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NOTE

Regulation of *p16* gene expression by histone H3 acetylation in canine lymphoid tumor cell lines

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Abstract

In order to investigate whether suppression of the *p16* gene is mediated by histone H3 acetylation in 4 canine lymphoid tumor cell lines, the gene's acetylation status was examined. In 2 canine lymphoid tumor cell lines with low *p16* mRNA expression (GL-1 and UL-1), the acetylation level was lower than that in CL-1 cells with high *p16* mRNA expression. The expression of the *p16* gene in these 2 cell lines was markedly restored after culture in the presence of a histone deacetylase inhibitors trichostatin A, indicating that *p16* was inactivated by hypoacetylation. Findings obtained this study will add new insights and lead to the better understanding of the disease pathogenesis and future development of epigenetic therapeutic strategies.

Key Words: canine lymphoid tumor, histone H3 acetylation, *p16*

Epigenetic events comprise various mechanisms including DNA methylation, histone modification, and modulation by regulatory RNA^{9,10}. In humans, epigenetic aberrations have been associated with various types of cancers^{8,23}. Histone acetylation is a type of histone modification; other such modifications include phosphorylation, methylation, ubiquitination, sumoylation, and

biotinylation^{11,12}. H3 and H4 histone N-terminal acetylation can alter chromatin structure and patterns of transcription^{11,13}. Aberrant histone acetylations have been reported in various tumors in humans such as hematopoietic malignancies, alimentary cancer, prostate cancer, and lung adenocarcinoma^{8,12,13}. Histone acetylation is catalyzed by histone acetyl transferase, and the

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Table 1. Primer sequences for PCR amplification

Primer name	Primer sequence (5'-3')	GenBank no.	Nucleotide no.
p16EXON1-F148	GGTCGGAGCCCGATTCA	AB675384	148-169
p16EXON1-R242	ACGGGGTCGGCACAGTT		242-226
RPL13A-F	GCCGGAAGGTTGTAGTCGT	AJ388525	87-105
RPL13A-R	GGAGGAAGGCCAGGTAATTC		173-154
p16EXON1-F36	GCAGCATGGAGCCCTTC	AB675384	36-51
p16EXON1-R165	CTGAATCGGGCTCCGACCCA		165-146

RPL13A: Ribosomal protein L13A

acetyl groups are removed by histone deacetylases (HDACs)^{13,15}. HDAC inhibitors (HDACi) can alter gene transcription by increasing the accumulation of hyperacetylated histones H3 and H4; therefore, various HDACi have been tested in preclinical and early clinical studies¹⁵. Vorinostat is the most advanced HDACi drug that was approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma in the United States. Clinical trials using some HDACi have been conducted in human patients with hematological malignancies^{1,18,24}.

p16 is widely known as a tumor suppressor gene, and P16 induces G1-phase cell-cycle arrest^{7,21}. Inactivation of *p16* mediated by gene deletion or DNA methylation has been reported in various human hematopoietic malignancies³. However, regulation of *p16* gene expression by histone acetylation has not been reported in lymphoid tumors.

We have previously found that inactivation of *p16* is mediated by wide-ranging deletions of the *p15-p14-p16* locus in 2 canine lymphoid tumor cell lines (Nody-1 and Ema)⁴. Additionally, *p16* gene expression was suppressed by DNA methylation of CpG islands in 3 other canine lymphoid tumor cell lines (CLBL-1, GL-1, and UL-1)⁵. Although *p16* expression was certainly suppressed via promoter hypermethylation, the *p16* expression level was still different among in these 3 cell lines⁵. Furthermore, the expression level of the *p16* did not entirely coincide with the methylation status in canine primary lymphoma samples, which suggest the involvement of

other epigenetic aberrations such as histone acetylation⁶. Therefore, the purpose of this study was to investigate whether the suppression of *p16* gene expression was mediated by histone H3 acetylation.

We used 4 canine lymphoid tumor cell lines, i.e., CLBL-1²², GL-1²⁰, UL-1²⁷, and CL-1¹⁹, which were established from canine patients with multicentric B-cell lymphoma, B-cell acute lymphoblastic leukemia, renal T-cell lymphoma, and mediastinal T-cell lymphoma, respectively. All cell lines were maintained in an atmosphere of 5% CO₂ and cultivated under normal conditions⁴ in the presence or absence of 25 ng/mL (82.7 nM) trichostatin A (TSA) (Upstate Biotechnology, US) for 24 hours. The optimal concentration of TSA and culture period were defined by preliminary assay. Extraction of total RNAs and complementary DNA synthesis were conducted by reference to our previous report⁴. This assay was performed in duplicate.

