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Leptin Modulates the Metastasis of Canine Inflammatory Mammary Adenocarcinoma Cells Through Downregulation of Lysosomal Proetective Protein Cathepsin A (CTSA)

렙틴의 라이소좀 내 Cathepsin A 효소 발현 저하를 통한 개의 악성 염증성 유선종양 전이 활성화 기전 연구

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ABSTRACT

Canine malignant breast cancers present with a poor prognosis due to metastasis to other organs, such as lung and lymph node metastases. Unlike in human studies where obesity

has been shown to increase the risk of breast cancer, this has not been well studied in veterinary science. In our preliminary study, I discovered that leptin downregulated cathepsin A, which is responsible for LAMP2a degradation. LAMP2a is a rate-limiting factor in chaperone-mediated autophagy and is highly active in malignant cancers. Therefore, in this study, alterations in metastatic capacity through cathepsin A by leptin, which are secreted at high levels in the blood of obese patients, were investigated. I used a canine inflammatory mammary gland adenocarcinoma (CHMp) cell line cultured with RPMI-1640 and 10% fetal bovine serum. The samples were then subjected to real-time polymerase chain reaction, Western blot, immunocytochemistry, and lysosome isolation to investigate and visualize the metastasis and chaperone-mediated autophagy-related proteins. Results showed that leptin downregulates cathepsin A expression at both transcript and protein levels, whereas LAMP2a, the rate-limiting factor of chaperone-mediated autophagy, was upregulated by inhibition of LAMP2a degradation. Furthermore, leptin promoted LAMP2a multimerization through the lysosomal mTORC2/PHLPP1/AKT pathway. These findings suggest that targeting leptin receptors can alleviate breast cancer cell metastasis in dogs.

Keywords : Leptin, Cathepsin A, Adenocarcinoma, Metastasis, Obesity

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LIST OF ABBREVIATIONS

CMT	**Canine mammary gland tumor**
FNA	**Fine needle aspiration**
OHE	**Ovariohysterectomy**
cIMC	**Canine inflammatory mammary carcinoma**
CTSA	**Lysosomal protective protein cathepsin A (PPCA)**
LAMP1	**Lysosomal-associated membrane protein 1**
LAMP2a	**Lysosomal-associated membrane protein 2a**
CMA	**Chaperone-mediated autophagy**
OBR	**Leptin receptor**
PHLPP1	**PH domain and leucine rich repeat protein phosphatase 1**
ACTB	**Beta-actin**
VIM	**Vimentin**
CDH1	**E-cadherin**
siRNA	**Small interfering ribonucleic acid**
cDNA	**Complementary deoxyribonucleic acid**
mRNA	**Messenger ribonucleic acid**
RNAi	**RNA interference**

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PUBLICATION PAPERS

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4. Lee H-S, Lee S-H, **Kim J-W**, Lee B-C, OH H-J, Kim J-H: Development of Novel Continuous and Interval Exercise Programs by Applying the FITT-VP Principle in Dogs. The World Scientific Journal, 2020; 2020, 3029591.

ABSTRACTS and PRESENTATIONS

1. **Kim J-W**, Mahiddine F-Y, Kim G-A: Canine leptin stimulates chaperone-mediated autophagy via regulation of protective protein cathepsin A (PPCA) in mammary gland tumors, KALAS International Symposium 2020. (Awarded as Excellent Poster)

2. **Kim J-W**, Kim E-H, Lee S-H, Kim G-A, Lee B-C: Serum Klotho Protein Concentration in Cloned Dogs. The Korean Society of Veterinary Science 2018.

3. **Kim J-W**, Won Y-K, Lee S-W, Lee H-J, Jo M-S, Kim M-J, Lee B-C: Similarity assessment of reproductive properties among cloned dogs based on their cell donor dogs. The Korean Society of Veterinary Science 2018.

4. **Kim J-W**, Kim G-A, Lee B-C: Comparison of mRNA expression of autophagy-related genes upon aging in peripheral blood mononuclear cells in dogs. The Korean Society of Veterinary Science 2019.

5. **Kim J-W**, Lee S-H, Ra K-H, Kumar R-P, Kim G-A, Lee B-C: Comparison of mRNA expression of autophagy-related genes between *Rectus abdominalis* and peripheral blood mononuclear cells(PBMCs). The Korean Society of Veterinary Science 2019.

