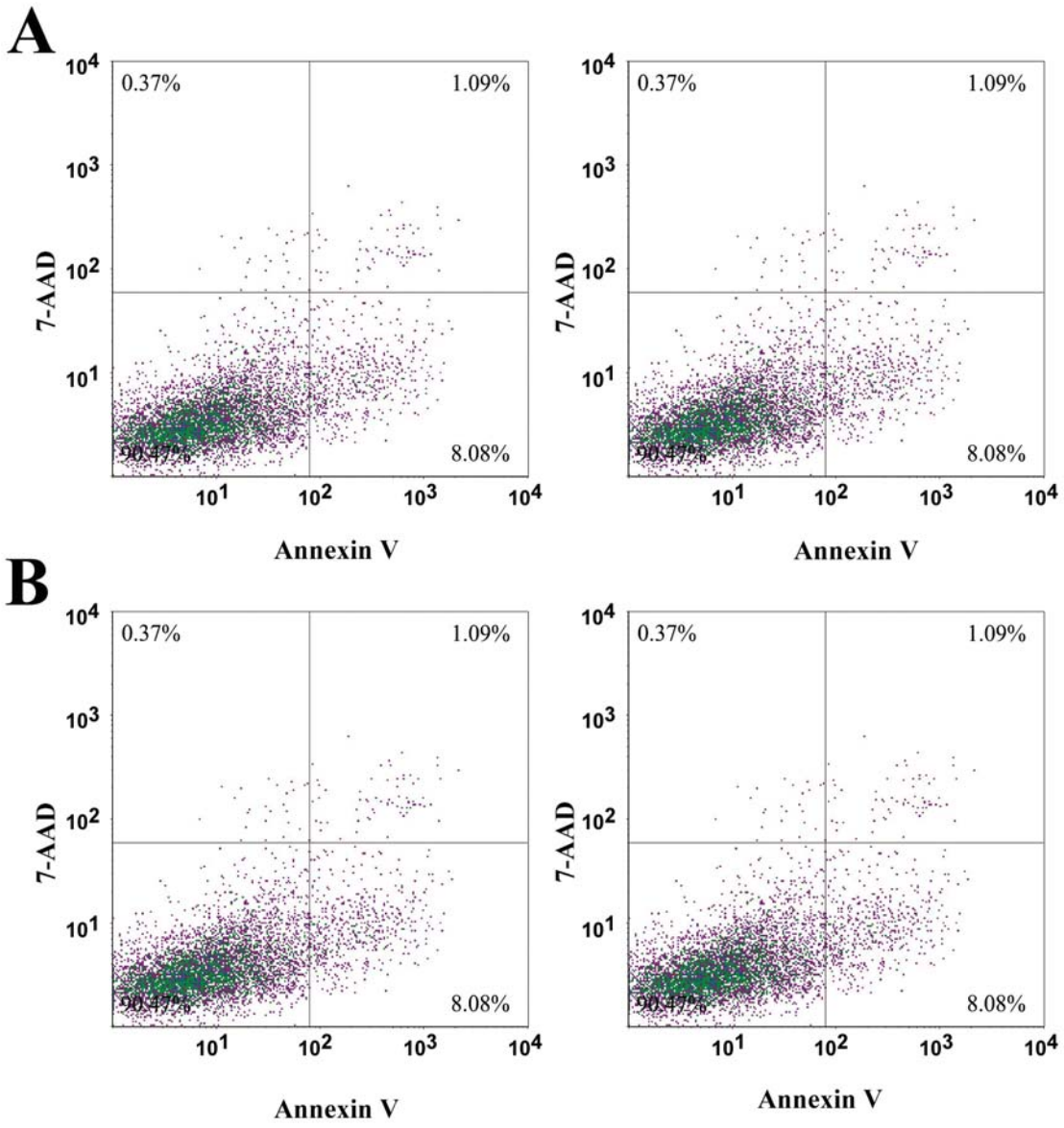

Figure 1. *Cellular effects during all-trans retinoic acid (ATRA) treatment, as shown by apoptosis assay. U937 (A) and HL-60 (B) cells were treated for 48 h (right panels) or left untreated (left panels), then stained with annexin V and 7-Aminoactinomycin D (7-AAD), and finally analysed by flow cytometry.*

assumed to serve as a DNA methylation-positive control, and DNMT3A as a negative control. Results for 15 CpG sites (except nine CpG sites for the cyclin A1 promoter region 2, due to limited data) were chosen for presentation based on a high a density of CpG sites and high technical quality.

