FULL PAPER

Physiology

DNA methylation inhibitor causes cell growth retardation and gene expression changes in feline lymphoma cells

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ABSTRACT. DNA methylation is an epigenetic mechanism controlling gene expression without affecting DNA sequences, and aberrant DNA methylation patterns are features of a number of diseases. Notably, epigenetic errors in cancer cells have been intensively studied over the last two decades in humans; however, little is known concerning dogs and cats. To analyze DNA methylation and gene expression changes in feline lymphoma cells, we added the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza) to three cell lines (3281 and FT-1 cells derived from T-cell lymphoma and MS4 cells derived from B-cell lymphoma). Adding 5-aza significantly retarded cell growth in a dose-dependent manner in all cell lines, and there were aberrant gene expression patterns. Transcription factor *Sox11* expression in 3281 cells was de-repressed by 5-aza treatment, and subsequent promoter DNA demethylation was analyzed by bisulfite sequencing. Cell cycle analysis suggested that inhibition of cell growth was due to DNA replication arrest, and this supported the result of increased expression of *p27kip1* gene which disturbed cells of 3281 and FT-1 entering the S phase. In this study, 5-aza suppressed the growth of feline lymphoma cells, but further experiments with normal lymph cells are necessary to confirm specificity of this drug treatment and to expand it for clinical use.

KEY WORDS: 5-aza-2′-deoxycytidine, apoptosis, DNA methylation, feline lymphoma, *Sox11*

Lymphoma is a malignancy in which lymphoid cells undergo neoplastic proliferation in lymphatic tissues, such as lymph node, spleen, intestinal tract and skin. In domestic cats, hematopoietic tumors, which account for approximately one-third of all tumors, are the most common, and 90% are lymphomas [3]. Retroviral infections, such as feline leukemia virus (FeLV) and feline immunodeficiency virus, are considered to be the main cause of feline lymphoma [1]. It was reported that FeLV infection is involved in the rearrangement of the *myc* gene and is strongly associated with the pathogenesis [13]. Recently, epigenetic error that has no effect on DNA sequences is receiving attention as a cause of human cancer in addition to gene mutation. In gastric mucosal cells of humans infected with *Helicobacter pylori*, advanced DNA methylation was confirmed in comparison to uninfected individuals [11]. In Hepatitis B virus infection, a viral protein, HBx, induces DNA methylation by activating DNA methyltransferases (DNMTs) [25]. It is believed that DNA methylation abnormality leads to acceleration of expression of oncogenes and inactivation of tumor suppressor genes and so induces cancer [2]. In this way, carcinogenesis caused by epigenetic abnormality is being clarified, and the importance of epigenetic changes in cancer is widely recognized. In fact, DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza) is approved by the Food and Drug Administration in the U.S.A. to be used clinically for acute myelodysplastic leukemia [7]. However, little is known about epigenetics in relation to feline cancer.

Mochizuki *et al.* recently reported that the tumor suppressor gene *p16* was mutated or methylated in feline primary neoplastic diseases and lymphoma cell lines [14]. They also showed that 5-aza treatment restored mRNA expression of the repressed *p16*. However, cell growth and other gene expression changes were not reported. Harman *et al.* treated mammary tumor cell lines derived from dogs, cats and humans with 5-aza and found *in vitro* reduction of tumorigenicity in all three species [4]. These results indicate therapeutic potential of DNA methylation inhibitors for feline tumors. Therefore, the aims of the present study were to evaluate the effects of the DNA methylation inhibitor 5-aza on feline lymphoma cell lines *in vitro*, by analyzing total live cell number, apoptosis ratio, cell cycle, gene expression and DNA methylation changes.