6. **Kim J-W**, Kim G-A, Lee B-C: Preliminary Investigation on the Expression of Autophagy and Anti-aging Related Genes in Peripheral Blood Mononuclear Cells(PBMCs) of Three Male *Beagle* Dogs. Korean Society of Life Science 2019.

7. Mahiddine F-Y, **Kim J-W**, Kim M-J: Canine Amniotic Membrane-Derived Mesenchymal Stem Cells Conditioned Media Enhances Frozen-Thawed Canine Sperm Parameters. The Korean Society for Microbiology and Biotechnology Conference 2020.

8. Mahiddine F-Y, **Kim J-W**, Kim M-J: Establishment of canine primary myoblast culture and myotubes differentiation. Korean Society for Biochemistry and Molecular Biology Conference 2020.

LITERATURE REVIEW

1. Canine Mammary Gland Tumor and Metastasis.

1.1. General information

Cancer is the number one cause of death in dogs, with mammary gland having the highest incidence in female dogs[1]. Approximately half of the patients diagnosed with mammary gland tumors are diagnosed as malignant [2] with the incidence of less than 0.5% for neutering before the first heat, 8% after the first, and 26% after the second onset[3].. If spaying is not done, the average CMT incidence increases by about four to dozens times [4, 5]. Although several studies exist on whether the timing of ovariohysterectomy (OHE) affects the occurrence of CMT, it is definitely recommended for prevention and auxiliary treatment purposes because it can reduce the probability of newly developed CMTs by 50 percent in mastectomy [6]. This phenomenon has led to the discovery that exposure to progesterone after puberty increases the chance of developing mammary gland tumors.

1.2. Types of Canine Malignant Mammary Gland Tumors

A typical malignant mammary gland is simple mammary adenocarcinoma, which in the case of such malignant tumors spreads to the lungs, local lymph nodes, bones, and sometimes brain. Patients suspected of malignant tumors are evaluated for their prognosis through metastasis assessment and its grading [7]. In the case of multiple CMTs, each

mammary gland is independent and all mammary glands should be evaluated in the evaluation of malignancy through fine needle aspiration (FNA) examination. In patients with metastasis, euthanasia is sometimes indicated because of poor prognosis. Therefore, in patients with malignant CMTs, it is important to develop novel drugs that can prevent metastasis with surgical resection in the early stages. In recent studies, CMTs are suggested as a human breast cancer research model because of the similarity in their etiology, cancer progression, clinical and molecular profile [8, 9].

1.3. Is Obesity related with CMTs?

The studies on human breast cancers have shown that the obesity calculated with body mass index (BMI) is positively correlated with the higher incidence rate of breast cancers [10, 11]. Moreover, free fat acids (FFAs) produced by fat lipolysis continuously activates NF-kB increasing the number of cancer stem cells [12], which also can be said obesity stimulates cancer invasion ability. Similarly, a study has demonstrated that obesity at the time of puppy and one-year prior to the diagnosis may increase the chance of developing CMTs [13]. It suggests that obesity in dogs measured by body condition score (BCS) acts as a major risk factor in the development of CMTs.

An increase in body fat caused by obesity induces the secretion of hormones and cytokines produced by adipose tissues. Among them, leptin, a well-known obesity hormone, increases in serum concentration with obesity [14]. This tendency is consistent with a canine obesity research [15]. Leptin is known to control energy homeostasis, food

intakes [16], and angiogenesis [17]. It has also been found to increase breast cancer proliferation and metastases with upregulated secretion of proinflammatory cytokines [18].

2. Cathepsins and Chaperone Mediated Autophagy in cancers

2.1. Cathepsins and Cancer Metastasis

Many studies have conducted to control tumors by targeting intracellular proteases. Among them, cathepsin enzymes (A, B, C, D, E, F, G, H, L, K, O, S, V and W) are mainly located within lysosomes. They can be subdivided into three types (1) cysteine proteases (B, C, F, H, L, K, O, S, V, W, and X); (2) serine proteases (A and G); (3) aspartic proteases (D and E). The cysteine cathepsin group is relatively well-studied than others. Cathepsins are known to be highly expressed in malignant invasive tumors. For e example, cathepsin B was found to have high activity at the edges of invasive tumors and cathepsin D was found to be related to local relapse and metastasis in human breast cancer [19]. Moreover, there is a study that high levels of cathepsin D expression can be used as biomarker for invasion and aggressiveness in human salivary gland carcinomas [20]. As such, there has been many correlation studies between cysteine cathepsins and cancer, but the correlation with serine cathepsins is poorly studied yet. There is a lack of researches going on serine cathepsin and cancer metastases.