Expression of the *p16* gene was measured by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) method as reported in our previous study⁴. The primers for real-time RT-PCR were prepared (Table 1). The amount of *p16* mRNA was quantified relative to that of the internal control gene, *ribosomal protein L13A* (*RPL13A*). All samples were evaluated in triplicate. Expression of *p16* mRNA in cell lines treated with or without TSA was compared between groups using Student's t test. Any *P*-value less than 0.05 was considered to be significant.

In order to evaluate histone H3 acetylation

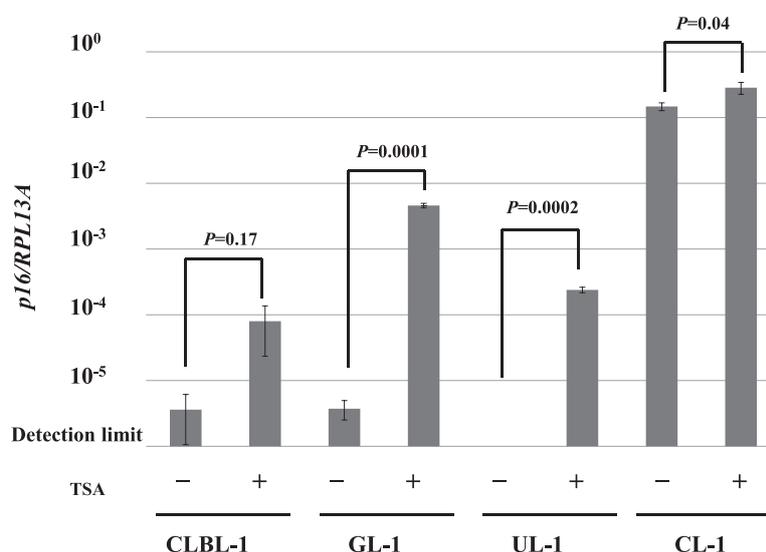


Fig. 1. Changes of the amount of *p16* transcript in CLBL-1, GL-1, UL-1, and CL-1 cells after culture in the absence or presence of TSA. The amount of *p16* transcript was quantified relative to that of *RPL13A* as an internal standard. Error bars indicate the standard deviations. N. D. shows not detected.

status in the 4 cell lines, the chromatin immunoprecipitation (ChIP) assay was performed using the Simple ChIP Enzymatic Chromatin IP Kit (Cell signaling technology, US) according to the manufacturer's instructions. The cell lysate was divided into 3 fractions. The first lysate was incubated with 2.5 μ g of anti-acetyl Histone H3 antibody (EMD Millipore, US). Recognition of canine acetyl histone H3 protein by this antibody was validated in our laboratory by western blotting analysis²⁵. The second lysate was incubated with 2.5 μ g of normal rabbit IgG (Cell signaling technology, US) as a negative control. The third lysate was used as a loading control. The ChIP assay was performed in duplicate. Real-time PCR using the immunoprecipitated DNA was performed to measure the relative abundance of the particular DNA sequence enriched by protein-specific immunoprecipitation versus immunoprecipitation using a non-specific antibody control. The primers for real-time PCR were named p16EXON1-F36 and p16EXON1-R165 and were designed to specifically amplify the genomic region in exon 1 of the *p16* gene (Table 1). Real-time PCR was performed as described above.

After cultivation for 24 hours in the presence of TSA, the expression of *p16* mRNA was

restored in all cell lines. The relative quantity of the *p16* transcript increased by 22-fold in CLBL-1 cells after culture with TSA ($P = 0.17$). The amount of *p16* mRNA increased by 1,200-fold in GL-1 cells after culture in the presence of TSA ($P = 0.00010$). For UL-1 cells, the amount of the *p16* transcript was below the detection limit before treatment with TSA, and it became detectable after culture in the presence of TSA ($P = 0.00020$). The relative quantity of the *p16* transcript increased by 2.0-fold in CL-1 cells after culture with TSA ($P = 0.040$) (Fig. 1).

Histone H3 acetylation was examined using a ChIP assay. The level of histone H3 acetylation was determined relative to the total amount of input DNA. Before treatment with TSA, the exon 1 region of the *p16* gene showed a lower level of H3 acetylation in CLBL-1 (0.47%), GL-1 (0.17%), and UL-1 cells (0.63%) than that in CL-1 cells (12%) (Fig. 2) (Table 2). After treatment with TSA, H3 acetylation level of the exon 1 region of the *p16* gene was 0.11%, 0.78%, 0.84%, and 6.0% in CLBL-1, GL-1, UL-1, and CL-1, respectively (Table 2).