A different level of promoter DNA methylation in cells exposed to ATRA, as compared to a parallel untreated culture, was seen for *p16*, with a higher methylation level in the HL-60 cells (Figure 4). There was a higher fraction of methylated molecules at eight of the analysed 15 CpG sites in the sequenced *p16* gene promoter PCR product from ATRA-treated HL-60 cells, while there was 0% methylation at six of the sites, and the single-remaining site exhibited 8% in both treated and untreated cells. The ATRA-incubated HL-60 cells exhibited 4-21% methylation at the eight differing *p16* CpG sites, whereas the HL-60 cells with no exposure to ATRA exhibited a clearly lower level with 0% at six sites and 2-4% at the remaining two CpG sites. Interestingly, the analysed *p16* sequence contains both upstream stimulatory factor (USF), specificity protein 1 (SP1) and the myeloid

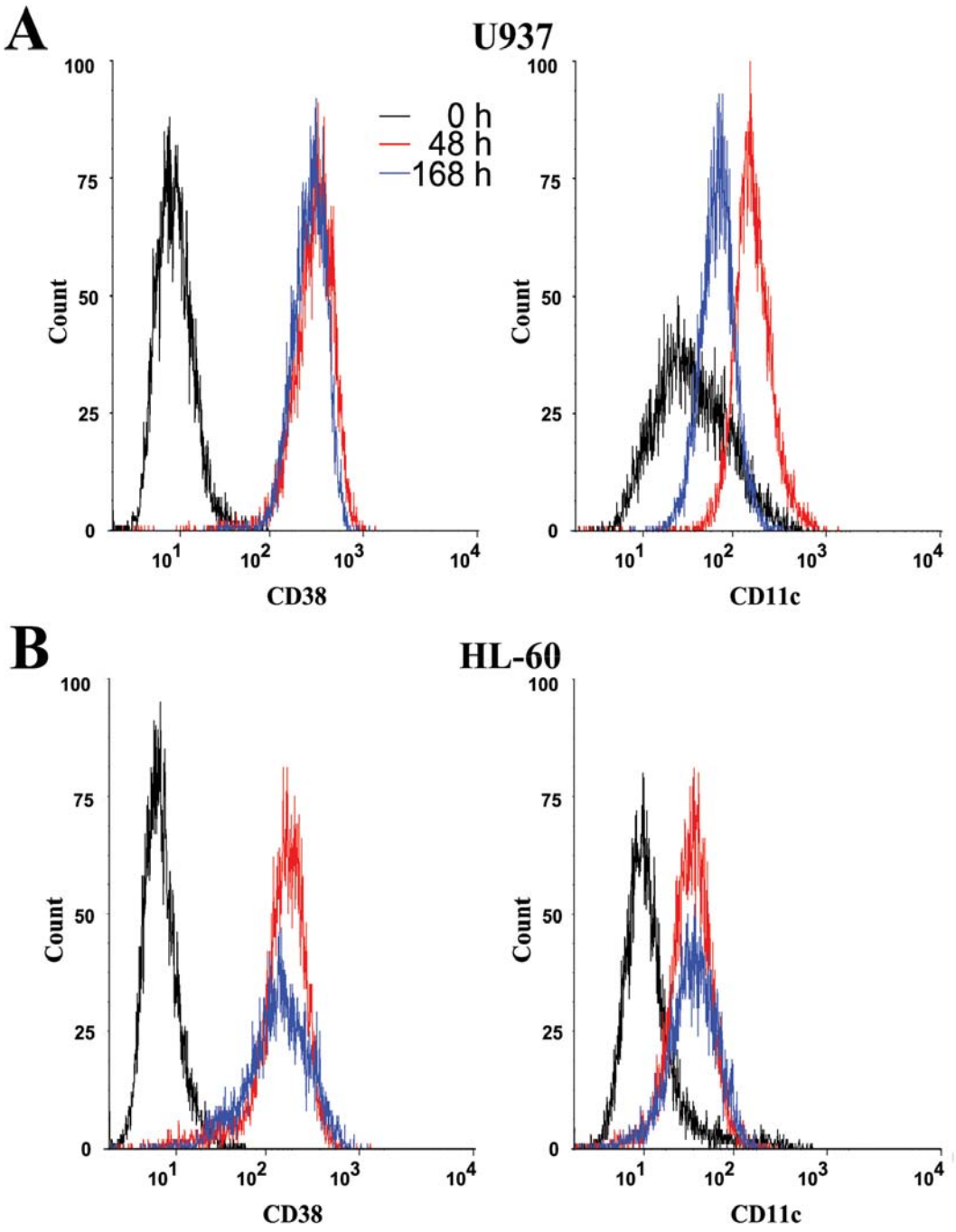

Figure 2. *Cellular effects of all-trans retinoic acid (ATRA) treatment, as expressed by cell surface CD markers. U937 (A) and HL-60 (B) cells were treated for 48 h (red) and 168 h (blue) and then labeled with antibodies to CD38 and CD11c as indicated; the left hand curve (in black) represents the expression level of untreated cells.*