2.2. Chaperone Mediated Autophagy in cancer physiology

Autophagy, meaning “self-devouring” mechanism that endogenously expresses in mammalian species [21], is divided into three subtypes; macroautophagy, microautophagy,

and chaperone-medicated autophagy (CMA). CMA is a mechanism that maintains homeostasis by selective degradation of peptides with KFERQ motif inside their amino acid sequences unlike non-specific macroautophagy (MA) [22]. CMA is known to be constantly active in malignant tumors, and is essential for tumor proliferation and metastasis [23]. In addition, inhibiting CMA decreases the rate of growth of tumors and the number of cells metastasis [23]. This feature has led researchers to consider it as a therapy target that may inhibit tumor progression. As expected, CMA can be modulated by small molecules such as anisomycin, cycloheximide and etc. as rapamycin inhibits MA [24]. In addition, endogenous cathepsin A (CTSA) located within lysosomes controls the half-life of LAMP2a, the rate-limiting factor for CMA through its degradation [25]. Also, the multimerization of LAMP2a is found to be stimulated by mTORC2/PHLPP1/AKT1 pathway [26].

I. Introduction

Leptin is a peptide hormone produced and secreted mainly by adipose cells and plays a crucial role in reproduction [27], appetite [28], and cancer environment [29]. Previous studies described leptin as a tumor metastasis-promoting factor that induces inflammation [30] and induces epithelial-mesenchymal transition (EMT), mostly in malignant breast [31, 32] and lung cancers [33, 34]. Thus, many studies have been conducted to find novel antagonists against the leptin receptor (OBR) [35]. Of these, Allo-aca, a leptin antagonist binding selectively to the C-terminus of OBR without any agonistic effects, has been shown to be effective against triple-negative breast cancer (TNBC) by increasing survival rates [36]. As the blood leptin concentration increases proportionally to the degree of obesity and aging in both humans and dogs [37-41], the regulation of leptin activity is crucial for managing and inhibiting cancer. Given the potential similarity between them, canine mammary gland tumors (CMTs) are considered as a model for investigating human breast cancer [42]. Similar to female humans, breast cancers are the most common tumor in domestic intact bitches, and approximately half of them are diagnosed as malignant [43]. Canine inflammatory mammary carcinoma (cIMC) is a fast-growing, malignant form that shows poor prognosis (mean survival time; 25 days) because of its high incidence of metastasis to regional lymph nodes [44], which has risk factors that are similar to those of human breast cancers (e.g., age, progesterone, obesity, etc.) [8, 45].

It has been suggested that leptin upregulates autophagy and promotes lysosomal degradation of long-lived proteins in adipose cells [46] and MCF-7 cells [30]. Lysosomal

activity is closely related to autophagy, especially chaperone-mediated autophagy (CMA), selective autophagy targeting substrate proteins bearing KFERQ-motifs (e.g., GAPDH) [47]. It is involved in cellular homeostasis and is highly expressed in cancer, promoting proliferation, anti-apoptosis, and metastasis [23, 48]. The multimerization of LAMP2a on the lysosomal membrane is crucial for binding with the chaperone protein HSP70 through the lysosomal mTORC2/PHLPP1/AKT pathway [26]. Cuervo and her colleagues demonstrated that the lysosomal enzyme cathepsin A (CTSA) regulates the half-life of LAMP2a, the rate-limiting factor for CMA, through its degradation [49]. Mutations in the CTSA gene cause the lysosomal storage disorder galactosialidosis both in humans and dogs [50, 51]. However, the association between leptin and CMTs has not yet been described. Moreover, although previous studies have described the relationship between cathepsins and leptin [52, 53], CTSA has not been extensively studied in cancer metastasis. Therefore, in this study, I mainly focused on the alteration of metastasis of CHMp cells with leptin (with or without Allo-aca) and CTSA and further evaluated the alteration of CMA activity.