Of the 4 canine lymphoid tumor cell lines used in this study, CLBL-1, GL-1, and UL-1 cells showed low expression of the *p16* gene together

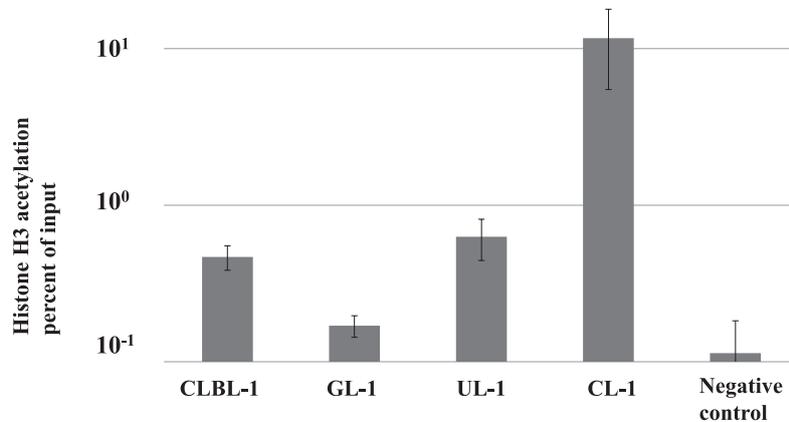


Fig. 2. Results of ChIP assay for histone H3 acetylation at *p16* exon 1 region in canine lymphoid tumor cell lines. Error bars indicate the standard deviation.

Table 2. The summary of the TSA treatment and ChIP assay

	CLBL-1	GL-1	UL-1	CL-1
<i>p16</i> expression	Low	Low	Undetectable	High
<i>p16</i> expression+TSA	22-fold (<i>P</i> = 0.17)	1200-fold (<i>P</i> = 0.00010)	<i>P</i> = 0.00020	2.0-fold (<i>P</i> = 0.040)
Acetylation level	0.47%	0.17%	0.63%	12%

a) Fujiwara-Igarashi *et al.* reported⁴⁾.

with hypermethylated CpG islands in our previous study⁵⁾. Furthermore, histone H3 at *p16* exon 1 was hypoacetylated in these 3 cell lines relative to CL-1. *p16* expression level was increased by culture in the presence of the HDACi, TSA, in these 3 cell lines. In contrast, we previously found that *p16* gene expression was high in CL-1 cells with hypomethylated CpG islands⁵⁾. While the expression of *p16* in CL-1 cells increased after TSA treatment, it might be directly influenced by TSA through its effect on the expression of *p16* itself or indirectly influenced through the effects on the expression of other genes. Besides, acetylation of histone H4 can affect its expression level.

In the 3 cell lines with low expression of *p16* mRNA, i.e., CLBL-1, GL-1, and UL-1 cells, *p16* expression appeared to be regulated through a unique mechanism. In CLBL-1 cells, although the amount of *p16* transcript was increased on culture with 5-Aza-2'-deoxycytidine (5-aza-dC)⁵⁾ (560-fold) or TSA (22-fold), the amplification rate was lower than that in GL-1 cells (5-aza-dC,

14,000-fold; TSA, 1,200-fold). DNA methylation may play an important role in *p16* suppression in CLBL-1 cells because *p16* expression increased after 5-aza-dC treatment with lower concentration than GL-1 cells⁵⁾. Although the amount of *p16* transcript increased 22-fold, the acetylation level of histone H3 after TSA treatment did not increase. Involvement in acetylation of histone H4 or of other non-histone protein can be also suggested. In GL-1 cells, *p16* expression was markedly increased by culture with TSA and 5-aza-dC. The amount of *p16* transcript in UL-1 cells was initially below the detection limit but increased upon culture with TSA or 5-aza-dC. While the acetylation level of histone H3 after TSA treatment slightly increased in GL-1 and UL-1 cells, statistical analysis could not be performed because of limited test sample. As described above, P16 is tumor suppressor gene and induces G1-phase cell cycle arrest. Since lymphoid tumor cells usually grow under the condition with low expression of *p16* gene in GL-1 and UL-1, these cells can not proliferate

under the condition with P16 overexpressed by TSA treatment. Compared to untreated cells, approximately 50% of proliferating cells were suppressed after TSA treatment in our study (data not shown). Therefore, the accurate acetylation status could not be detected due to many suppressed cells. Consequently, only slight increase of histone H3 acetylation level could be detected. Histone H3 acetylation in addition to DNA methylation may be the major mechanism to suppress *p16* gene expression in GL-1 and UL-1 cells.