Table 1. Concentration of 5-aza and culture period

Cell line	5-aza concentration (μM)	Days of culture
3281	0, 1, 5, 10	4
FT-1	0, 5, 10, 20	3
MS4	0, 0.5, 1, 2	7

Table 2. Primer sequences and annealing temperature used in this study

Gene	Forward and reverse primer sequences	Product (bp)	Annealing (°C)
Primers for real-time PCR			
Gapdh	5′TGTGAACGGATTTGGCCGTA3′ 5′CCGTTCTCAGCCTTGACTGT3′	173	60
Bcl2	5′GGATGCCTTTGTGGAACTGT3′ 5′CGTTTCATGGGACATCACTG3′	223	60
Bcl-xL	5′AATGTCTCAGAGCAACCGGG3′ 5′ACCGACTCCAGCTGTATCCT3′	80	60
p27kip1	5′GCGGTGCCTTTAATTGGGTC3′ 5′CTAACCCCGTCTGGTTGTCC3′	91	60
Ezh2	5′GTTGATGGGGTGTGGGAGTAG3′ 5′TGGCCATTGCAGATCTAGGAC3′	84	60
Sox11	5′GAGTTCCCCGACTACTGCAC3′ 5′CGCCCCTCTCAATACGTGAA3′	101	60
Osm	5′GCTGGCCTCTTATGCCTCAT3′ 5′AGGTAAGGTGGTCTTCCCGA3′	191	60
Primers for bisulfite PCR			
Sox11 5′flanking	5′GGAGAAAAGTAGGTGTATTTTAATTTT3′ 5′AAAAACAAATTTTCTTTCAATCTCC3′	245	50

MATERIALS AND METHODS

Cells

The feline lymphoma cell line, FELV3281-AD (RCB2610), was provided by the RIKEN BioResource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The FT-1 and MS4 cells were kindly gifted by Prof. Tsujimoto (Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan). The 3281 and FT-1 cells were derived from T-cell lymphoma, whereas MS4 cells were derived from B-cell lymphoma [13, 15, 18]. The cells were incubated in RPMI1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan) medium supplemented with 10% heat-inactivated Fetal Bovine Serum and Penicillin–Streptomycin liquid (Thermo Fisher Scientific, San Jose, CA, U.S.A.) at 37°C in a humidified atmosphere of 5% CO_2. Each culture was performed with six-well plates in triplicate, and each experiment was performed three times. The total and living cell numbers were analyzed by Tali® Image-Based Cytometer (Thermo Fisher Scientific) using the Tali® Viability Kit-Dead Cell Red (Thermo Fisher Scientific). Cell cycle and apoptosis were analyzed using a Tali® Cell Cycle Kit (Thermo Fisher Scientific) and Tali® Apoptosis Kit-Annexin V Alexa Fluor 488 & Propidium Iodide (Thermo Fisher Scientific), respectively. DNA methylation inhibitor, 5-aza, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The concentrations of 5-aza and culture periods of each cell line are listed in Table 1.

RNA extraction and real-time PCR

Total RNA was isolated using an RNeasy Protect Mini Kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. RNA (1 μg) was used for cDNA synthesis by ReverTra Ace® qPCR RT Kit (TOYOBO, Osaka, Japan). The cDNA (2 μl) was mixed with THUNDERBIRD® SYBR qPCR Mix (TOYOBO) containing 50 pmol of each primer (Table 2) and, real-time PCR was performed with a StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific) using the following conditions: 94°C for 1 min, followed by 40 cycles at 94°C for 15 sec, and 60°C for 30 sec. *Gapdh* was used as a reference gene, and the ΔΔCt method of quantification was used to obtain fold-change relative to non-treated cells. Relative gene expression changes between non-treated, and treated cells were compared by one-way ANOVA, and differences were considered significant at $P<0.05$.

DNA extraction and methylation analysis

Genomic DNA was extracted by NucleoSpin® Tissue (TaKaRa Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Bisulfite conversion was carried out using an EZ DNA Methylation-Lightning™ Kit (Zymo Research, Orange, CA,

U.S.A.) according to the manufacturer's instructions. The PCR amplifications were performed in 20 μl containing 50 pmol of each primer (Table 2) using EmeraldAmp® PCR Master Mix (TaKaRa Bio Inc.) with the following conditions: 94°C for 2 min, followed by 35 cycles at 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 5 min. The amplified PCR products were then cloned into pGEM-T-easy vector (Promega, Madison, WI, U.S.A.) and sent to a sequence service (Greiner Bio-One, Frickenhausen, Germany). At least 12 clones were sequenced from each sample. Sequenced clones were analyzed by QUMA (QUantification for Methylation Analysis) program [10]. The Mann–Whitney U-test was used for statistical analysis with $P<0.05$ denoting a significant difference.