II. Materials and Methods

2.1. Cell line and Cell Culture Methods

The CHMp cell line was generously donated by Professor So Young Lee from the Department of Veterinary Pharmacology, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea. The cells were originally isolated from the primary lesions of 12-year-old female inflammatory adenocarcinoma and established as a cell line by Professor Nobuo Sasaki from the University of Tokyo. The cells were cultured in RPMI-1640 supplemented with 10% charcoal-dextran treated fetal bovine serum (both from Gibco, Waltham, MA), and 0.5% gentamicin (Merck), in a 5% carbon dioxide 37 °C incubator.

2.2. Chemicals and Antibodies

Canine leptin was purchased from Peprotech, Rocky Hill, NJ, USA, and Allo-aca (H-alloThr-Glu-Nva-Val-Ala-Leu-Ser-Arg-Aca-NH_2) developed by Otvos et al. [54], was synthesized by Koma Biotechnology, Seoul, Republic of Korea. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The antibodies used in this study are summarized in Table 1.

Table 1. The information of antibodies used in this study

Target	Catalog#	Host	Clone	Dilution	Manufacturer
LAMP2a	ab18528	Rabbit	Polyclonal	1:1000	Abcam
LAMP1	ab24170	Rabbit	Polyclonal	1:1000	Abcam
HSP70	ab53496	Mouse	Monoclonal	1:1000	Abcam
Vimentin	ab1316	Mouse	Monoclonal	1:400	Abcam
MMP9	ab38898	Rabbit	Polyclonal	1:1000	Abcam
pAKT1 (Ser473)	ab81283	Rabbit	Monoclonal	1:5000	Abcam
ACTB	ab8227	Mouse	Monoclonal	1:5000	Abcam
CDH1	14-3249-82	Rat	Monoclonal	1:250	Invitrogen
AKT1	sc-135829	Mouse	Monoclonal	1:1000	SCBT
PHLPP1	sc-390129	Mouse	Monoclonal	1:500	SCBT
mTOR	sc-517464	Mouse	Monoclonal	1:200	SCBT
CTSA	139645	Rabbit	Polyclonal	1:1000	US Biological

2.3. Reverse siRNA Transfection

Cathepsin A-siRNA (CTSA-siRNA) was transfected using Lipofectamine RNAiMAX (Invitrogen, Waltham, MA). Before seeding the cells in 6-well dishes, 50 pmol of CTSA-siRNA was diluted in 500 µL Opti-MEM (Gibco, Waltham, MA) with the addition of 7 µL of Lipofectamine RNAiMAX (Invitrogen, Waltham, MA). After 20 min of incubation at room temperature, 300,000 CHMp cells were seeded and incubated for 24 h and then used for further experiments. The siRNA sequences are listed in Table 2.

Table 2. CTSA siRNA sequences used in this study. The selected siRNA was marked as bold

siRNA	Accession No.	Sequences
CTSA_siRNA_1	NM_001109915.1	CGUGACAAAUCUGCAGGAA
		UUCCUGCAGAUUUGUCACG
CTSA_siRNA_2		CACAGAAAUACCGGAUCUU
		AAGAUCCGGUAUUUCUGUG
CTSA_siRNA_3		UGUUCUCAGAACAAGUGUA
		UACACUUGUUCUGAGAACA

2.4. Cell Proliferation Assay

Five thousand cells per well were seeded in a 96-well dish with RPMI-1640 and 10% FBS and treated with 12 nM leptin and 100 nM Allo-aca for five days. Each day, the cells were treated with 10 µL of 12 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen, Waltham, MA) in 100 µL phenol red-free RPMI-1640 (Gibco, Waltham, MA) and incubated for 4 h at 37 °C. After incubation, 85 µL of MTT-containing media was discarded, and 80 µL of DMSO was added to dissolve the formazan for 10 min at 37 °C following horizontal shaking for 30 min in the dark. The samples were analyzed at 450 nm using a Tecan Sunrise Microplate Reader (Tecan, Männedorf, Switzerland).

