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<td>Author(s)</td>
<td>Fujiwara-Igarashi, Aki; Tomiyasu, Hirotaka; Igarashi, Hirotaka; Yu, Yoshihiko; Goto-Koshino, Yuko; Ohno, Koichi; Tsujimoto, Hajime</td>
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Regulation of \(p16\) gene expression by histone H3 acetylation in canine lymphoid tumor cell lines

Aki Fujiwara-Igarashi\(^1,2,\ast\), Hirotaka Tomiyasu\(^2\), Hirotaka Igarashi\(^2\), Yoshihiko Yu\(^1\), Yuko Goto-Koshino\(^2\), Koichi Ohno\(^2\) and Hajime Tsujimoto\(^2\)

\(^1\)Division of Therapeutic Science I, Department of Clinical Veterinary Medicine, Nippon Veterinary and Life Science University, Musashino-shi, Tokyo, Japan
\(^2\)Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

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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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Acetyl groups are removed by histone deacetylases (HDACs\textsuperscript{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 studies\textsuperscript{15}. 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\textsuperscript{1,18,24}. p16 is widely known as a tumor suppressor gene, and P16 induces G1-phase cell-cycle arrest\textsuperscript{7,21}. Inactivation of p16 mediated by gene deletion or DNA methylation has been reported in various human hematopoietic malignancies\textsuperscript{3}. 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)\textsuperscript{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)\textsuperscript{5}. Although p16 expression was certainly suppressed via promoter hypermethylation, the p16 expression level was still different among in these 3 cell lines\textsuperscript{5}. 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 acetylation\textsuperscript{6}. 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-1\textsuperscript{22}, GL-1\textsuperscript{20}, UL-1\textsuperscript{27}, and CL-1\textsuperscript{19}, 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% CO\textsubscript{2} and cultivated under normal conditions\textsuperscript{4} 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 report\textsuperscript{4}. 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 study\textsuperscript{4}. 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

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>GenBank no.</th>
<th>Nucleotide no.</th>
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<tr>
<td>p16EXON1-F148</td>
<td>GGTCGGAGCCGATTCA</td>
<td>AB675384</td>
<td>148-169</td>
</tr>
<tr>
<td>p16EXON1-R242</td>
<td>ACCGGGTCGGCACAGTT</td>
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<tr>
<td>RPL13A-F</td>
<td>GCCGGAAGGTTGTTAGTCGT</td>
<td>AJ388525</td>
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<td>RPL13A-R</td>
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<tr>
<td>p16EXON1-F36</td>
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<td>36-51</td>
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<tr>
<td>p16EXON1-R165</td>
<td>CTGAATCGGGCTCCGACCCA</td>
<td></td>
<td>165-146</td>
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RPL13A: Ribosomal protein L13A

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Table 1. Primer sequences for PCR amplification
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 μg 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 analysis\(^\text{25}\). The second lysate was incubated with 2.5 μg 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...
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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 islands\(^5\). 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’-deoxycitidine (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 cells\(^5\). 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

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

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

- Fuwara-Igarashi et al. reported\(^4\).
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 gastric\textsuperscript{16} and prostate cancers\textsuperscript{17}. 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\textsuperscript{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.

Acknowledgements

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