zinc finger 1 (MZF1) transcription factor-binding motifs, compatible with a role for the observed effect of ATRA on *p16* DNA methylation in gene expression. In contrast, there was almost complete *p16* methylation in the U937 cells in both untreated and ATRA-exposed cultures (ranging from 75.5% to 100% at the 15 CpG sites), thus with limited potential for an increase in methylation; a typical result is illustrated by the CpG site located at position 223, with 95% and 94% of all sequenced molecules being methylated in untreated and treated cells, respectively. The *p16* promoter

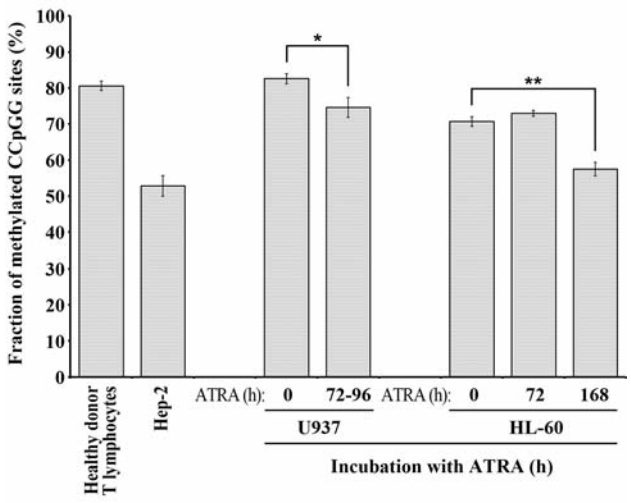

Figure 3. *Increased total genomic CCGG methylation level during all-trans retinoic acid (ATRA) treatment. Methylation at CpG located in the total genome of proliferating normal human T-lymphocytes, human laryngeal carcinoma Hep-2 cells, and in U937 and HL-60 cells incubated with ATRA as indicated. The mean and standard error of the mean (SEM) of 4-7 separate experiments (each performed in triplicate) are shown. *p<0.05; **p<0.01.*

methylation at eight of the CpG sites was higher in U937 cells treated with ATRA, while in control cells, *p16* promoter methylation was higher at four sites. The remaining 3 sites had 100% methylation in U937 cells, both in the absence and presence of ATRA treatment.