RESULTS

Total live cell number

The 5-aza treatment resulted in significantly decreased total live cell numbers in all cells examined (Fig. 1). In 3281 cells, 1 μM 5-aza was enough to suppress cell growth, whereas 20 μM 5-aza was needed to suppress FT-1 cell growth (Fig. 1A and 1B). In MS4 cells, there was a decrease in the number of viable cells depending on the 5-aza concentration (Fig. 1C). For cell cycle analysis, the number of live cells with tetraploid chromosomes showed no significant change in all samples examined (data not shown).

Apoptosis analysis

In 3281 cells, 5-aza treatment resulted in a dose-dependent increase in apoptotic cell ratio (Fig. 2A). No concentration-dependent changes were observed in FT-1 cells, but the apoptotic cell ratio was significantly higher in 10 μM 5-aza treated cells (Fig. 2B). In MS4 cells, the overall proportion of cells showing apoptosis compared to other cell lines was small, and no concentration-dependent change was observed, except for 0.5 and 1 μM 5-aza treatments (Fig. 2C).

Gene expression analysis

We analyzed six genes using real-time PCR. *Bcl2* and *Bcl-xL* are anti-apoptosis genes. *p27kip1*, also known as *Cdkn1b*, is the gene for a cyclin-dependent kinase inhibitor, which inhibits the cell cycle. *Ezh2* is the gene for a histone methyltransferase and is reported to be closely related to human lymphoma. *Sox11* is the gene for a transcription factor involved in embryonic development and tumorigenesis. *Osm*, also known as *Oncostatin M*, is the gene for a cytokine belonging to the interleukin 6 group. In 3281 cells with 5-aza treatment (Fig. 3A), *Ezh2* was significantly and dose-dependently over-expressed. *p27kip1* was also significantly over-expressed in 10 μM treated cells, whereas *Osm* was down-regulated in 1 and 5 μM treatment. Interestingly, de-repression of *Sox11* by 5-aza treatment was observed only in 3281 cells. In FT-1 cells treated with 5-aza (Fig. 3B), *Bcl2* and *Bcl-xL* were significantly up-regulated at every concentration, whereas *p27kip1* was significantly up-regulated only for 20 μM treatment. *Sox11* expression was not changed in cells treated with 5-aza, and *Osm* was significantly down-regulated only for 20 μM treatment. In MS4 cells treated with 5-aza (Fig. 3C), *p27kip1* was significantly down-regulated at every concentration, whereas *Ezh2* was significantly over-expressed only in the 2 μM treatment.

DNA methylation analysis

Finally, to confirm whether changes in gene expression were directly caused by DNA methylation inhibition, we analyzed DNA methylation levels in the *Sox11* gene promoter regions, because *Sox11* expression was de-repressed by 5-aza treatment in 3281 cells. We found a CpG island in the *Sox11* 5′-flanking region (3.6 kb upstream from the transcription start site) and analyzed the DNA methylation levels by bisulfite sequencing. The *Sox11* upstream region was highly methylated in non-treated 3281 cells (93.3%), whereas methylation level was significantly reduced to 51.7% in 10 μM 5-aza treated cells (Fig. 4A). In FT-1 cells, methylation levels were significantly decreased from 92.2 to 75.2% by 20 μM 5-aza treatment (Fig. 4B). In MS4 cells, methylation levels were unexpectedly increased from 11.7 to 64.4% by 2 μM 5-aza treatment (Fig. 4C).

