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The DNA methylation inhibitor 5-aza-2'-deoxycytidine retards cell growth and alters gene expression in canine mammary gland tumor cells

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Abstract

Disruption of gene expression by DNA methylation changes is widely involved in tumorigenesis. Here, to investigate DNA methylation changes in canine, we treated a canine mammary gland tumor cell line with a DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza). Cell growth was significantly retarded following 5-aza treatment and the epithelial marker genes *CDH1* and *KRT18* were significantly up-regulated, whereas the mesenchymal marker genes *CDH2* and *VIM* were significantly down-regulated. We also found a significant decrease in DNA methylation level in the *CDH1* promoter region by 5-aza treatment. These results showed for the first time in canine mammary gland tumor cells that inhibition of DNA methylation caused cell growth retardation and affected epithelial mesenchymal transition-related gene expression via changes in DNA methylation level.

Key Words: canine mammary gland tumor, DNA methylation, E-cadherin

Mammary gland tumors account for approximately half of all tumors in dogs, making them the most common tumor type; therefore, establishing effective diagnostic and therapeutic methods is important[1,5]. Approximately half of these tumors are malignant, and because of their strong potential for invasion and metastasis, they often affect other organs such as the lungs and regional lymph nodes[16]. Various genes are involved in invasion and metastasis of cancer cells, but those involved in epithelial mesenchymal transition (EMT), a process that confers migratory ability by promoting differentiation of epithelial cells into mesenchymal cells, is particularly important[20]. EMT is characterized by decreased expression of the tumor suppressor gene E-cadherin (*CDH1*), which is involved in cell adhesion. In human mammary gland tumors (MGT), the expression of *CDH1* decreases as malignancy increases, abnormally high CpG methylation is observed in the promoter region of the gene, and CpG methylation induces EMT in cultured cells[15]. In dogs as well, *CDH1* expression is known to be decreased in tumors with high invasiveness in MGT, but its methylation patterns as they relate to malignancy have not been

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Table 1. Primer sequences used in this study

Gene		Primer sequence (5´-3´)	PCR product size (bp)
ACTB	forward	GGCATCCTGACCCTGAAGTA	81
	reverse	GGTGTGGTGCCAGATCTTCT	
CDH1	forward	AGATGCGGATGATGATGTGA	92
	reverse	TGAACATCATGCTGCTAGGC	
KRT18	forward	TGAACGTCAAGGTCAAGCTG	91
	reverse	TCCAGGGCGTCAGTAAGACT	
CDH2	forward	TGTGAACGGGCAAATAACAA	137
	reverse	AGATCTGCAGCGTTCCTGTT	
VIM	forward	CCGACAGGATGTTGACAATG	116
	reverse	GCTCCTGGATTTCCTCATCA	
SNAI1	forward	CCTTCTGAGTGCCCCCATTT	119
	reverse	ATCAGCCGTCCACAGAAAGG	
SNAI2	forward	GAGCATTTGCAGACAGGTCA	85
	reverse	TTGGAGCAGTTTTTGCACTG	
CDH1 (for bisulfite analysis)	forward	TTAACTCAAAACCTACTTAAAACTCTCTCC	207
	reverse	AAAAGTAGGTTTAGTATTTTTGGTGGGG	

investigated[6]. The DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-aza) is reported to inhibit cell growth and invasion in several human MGT cell lines[3,4]. In canine urothelial carcinoma, it is reported that subcutaneous 5-azacitidine treatment showed antitumor activity[9]. However, the epigenetic aspects of canine MGT have not been well studied, especially the potential for use of DNA methylation inhibitors as anti-tumor drugs, although naturally occurring MGT in canines can be used as a model for human breast cancer.

In this study, we treated canine MGT cells with the DNA methylation inhibitor 5-aza and analyzed changes in the expression of EMT-related genes. Furthermore, we examined whether or not inhibition of DNA methylation directly caused up-regulation of the epithelial marker gene *CDH1*.