For cyclin A1, two separate promoter regions were analysed; the first showed no methylation among all 15 CpG sites (Figure 5A); in the second region, there was a modest level of methylation in the nine CpG sites of HL-60 (0-25%, with data only for ATRA-treated cells for the first three sites), and an intermediate level of 40-80% among the seven CpG sites scored for U937 (Figure 5B), with no apparent difference due to ATRA at the cyclin A1 promoter. The *CDK2* (data not shown) and *DNMT3A* promoters displayed virtually no CpG methylation (Figure 5E). The results for the remainder of the analysed promoter sequences likewise revealed no indication of an influence of ATRA on promoter methylation (Figure 5C, D and F); here the level of methylation was clearly higher for RARα2 and ERα in U937 cells as compared with HL-60 cells, while for *DNMT3A* there was only 0-10% methylation in HL-60 cells and 0-12% in U937cells. For heavily methylated *CDX1*, most CpG sites exhibited >80% methylation, even in HL-60 cells. Thus, our promoter DNA methylation results show that in the set of 7 analysed genes, ATRA affects the level of CpG methylation only in the *p16* gene of HL-60 cells.

Discussion

The ability of ATRA to counteract a neoplastic phenotype is not restricted to PML, the only form of cancer, as yet, where ATRA has a place in common clinical practice. ATRA has been shown to reduce proliferation in several cell lines derived from both solid carcinomas and non-PML leukemias. Epigenetic modulation is now gaining a prominent role in anti-leukemic therapy, prompting us to seek evidence of DNA methylation changes during ATRA-induced differentiation in leukemia cell lines. It may be speculated that the addition of an epigenetic drug might be a tool to potentiate the anti-leukemic influence of ATRA, and thus extend the clinical indications of ATRA to a wider spectrum of leukemic disorders.

What is the relevance of our observed changes in ATRA-induced DNA methylation? For most of the gene expression changes occurring during ATRA treatment, the available data suggest no major impact of promoter methylation (6, 9). However, histone modifications have been documented, suggesting to us that other types of epigenetic modulations (commonly seen to manifest together with histone changes) may also become induced (6, 17). It is known that DNA methylation may not become evident until after prolonged exposure to an epigenetic modulator, and that this transition occurs gradually, a process forming 'seeds' of methylation. Interestingly, the acquisition of *p16* promoter methylation has been demonstrated to evolve in such a slow fashion, not becoming apparent until after several cell divisions, and then much later (more than twenty cell cycles) engaging a high enough number of CpG sites for *p16* gene expression to be affected (18). The previous reports on the 'seeds' of methylation phenomenon (10-13) detail a modest amount of CpG methylation, similar to our observation (Figure 4) on the *p16* promoter in seven days (corresponding to approximately five cell generations in ATRA-treated U937 cells). We therefore argue that our combined findings of this effect on *p16* and of the changes in the methylation level among the total CCGG stretches of the genome (Figure 3), justify the conclusion that ATRA can affect DNA methylation in non-PML forms of leukemia. It remains for future work to determine if this effect encompasses a wider epigenetic re-organization with phenotypic consequences, and eventually, whether it can help design a non-cytotoxic and clinically-successful combined use of ATRA plus an epigenetic drug.

Conflicts of Interest

The Authors report no potential conflicts of interest.