2.5. Cell Invasion Assay

The cultured cells were treated with non-enzymatic cell dissociation solution (Sigma-Aldrich, St. Louis, MO, USA) for 5 min to detach cells without damaging cell surface receptors. Suspended cells were subjected to a cell invasion assay using 8 µm BioCoat Matrigel invasion chambers (Corning, Bedford, MA, USA). Cells (2×10^5) were seeded in each well with RPMI-1640 + 10% FBS and incubated for 22 h. After incubation, the upper membrane was swabbed with sterile cotton swabs and stained with Diff-Quick solution (Sysmax, Tokyo, Japan). The invaded cells were counted under a 40X bright microscope and counted with ImageJ software (NIH, Bethesda, MD, USA).

2.6. RNA Extraction and Real-Time Quantitative PCR analysis (RT-qPCR)

After 24 h of incubation with the addition of leptin and/or Allo-aca, the cells were collected for RNA extraction. I used an easy-spin Total RNA Extraction Kit, and complementary DNA (cDNA) synthesis was performed using the Maxime RT PreMix Kit (both kits from Intron Biotechnology, Gyeonggi, Republic of Korea) according to the manufacturer's instructions. For RT-qPCR analysis, I used 2× SYBR Green PCR Master Mix (Applied Biosystems, MA, USA) as a probe, and the primer sequences are listed in Table 1. Amplification was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, MA, USA) with reactions containing 50 ng cDNA, 10 pmol of forward and reverse primers, 10 μl of SYBR green premix, and nuclease-free water in each well. The thermocycling protocol was 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 40 s. All reaction cycles were conducted in a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems, MA, USA). To compare mRNA expression based on leptin and Allo-aca treatment in CHMp cells, mRNA expression was normalized to the housekeeping gene β-actin (ΔCt), and the relative amount of each transcript was quantified in at least three replicates using the following equation: relative quantity (R) = $2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$

Table 3. Primers and their sequences used in this study

Gene	GenBank Accession No.	Primer Sequences	Product Size (bp)
CTSA	NM_001109915.1	F: 5'-CAG ACC CAC TGC TGT TCT CA-3' R: 5'-CTG CAG ATT TGT CAC GCA TT-3'	56
PHLPP1	XM_005615667.3	F: 5'-CCC ACA CCA CTG ACT TTC CT-3' R: 5'-GGG GTG CAG GTT TGT TTC TA-3'	128
AKT1	XM_022422422.1	F: 5'-GGT GAT CCT GGT CAA GGA GA-3' R: 5'-GAG TAC TTC AGG GCC GTC AG-3'	159
VIM	NM_001287023.1	F: 5'-CCG ACA GGA TGT TGA CAA TG-3' R: 5'-GCT CCT GGA TTT CCT CAT CA-3'	116
CDH1[1]	NM_001287125.1	F: 5'-AAT GAC CCA GCT CGT GAA TC-3' R: 5'-CAC CTG GTC CTT GTT CTG GT-3'	108
ACTB	NM_001195845.2	F: 5'-GCG CAA GTA CTC TGT GTG GA-3' R: 5'-ACA TTT GCT GGA AGG TGG AC-3'	65

2.7 Immunocytochemistry (ICC)

The cells were seeded in 8-well SPL Cell Culture (SPL Life Science, Gyeonggi, Republic of Korea) at a density of 5 000 cells/well. After 6 h of incubation, cells were fixed with 4% paraformaldehyde and washed with ice-cold phosphate-buffered saline (PBS). The cells were then permeabilized with 0.1% Triton X-100 diluted in PBS for 10 min. One percent bovine serum albumin (BSA) in PBS-Tween 20 was used for blocking for 1 h, and primary antibodies against LAMP1 (diluted 1:200 in PBS-T with 1% BSA) were incubated overnight at 4 °C. The next day, goat anti-rabbit IgG H&L Alexa Fluor 488 antibody against LAMP1 primary antibodies was added for 1 h at room temperature in the dark. For the anti-LAMP2a antibody, the antibody was manually conjugated with Alexa Fluor 647 Conjugation Kit-Lightning-Link (Abcam, Cambridge, United Kingdom) and incubated overnight in the dark. The slides were then washed with ice-cold PBS and then mounted with Vectashield Antifade Mounting Medium (Vector Laboratories, Peterborough, United Kingdom). All images were obtained using a Ti2-E confocal microscopy system (Nikon, Tokyo, Japan).