DISCUSSION

The inhibition of cell proliferation is thought to be caused by hypomethylation of cell proliferation-related genes or hypomethylation of upstream genes. In this study, we found that treatment with 5-aza (the most common DNA methylation inhibitor) inhibited cell proliferation in all three feline lymphoma cell lines examined: 3281, FT-1 and MS4. The 5-aza has DNA methyltransferase inhibitory action and, when it enters the cell, binds to newly synthesized DNA and RNA. Upon incorporation into RNA, it exhibits a cytocidal effect by protein synthesis inhibition [6]. However, when incorporated into the DNA strand during DNA replication, it binds irreversibly to DNMT and inhibits dissociation of DNMT from DNA. As a result, depletion of DNMT and breakdown of the DNA methylation maintenance mechanism causes passive DNA demethylation [9]. The effective concentration depends on the cell line. FT-1 cells were the most resistant to 5-aza, as their proliferation was significantly inhibited only at 20 μM-compared to 1 μM in 3281 cells and 0.5 μM in MS4 cells. As it is reported that DNA demethylation effect of 5-aza treatment on genomic DNA is not random and restricted to specific regions in human fragile X syndrome cells, cell type-specific and gene-specific differences by 5-aza treatment were also found in our study [21].

Bcl2 and Bcl-xL are Bcl2 family proteins and inhibit apoptosis. Cells over-expressing them show resistance to apoptotic stimuli. Both are present in the outer membrane of mitochondria and are thought to induce apoptosis by controlling the release of apoptosis-inducing proteins, such as cytochrome c, to the cytoplasm [23]. In this study, apoptotic cell ratio was dose-dependently increased
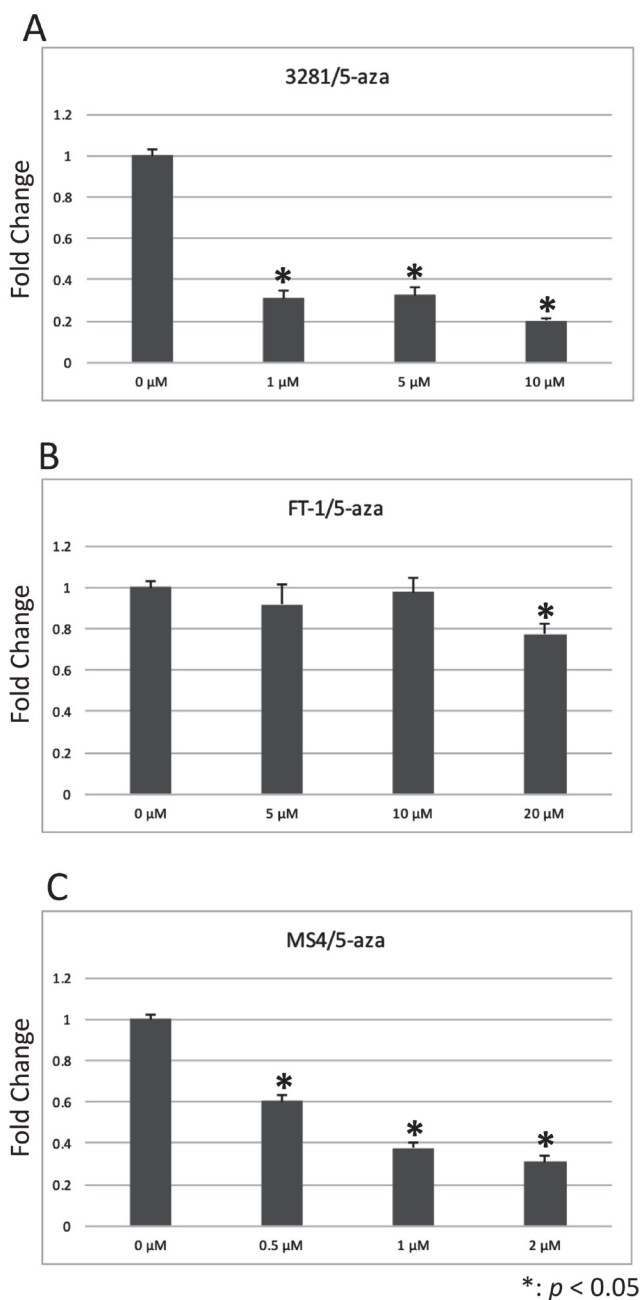
Fig. 1. Relative change of total live cell number for 3281 (A), FT-1 (B) and MS4 (C) cells after treatment with different concentrations of 5-aza. Results represent the mean ± standard error of three independent experiments. *Significantly different ($P<0.05$) compared with non-treated controls (0 μM).
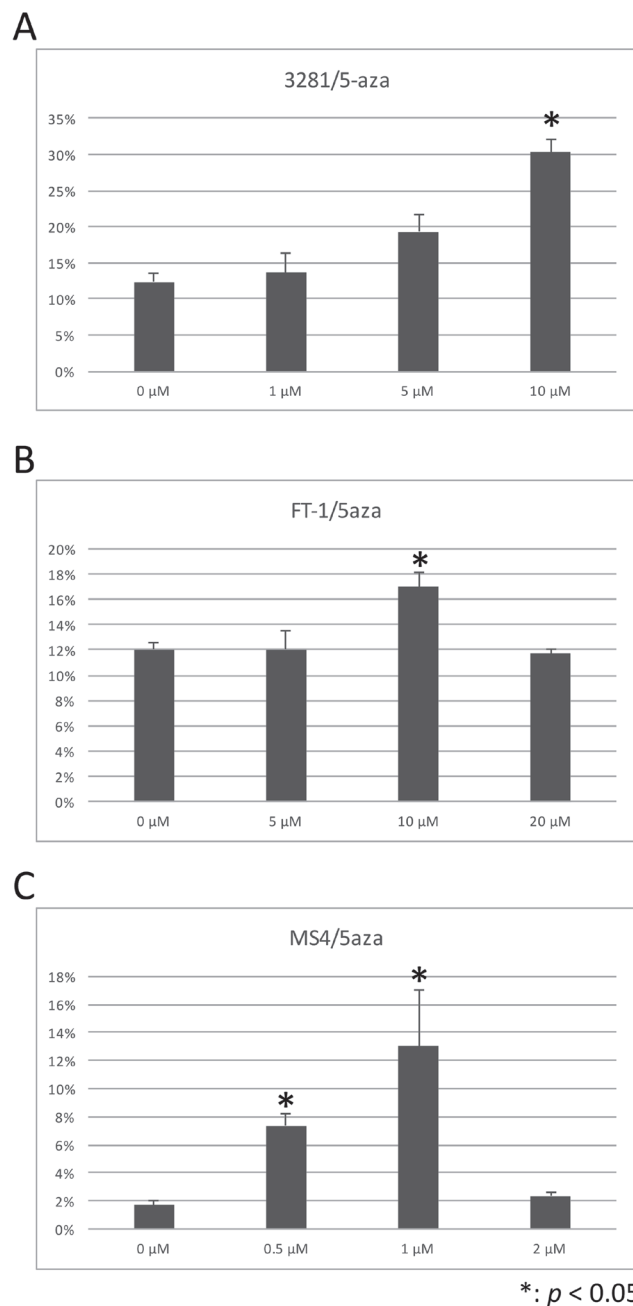
Fig. 2. Apoptotic cell ratio (%) in 3281 (A), FT-1 (B) and MS4 (C) cells treated with different concentrations of 5-aza. Results from triplicate plates represent the mean percentage ± standard error. *Significantly different ($P<0.05$) compared with non-treated controls (0 μM).