The histone H3 acetylation level of *p16* gene was not always coincident with response after TSA treatment in the lymphoid tumor cell lines. Furthermore, TSA can influence on acetylation status of histone H4 and other non-histone proteins. Although p53 and p21 were cell-cycle regulators and non-histone proteins, they have been known to be acetylated by TSA treatment. Examination of other mechanisms including acetylation of histone H4 and other non-histone proteins can be needed.

Regulation of *p16* gene expression by histone acetylation has been reported in human gastric¹⁶⁾ and prostate cancers¹⁷⁾. These studies showed that histone H3 hypoacetylation and DNA methylation were both associated with silencing of the *p16* gene. Furthermore, aberrant regulation of histone acetylation is considered to occur in canine tumor cells^{2,14,25,26)}. In the present study, we demonstrated that differences in the level of histone H3 acetylation level as well as DNA methylation among cell lines were related to changes in *p16* expression upon treatment with TSA, which suggest that *p16* expression is regulated through histone H3 acetylation in the part of canine lymphoid tumor cell lines.

Histone H3 hypoacetylation may thus represents a molecular mechanism that can be used to suppress *p16* gene expression in canine lymphoid tumors. In the present study, histone H3 acetylation was examined only in the cultured tumor cell lines; further studies are warranted to examine primary lymphoid tumor samples. In

addition, acetylation status of histone H4 will be needed. As described above, novel drugs that target epigenetic pathway have been tested in humans; therefore, their clinical applications in veterinary medicine are expected. Findings obtained this study will add new insights on the epigenetic aberrations of the *p16* gene in canine lymphoid tumors, which will lead to the better understanding of the disease pathogenesis and future development of epigenetic therapeutic strategies.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

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References

- 1) Claus, R. and Lubbert, M. 2003. Epigenetic targets in hematopoietic malignancies. *Oncogene*, **22**: 6489–6496.
- 2) Dhawan, D., Ramos-Vara, J. A., Hahn, N. M., Waddell, J., Olbricht, G. R., Zheng, R., Stewart, J. C. and Knapp, D. W. 2012. DNMT1: an emerging target in the treatment of invasive urinary bladder cancer. *Urol. Oncol.*, **31**: 1761–1769.
- 3) Drexler, H. G. 1998. Review of alterations of the cyclin-dependent kinase inhibitor INK4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells. *Leukemia*, **12**: 845–