2.8. Lysosome Isolation and Immunoblot

CHMp cells were cultivated in 148.2 mm dishes at a density of 5×10^6 and then treated with 0.01% DMSO (control), leptin (12 nM), Allo-aca (100 nM), or combination of leptin and Allo-aca. After treatment with chemicals, the cells were collected with disposable cell

scrapers. Lysosomes were then isolated from cells using the Minute Lysosome Isolation Kit (Invent Biotechnologies, Plymouth, MN, USA) based on the spin-column method. Approximately 1.0×10^8 cells were subjected to lysosome isolation. The isolated lysosomes were resuspended in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with a protein inhibitor cocktail and phosphatase inhibitor to prevent protein degradation, blocking the activity of phosphatases, and centrifuged at 16,000 × g for 30 min. The supernatant was transferred to a new 1.5 mL tube and subjected to protein quantification using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Lysosome lysates (10 μg) were loaded onto 12 % Mini-PROTEAN TGX Precast gels. After transfer and blocking, the membranes were incubated with primary overnight and developed with HRP-conjugated secondary antibodies.

2.9. Western Blot Analysis

The samples were lysed with PRO-PREP Protein Extraction Solution (Intron Biotechnology, Gyeonggi, Republic of Korea), and their protein concentration was measured by Bradford assay. For SDS-PAGE, 5 μg of each sample was loaded onto each well of 12% Mini-PROTEAN TGX Precast Gel (Bio-Rad, Hercules, CA) and paged at 100 V for 1 h 30 min. The samples were then transferred to 0.45 μm methanol-activated polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA) at 350 mA for 1 h 10 min. The membranes were blocked with 5% skim milk solution and washed three times with TBS-T for 10 min for 3 min, followed by primary antibody incubation

overnight. The next day, the membranes were incubated with HRP-conjugated secondary antibody for an hour. For primary antibodies, I used rabbit anti-LAMP1, LAMP2a, cathepsin A (PPCA), mouse anti-hsp70, β-actin, PHLPP1, AKT1, pAKT1(ser473), and rat anti-CDH1.

2.10. Statistical Analysis

The raw data were analyzed with GraphPad Prism 8.0 (San Diego, CA) with one-way ANOVA followed by Bonferroni's test for further analysis. The Western blot and immunocytochemistry images were analyzed with ImageJ software (National Institute of Health, Bethesda, MD) according to NIH guidelines.

III. Results

3.1 Leptin downregulates CTSA (Cathepsin A) and upregulates LAMP2a in CHMp cells.

To determine the optimal concentration of leptin and Allo-aca, I introduced leptin into CHMp cells. The MTT assay showed that 6 nM leptin had a cell proliferation effect, while Allo-aca did not show this effect even at 100 nM (the highest concentration) (Figure 1 a,b). Interestingly, CTSA mRNA was significantly decreased with 12 nM leptin treatment and increased at the lowest concentration (1 nM) with Allo-aca (Figure 1 c,d). Thus, I treated cells with 12 nM leptin and/or 100 nM Allo-aca to maximize its antagonistic effect. Real-time PCR analysis showed that gene expression was upregulated following treatment with Allo-aca, disregarding the presence of leptin (Figure 2a). Subsequently, the time-course immunoblot of CTSA and LAMP2a showed that leptin gradually downregulates CTSA from 6 h while significantly upregulating LAMP2a after 24 h (Figure 2b). Conversely, Allo-aca upregulated CTSA after 6 h of treatment, but no significance was observed in LAMP2a (Figure 2c).

As shown in Figure 1d, Allo-aca antagonized the effect of leptin in CHMp cells, resulting in decreased LAMP2a expression. To elucidate the role of leptin in CMA, I transfected CHMp cells with CTSA-siRNA (Figure 3). Immunoblot analysis showed that Allo-aca upregulated CTSA in the transfected cells, but the expression of LAMP2a was not significantly altered.