in 3281 and MS4 cells treated with 5-aza; however, *Bcl2* and *Bcl-xL* expression levels were not changed in either cell type. In FT-1 cells treated with 5-aza, *Bcl2* and *Bcl-xL* were significantly up-regulated at every concentration, and apoptosis ratio was unchanged except for 10 μM treatment, suggesting that over-expression of *Bcl* genes inhibited the induction of apoptosis. The p27kip1 binds to a complex of cyclin-dependent kinase 2 and cyclin E, regulates cell cycle progression and suppresses cell proliferation by inhibiting the transition from G1 phase to S phase [8]. Over-expression of *p27kip1* was significant for 10 μM (3281 cells) and 20 μM (FT-1 cells) 5-aza treatment, suggesting some role in inhibiting cell proliferation in both cell lines. However, *p27kip1* expression significantly decreased in MS4 cells, suggesting that the decreased number of viable cells was due to other factors. It is conceivable that a significant increase in tetraploidy indicates an inhibition in cell division, but this was not found for all cell lines treated with 5-aza. This implies that the decrease in cell number was not a result of inhibited cell division but inhibited DNA replication. Particularly in
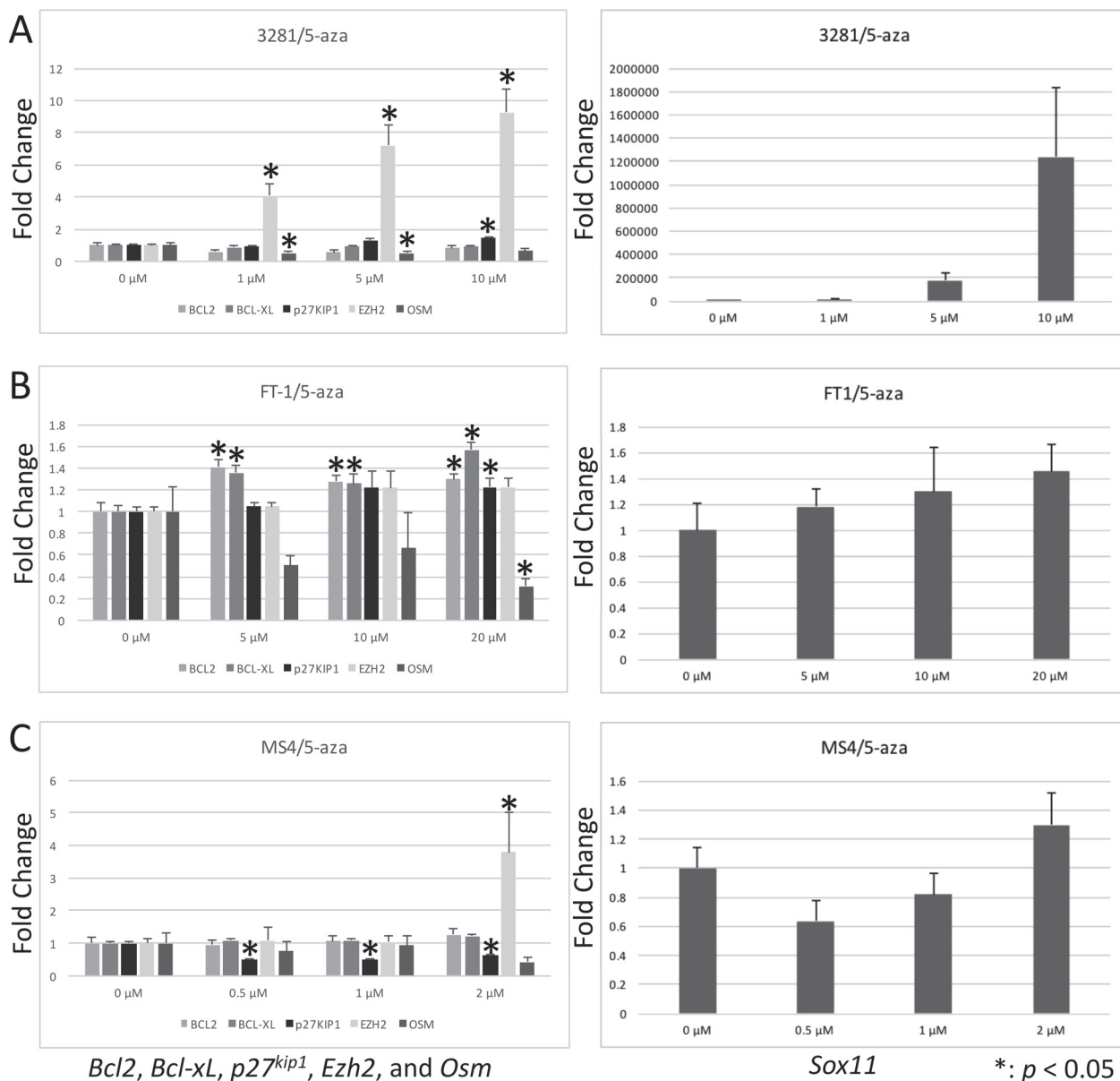
Fig. 3. Relative change of gene expression levels normalized by *Gapdh* expression for 3281 (A), FT-1 (B) and MS4 (C) cells treated with different concentrations of 5-aza. Because *Sox11* expression was greatly up-regulated in 3281 cells by 5-aza treatment, the graphs are provided separately; the other five genes (*Bcl2*, *Bcl-xL*, *p27kip1*, *Ezh2* and *Osm*) are presented together. Results from three independent experiments with triplicate plates represent the mean ± standard error. *Significantly different ($P<0.05$) compared with non-treated controls (0 μM).