The canine MGT cell line AZACB was purchased from Cosmo Bio (Tokyo, Japan). Cells were cultured in DMEM medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) and Penicillin-Streptomycin-Amphotericin B Suspension (Wako Pure Chemical Industries Ltd.), and incubated at 37°C in a humidified 5% CO_2 atmosphere. Total live cell number was counted using a Tali™ Image-Based Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) using the Tali™ Viability Kit - Dead Cell Red (Thermo Fisher Scientific). The DNA methylation inhibitor 5-aza-2'-deoxycytidine was purchased from Wako Pure Chemical Industries, Ltd. Cells were treated with 0, 1, and 10 μM of 5-aza for four days in triplicate. The NucleoSpin® RNA kit (TaKaRa Bio Inc., Shiga, Japan) was used to extract total RNA according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis using the ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). Two microliters of cDNA was mixed with THUNDERBIRD® SYBR qPCR Mix (Toyobo) containing 50 pmol of each primer (Table 1) and real-time PCR was performed under the following conditions: 94°C for 1 min, followed by 40 cycles at 94°C for 15 sec and 60°C for 30 sec. mRNA expression was normalized to expression of *ACTB* and the ΔΔCt method of

quantification was used to determine the fold change in expression relative to non-treated cells. Genomic DNA was extracted using a NucleoSpin® Tissue kit (TaKaRa Bio Inc.) according to the manufacturer's instructions. Bisulfite conversion was carried out using EpiTect Bisulfite Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification was performed in 20-μL volumes containing 50 pmol of each primer (Table 1) and EmeraldAmp® PCR Master Mix (TaKaRa Bio Inc.) under the following conditions: 94°C for 2 min, followed by 30 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec. The amplified PCR products were cloned with pGEM-T-easy vector systems (Promega, Madison, WI, USA), sent to a sequencing service (Greiner Bio-One, Frickenhausen, Germany) and analyzed using the QUMA program[12]. Bisulfite data were analyzed with the Mann–Whitney *U*-test and statistical differences in gene expression were determined using One-way ANOVA. *P*-values < 0.05 denotes a statistically significant difference between non-treated and treated cells.

The total live cell number was significantly decreased by 10% to 20% by 5-aza treatment, in a concentration-dependent manner (Fig. 1A). However, cell morphology was not altered (data not shown). We analyzed the expression of six EMT-related genes using real-time PCR. *CDH1* and Cytokeratin18 (*KRT18*) are epithelial marker genes, whereas N-cadherin (*CDH2*) and Vimentin (*VIM*) are mesenchymal markers. Snail (*SNAI1*) and Slug (*SNAI2*) are transcription factors and are reported to repress *CDH1* gene expression. The results of real-time PCR analysis are depicted in Fig. 1B. The expression of epithelial marker genes, such as *CDH1* and *KRT18*, was significantly higher ($P < 0.05$) in 5-aza-treated cells, and the changes were concentration-dependent. *CDH1* was more than 60-fold over-expressed and *KRT18* was more than 250-fold over-expressed in cells treated with 10 μM 5-aza, compared to non-treated cells. On the other hand, expression of the mesenchymal marker genes *CDH2* and *VIM* was significantly lower ($P < 0.05$) in 5-aza-treated cells. The relative mRNA levels of these genes in 5-aza-treated cells were approximately half those in non-treated cells. The expression of *SNAI1* was significantly up-regulated in a concentration-dependent manner, whereas *SNAI2* expression was not significantly altered by 5-aza treatment (Fig. 1B). E-cadherin protein was also over-expressed in 5-aza-treated cells (Supplemental Fig. 1). Next, to confirm whether or not the over-expression of *CDH1* was directly caused by 5-aza treatment, we analyzed DNA methylation levels at the *CDH1* promoter region. We identified one CpG island in the 5′ flanking region of *CDH1* (CanFam3.1, chr5: 80,834,648-80,835,145) using MethPrimer and analyzed DNA methylation levels using the bisulfite sequencing method[14]. The *CDH1* 5′ flanking region was highly methylated in non-treated cells (83.1%), whereas the methylation levels were 0% in both 1 μM- and 10 μM-treated cells (Fig. 1C).