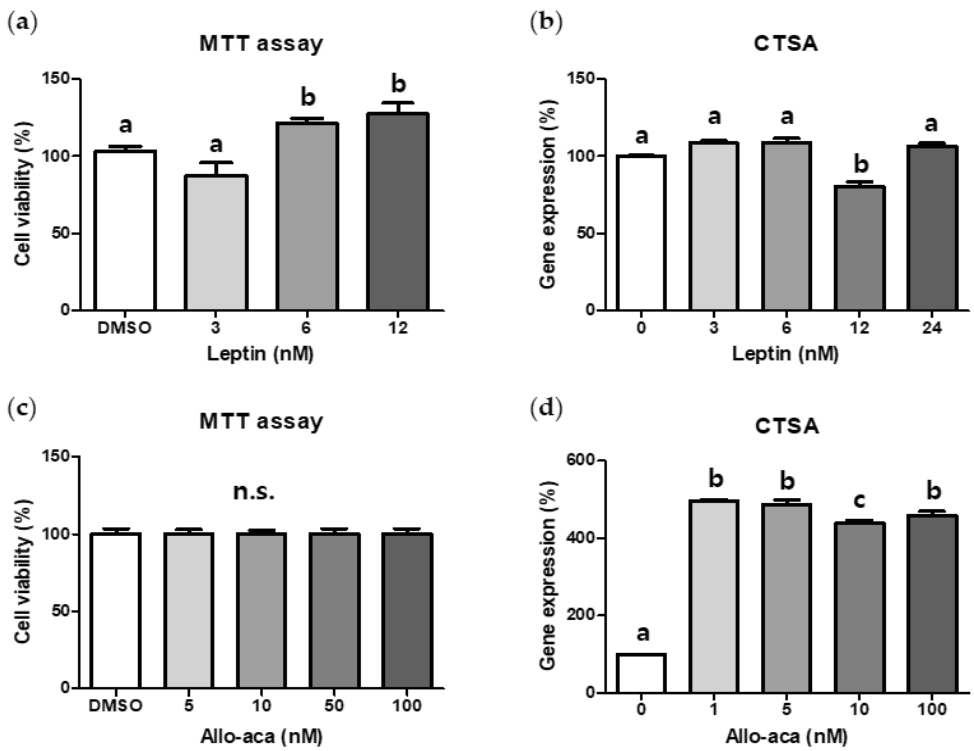


Figure 1. Preliminary studies on determining optimal concentration of leptin and Allo-aca

(a,b) MTT assay shows leptin significantly increases cell number from 6 nM and downregulates CTSA gene at 12 nM dose. (c,d) Although, Allo-aca did not alter cell number among different concentrations nor show any cytotoxic effects, it significantly upregulates CTSA gene from the lowest dose. * n.s.;not significant.