3281 and FT-1 cells, this is consistent with the increase in *p27kip1* expression which inhibited the transition to S phase.

Ezh2 is known as a histone H3K27 methylation enzyme-in human B-cell lymphoma, activated mutation of the 641st tyrosine residue (Y641) has been reported [16] as well as over-expression in prostate, stomach, breast and prostate cancers [12]. Both in MS4 cells derived from B cells and 3281 cells derived from T cells, *Ezh2* expression increased significantly with 5-aza addition, suggesting that DNA methylation and other factors controlled *Ezh2* expression in feline lymphoma cells. In human cancer cells, many of the genes in which abnormal DNA methylation is observed are methylated at histone H3K27 in normal cells [20]. Further analysis is needed to clarify whether feline-induced gene mutation and/or DNA methylation changes are also preserved in feline lymphoma cells.

Sox11 is a transcription factor of the SoxC family and is involved in nervous system development, tissue remodeling and cell proliferation regulation [17]. *Sox11* is expressed in mantle cell lymphomas (MCL), subsets of Burkitt's lymphomas and B-cell lymphoblastic leukemia, but not in healthy B cells and other B-cell lymphomas in human. The promoter region of *Sox11* is in a hypomethylated state both in human MCL and normal B cells, and its expression was reported to be decreased by 5-aza treatment of MCL in which *Sox11* is expressed [24]. In our study, *Sox11* was expressed in MS4 cells (derived from B cells) with promoter hypomethylation, but was almost not expressed in 3281 and FT1 cells (derived from T cells) with promoter hypermethylation.
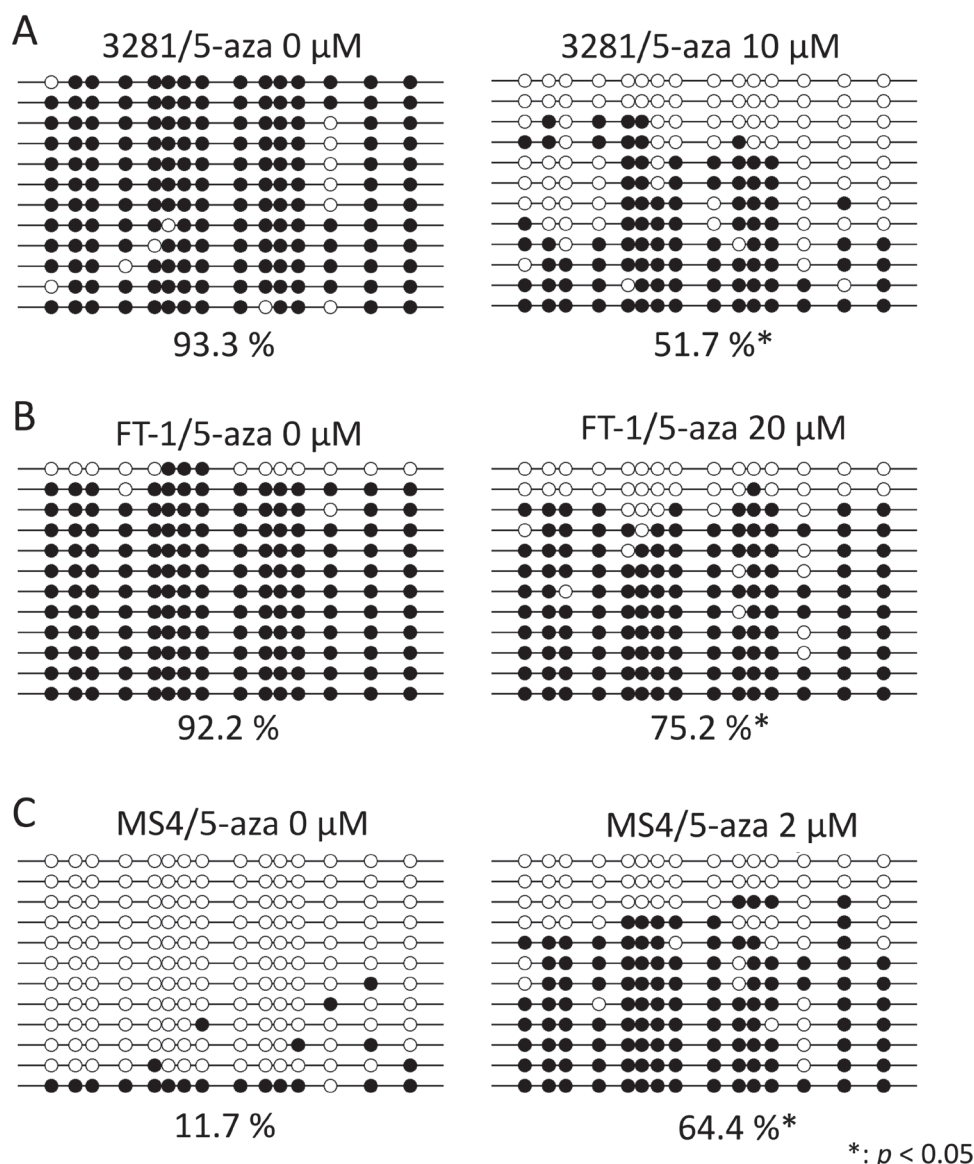
Fig. 4. DNA methylation patterns analyzed by bisulfite sequencing in 3281, FT-1 and MS4 cells treated with different concentrations of 5-aza. Each circle shows CpG dinucleotides. White circles represent unmethylated cytosine, and black circles represent methylated cytosine. Each line indicates an individual sequenced clone (allele). The methylation percentages are indicated below figures. *Significantly different ($P<0.05$) compared with non-treated controls (0 μM).