Acknowledgements

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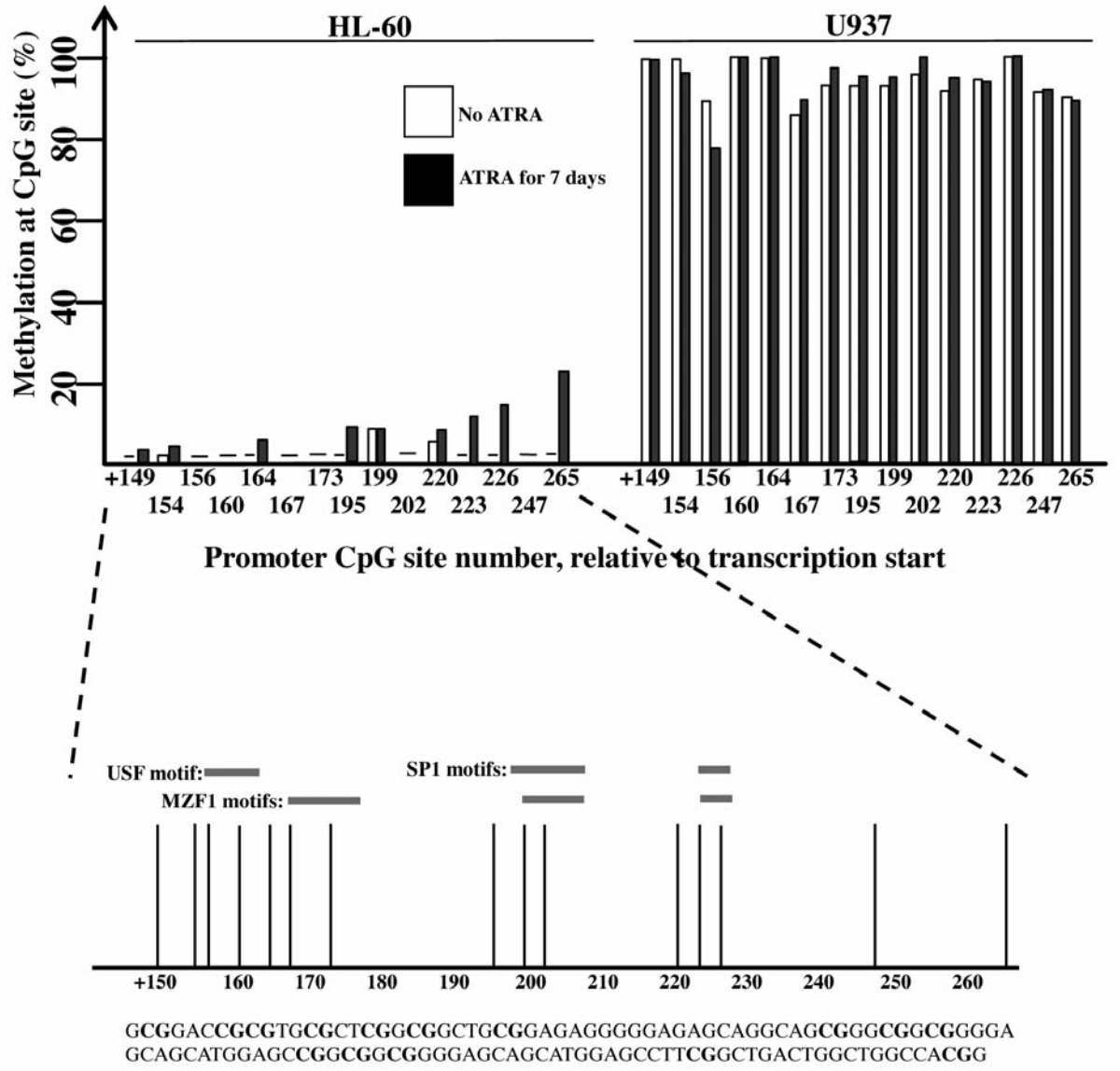

Figure 4. *Increased p16 promoter CpG methylation level in HL-60 cells during all-trans retinoic acid (ATRA) treatment. Data show the fraction of PCR-amplified promoter molecules with a methylated cytosine at the indicated gene promoter CpG sites of HL-60 and U937 cells incubated with ATRA for seven days (closed bars) or with no ATRA (open bars). A line indicates 0% methylation. Binding motifs for the transcription factors upstream stimulatory factor (USF), specificity protein-1 (SP1) and the myeloid zinc finger-1 (MZF1) are shown.*

Childhood Cancer Foundation, Malmö Hospital Cancer Foundation, Malmö Hospital Foundation, Gunnar Nilsson Cancer Foundation and Crafoord Foundation to JLP.