CDH1 is known to be a cell-cell adhesion molecule, characteristic of epithelial cells and an anti-tumor gene[22]. Aberrant DNA methylation patterns in the 5′ proximal promoter region of the *CDH1* gene have been reported in different types of human cancer cells, correlated with down-regulation of *CDH1* expression[2,7,11,23]. *CDH1* methylation has also been reported to increase during the progression of human breast cancer malignancy[18]. Another report showed that 5-aza treatment reduced mortality, increased *in vitro* aggregation, and suppressed metastasis with hypo-methylation of the *CDH1* promoter region and induction of re-expression of *CDH1* in human breast cancer cells[17]. Together with our results, it is suggested that hypo-methylation of the *CDH1* promoter region is a trigger that induces *CDH1* gene expression in both human and canine MGT cells. However, we also observed more than 3-fold over-expression of *CDH1* in 5-aza-treated (10 μM) cells with the same methylation level compared to 5-aza (1 μM) treatment. Because this difference cannot be explained by DNA methylation alone, we analyzed other factors

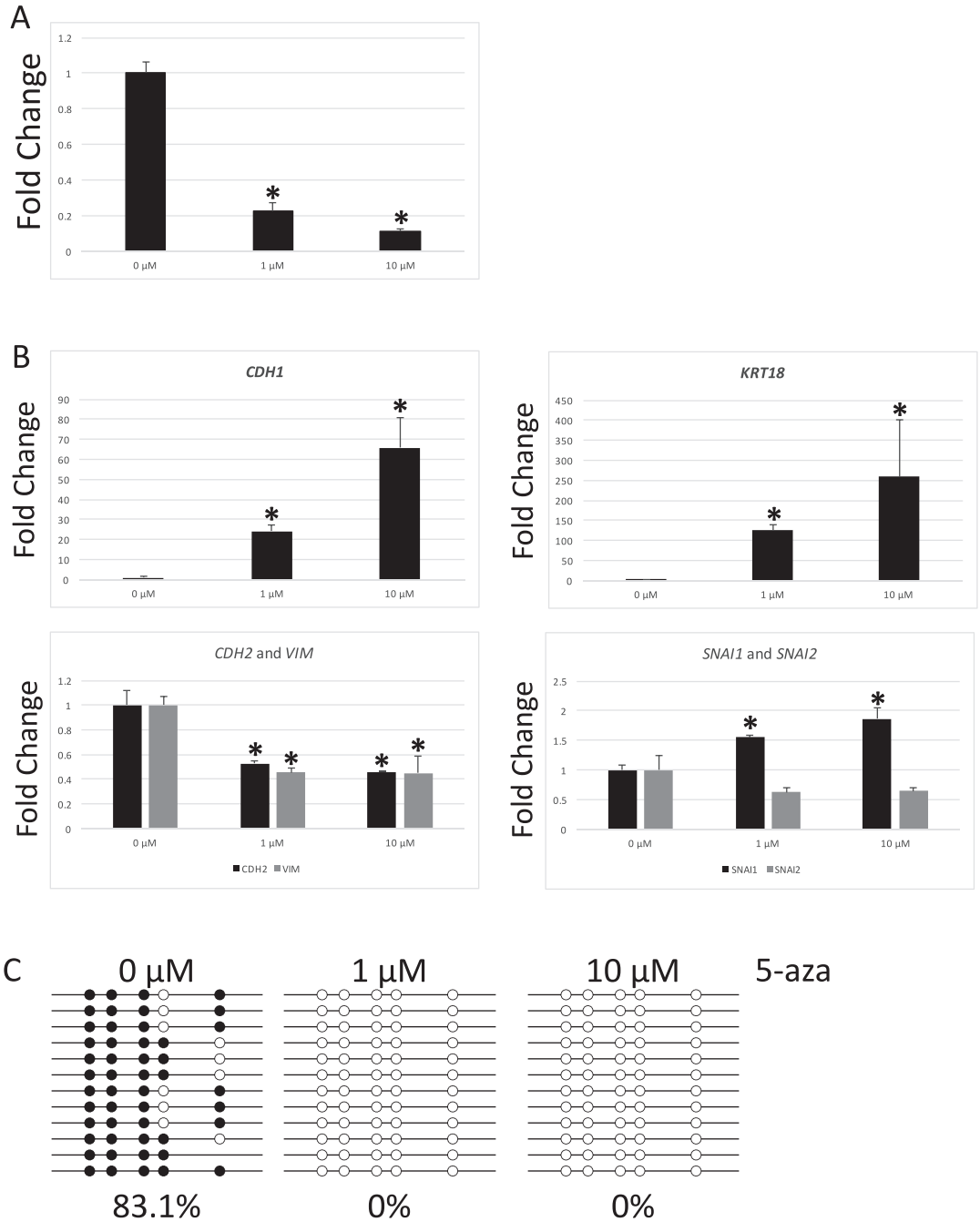

Fig. 1. Effects of 5-aza treatment in canine mammary gland tumor cells. A: Fold changes in total live cell number after four days of 5-aza treatment. Asterisks denote significant differences compared to 0 μM controls (*$P < 0.05$). Results from triplicate plates represent the mean ± standard error. B: Relative gene expression levels in 5-aza-treated cells. Asterisks denote significant differences compared to 0 μM controls (*$P < 0.05$). Results from triplicate plates represent the mean ± standard error. C: DNA methylation analysis of the *CDH1* 5′ flanking region. Each circle shows CpG dinucleotides. White circles represent unmethylated cytosines, and black circles represent methylated cytosines. The methylation level (as a percentage) is indicated below.

controlling *CDH1* gene expression. *SNAI1* is a transcription factor known to repress *CDH1* expression via recruiting the histone deacetylase complex[19]. We analyzed changes in the expression of *SNAI1* in 5-aza-treated cells and found that it was significantly up-regulated. This is contrary