- 859.
- 4) Fujiwara-Igarashi, A., Goto-Koshino, Y., Mochizuki, H., Maeda, S., Fujino, Y., Ohno, K. and Tsujimoto, H. 2013. Simultaneous inactivation of the p16, p15 and p14 genes encoding cyclin-dependent kinase inhibitors in canine T-lymphoid tumor cells. *J. Vet. Med. Sci.*, **75**: 733-742.
 - 5) Fujiwara-Igarashi, A., Goto-Koshino, Y., Mochizuki, H., Sato, M., Fujino, Y., Ohno, K. and Tsujimoto, H. 2014. Inhibition of p16 tumor suppressor gene expression via promoter hypermethylation in canine lymphoid tumor cells. *Res. Vet. Sci.*, **97**: 60-63.
 - 6) Fujiwara-Igarashi, A., Goto-Koshino, Y., Sato, M., Maeda, S., Igarashi, H., Takahashi, M., Fujino, Y., Ohno, K. and Tsujimoto, H. 2014. Prognostic significance of the expression levels of the p16, p15, and p14 genes in dogs with high-grade lymphoma. *Vet. J.*, **199**: 236-244.
 - 7) Gil, J. and Peters, G. 2006. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nat. Rev. Mol. Cell Biol.*, **7**: 667-677.
 - 8) Haluskova, J. 2010. Epigenetic studies in human diseases. *Folia. Biol., (Praha)* **56**: 83-96.
 - 9) Holliday, R. 1987. DNA methylation and epigenetic defects in carcinogenesis. *Mutat. Res.*, **181**: 215-217.
 - 10) Holliday, R. 2006. Epigenetics: a historical overview. *Epigenetics*, **1**: 76-80.
 - 11) Jenuwein, T. and Allis, C. D. 2001. Translating the histone code. *Science*, **293**: 1074-1080.
 - 12) Kanwal, R. and Gupta, S. 2010. Epigenetics and cancer. *J. Appl. Physiol.*, **109**: 598-605.
 - 13) Khan, S. N. and Khan, A. U. 2010. Role of histone acetylation in cell physiology and diseases: An update. *Clin. Chim. Acta.*, **411**: 1401-1411.
 - 14) Kisseberth, W. C., Murahari, S., London, C. A., Kulp, S. K. and Chen, C. S. 2008. Evaluation of the effects of histone deacetylase inhibitors on cells from canine cancer cell lines. *Am. J. Vet. Res.*, **69**: 938-945.
 - 15) Lane, A. A. and Chabner, B. A. 2009. Histone deacetylase inhibitors in cancer therapy. *J. Clin. Oncol.*, **27**: 5459-5468.
 - 16) Majid, S., Kikuno, N., Nelles, J., Noonan, E., Tanaka, Y., Kawamoto, K., Hirata, H., Li, L. C., Zhao, H., Okino, S. T., Place, R. F., Pookot, D. and Dahiya, R. 2008. Genistein induces the p21WAF1/CIP1 and p16INK4a tumor suppressor genes in prostate cancer cells by epigenetic mechanisms involving active chromatin modification. *Cancer. Res.*, **68**: 2736-2744.
 - 17) Meng, C. F., Zhu, X. J., Peng, G. and Dai, D. Q. 2009. Promoter histone H3 lysine 9 di-methylation is associated with DNA methylation and aberrant expression of p16 in gastric cancer cells. *Oncol. Rep.*, **22**: 1221-1227.
 - 18) Mercurio, C., Minucci, S. and Pelicci, P. G. 2010. Histone deacetylases and epigenetic therapies of hematological malignancies. *Pharmacol. Res.*, **62**: 18-34.
 - 19) Momoi, Y., Okai, Y., Watari, T., Goitsuka, R., Tsujimoto, H. and Hasegawa, A. 1997. Establishment and characterization of a canine T-lymphoblastoid cell line derived from malignant lymphoma. *Vet. Immunol. Immunopathol.*, **59**: 11-20.
 - 20) Nakaichi, M., Taura, Y., Kanki, M., Mamba, K., Momoi, Y., Tsujimoto, H. and Nakama, S. 1996. Establishment and characterization of a new canine B-cell leukemia cell line. *J. Vet. Med. Sci.*, **58**: 469-471.
 - 21) Russo, A. A., Tong, L., Lee, J. O., Jeffrey, P. D. and Pavletich, N. P. 1998. Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumour suppressor p16INK4a. *Nature*, **395**: 237-243.
 - 22) Rutgen, B. C., Hammer, S. E., Gerner, W., Christian, M., de Arespacochaga, A. G., Willmann, M., Kleiter, M., Schwendenwein, I. and Saalmuller, A. 2010. Establishment and characterization of a novel canine B-cell line derived from a spontaneously occurring diffuse large cell lymphoma. *Leuk. Res.*, **34**: 932-938.
 - 23) Santos-Reboucas, C. B. and Pimentel, M. M. 2007. Implication of abnormal epigenetic patterns for human diseases. *Eur. J. Hum. Genet.*, **15**: 10-17.
 - 24) Stimson, L., Wood, V., Khan, O., Fotheringham, S. and La Thangue, N. B. 2009. HDAC inhibitor-based therapies and haematological malignancy. *Ann. Oncol.*, **20**: 1293-1302.
 - 25) Tomiyasu, H., Goto-Koshino, Y., Fujino, Y., Ohno, K. and Tsujimoto, H. 2013. Epigenetic regulation of the ABCB1 gene in drug-sensitive and drug-resistant lymphoid tumour cell lines obtained from canine patients. *Vet. J.*, **199**: 103-9.
 - 26) Watanabe, M., Chen, I. Y., Ishikura, Y., Nakagawa, T., Mochizuki, M., Nishimura, R., Sasaki, N. and Sugano, S. 2009. Tumor cell growth inhibition and cell differentiation analysis in a canine mammary tumor cell line (MCM-B2) treated with four chemical

- reagents. *J. Vet. Med. Sci.*, **71**: 1413-1417.
- 27) Yamazaki, J., Baba, K., Goto-Koshino, Y., Setoguchi-Mukai, A., Fujino, Y., Ohno, K. and Tsujimoto, H. 2008. Quantitative assessment of minimal residual disease (MRD) in canine lymphoma by using real-time polymerase chain reaction. *Vet. Immunol. Immunopathol.*, **126**: 321-331.