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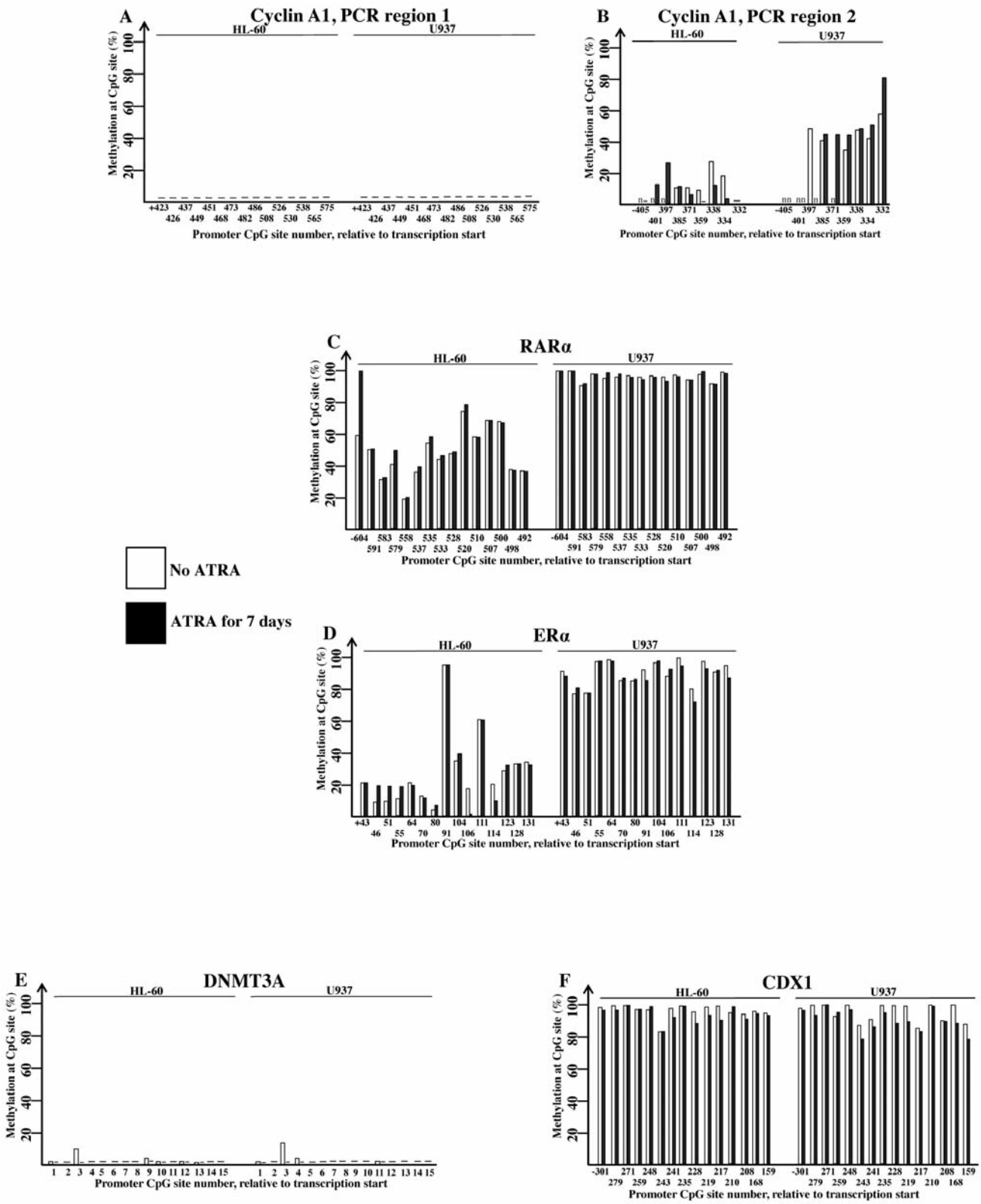

Figure 5. *No influence of seven days of all-trans retinoic acid (ATRA) treatment on CpG methylation in a set of leukemia-associated gene promoters. The letter n indicates that there is no data. A and B, cyclin A1 (two separate promoter regions); C, retinoic acid receptor (RAR)α; D, estrogen receptor (ER)α; E, DNA (cytosine-5-)-methyltransferase 3 A (DNMT3A); F, caudal type homeobox 1 (CDX1).*

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