to the over-expression of *CDH1* observed in 5-aza-treated cells. Another transcription factor, *SNAI2*, is also known to repress *CDH1* expression in human breast cancer cells[10]. However, no significant expression changes were observed in 5-aza-treated cells. *KRT18* is a member of the type I intermediate filament gene family and is expressed in single-layer epithelial tissues. It is reported that 5-aza-treated mouse utricle epithelial-derived progenitor cells and mouse and rat intestinal cells showed increased expression of *KRT18*, suggesting the involvement of DNA methylation in regulating gene expression[13,24]. We were unable to quantify changes in the methylation of the *KRT18* promoter region because of the difficulty of performing bisulfite PCR; however, significant up-regulation was observed following 5-aza treatment, suggesting the involvement of DNA methylation in regulating *KRT18* expression in canine MGT cells.

We also observed down-regulation of the mesenchymal marker genes *CDH2* and *VIM*. Methylation of *VIM* has been reported to be related to survival in human breast cancer cells[21]. However, no reports have been published on *VIM* and DNA methylation in canine tumors. Increased *CDH2* and reduced *CDH1* expression is known as the "cadherin switch," which is related to cancer progression and is a feature of EMT[8]. Our observation that *CDH1* and *KRT18* were up-regulated and *CDH2* and *VIM* were down-regulated suggested that 5-aza treatment induced an anti-EMT effect in canine MGT cells *in vitro*.

In this study, the DNA methylation inhibitor 5-aza induced retardation of cell proliferation, up-regulation of epithelial marker genes, and down-regulation of mesenchymal marker genes in canine MGT cells. The demethylation of *CDH1*, as determined by bisulfite sequencing, indicated that 5-aza-induced demethylation might play a role in activating the expression of *CDH1* in canine MGT cells. As our results were from *in vitro* experiments only, and further studies are needed to confirm the effect of 5-aza treatment *in vivo*.

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Supplemental data

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