In 3281 cells treated with 5-aza, *Sox11* expression was drastically de-repressed in a dose-dependent manner subsequent to DNA demethylation. This is the first evidence that *Sox11* may be directly regulated via DNA methylation with 5-aza treatment in feline lymphoma cells. However, even though a significant decrease in methylation level was also observed in FT-1 cells, *Sox11* expression was not up-regulated. This can be explained by the reduction in methylation level (75.2% in FT-1 cells compared to 51.7% in 3281 cells) being insufficient to trigger *Sox11* de-repression, or by factors other than DNA methylation being involved in this process. This is consistent with the result that unexpected increase of methylation (64.4%) did not trigger repression of *Sox11* in 2 μM of 5-aza treated MS4 cells.

Oncostatin M (Osm) is a multifunctional cytokine of the interleukin 6 family and is responsible for the differentiation induction and suppression of growth of melanoma and other solid cancers [19]. Furthermore, although Osm is known to be involved in various biological phenomena, such as inflammation, hematopoiesis and development [22], no relationships to lymphoma and epigenetic regulation mechanisms have yet been reported. Osm is thought to be produced from activated T cells [5]. In our study, in 3281 cells derived from T cells, the expression of *Osm* was faint, while in the FT-1 cell line (also derived from T cells), expression was at levels several million times those in other cell lines. This suggests that there was a cell line in which *Osm* was remarkably expressed even in neoplastic T cells. In addition, suppression of *Osm* expression by 5-aza treatment in FT-1 cells may be caused by the continued moderate gradient of decrease in the number of viable cells.

In this study, an inhibitory effect on cell proliferation by DNA methylation inhibitor, 5-aza, was observed in all feline lymphoma cell lines examined, and a number of gene expressions were changed. Notably, we for the first time found that *Sox11* expression was directly de-repressed by inhibition of DNA methylation. In order to apply DNA methylation inhibitors as anti-cancer drugs in the future, it is necessary to examine the effect on healthy cells and also examine the retardation of tumorigenesis *in vivo*.

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