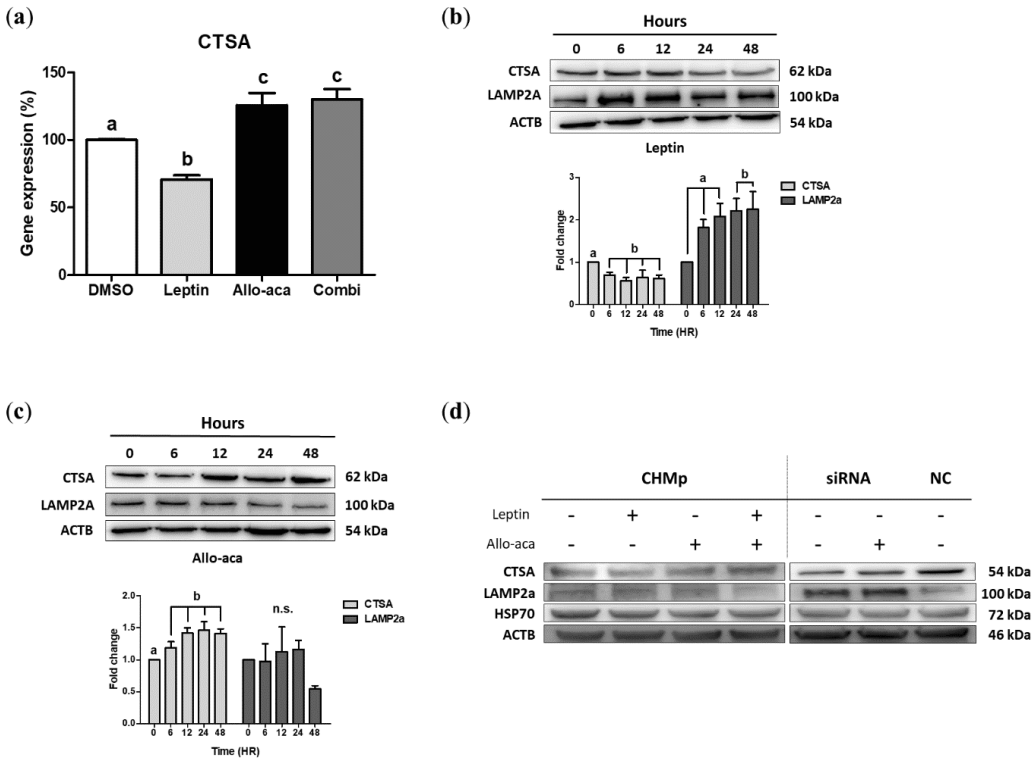


Figure 2. Leptin downregulates CTSA gene in CHMp cells

(a) CTSA gene expression in CHMp cells treated with leptin and Allo-aca was evaluated using real-time PCR. (b,c) Western blot analysis of time-course CTSA/LAMP2a alteration induced by leptin (b) and Allo-aca (c) treatment. (d) Western blot analysis of CMA related genes. Cells were incubated with the leptin and/or Allo-aca for 24 hours and CTSA siRNA was transfected 24 hours before chemical treatment. All graphs are visualized as mean ± SEM with at least 3 replicates. (Combi;Combination of leptin and Allo-aca, NC;Negative control siRNA)

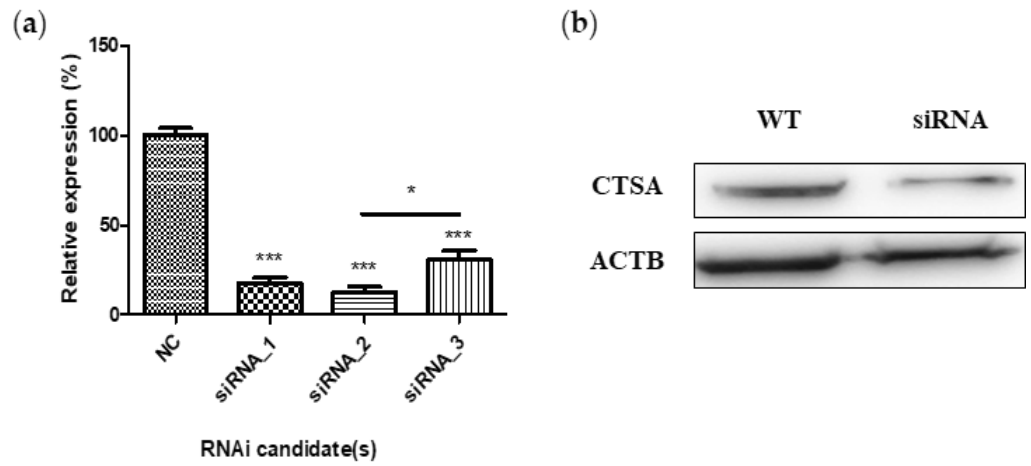


Figure 3. siRNA-induced knockdown of cathepsin A in CHMp cell line

Real-time reverse-transcription quantitative PCR(RT-qPCR) and western blot analysis confirm the knockdown of cathepsin A (CTSA) in CHMp cells. I used Lipofectamine RNAiMAX for transfection and followed reverse transfection protocol provided by the manufacturer. The cells were collected 24 hours after transfection and immediately processed for the analysis. a) siRNA_2 showed the highest efficacy among 3 candidates. b) Western blot analysis confirmed the downregulated expression of CTSA in siRNA_2 treated group. * NC;Negative control

3.2 Leptin promotes cell proliferation in CHMp cells

Cell proliferation assay showed that the proliferation capacity of CHMp cells was significantly increased with 12 nM leptin treatment, and Allo-aca antagonized its effect (Figure 4a), resulting in a significantly lower proliferation index. Moreover, Allo-aca alone did not show a negative effect on cell proliferation, as no significant difference was observed between the control and Allo-aca groups. In agreement with the previous results, I also found that the knockdown of CTSA using siRNA inhibited cell proliferation, and that Allo-aca did not affect cell proliferation in transfected cells (Figure 4b).

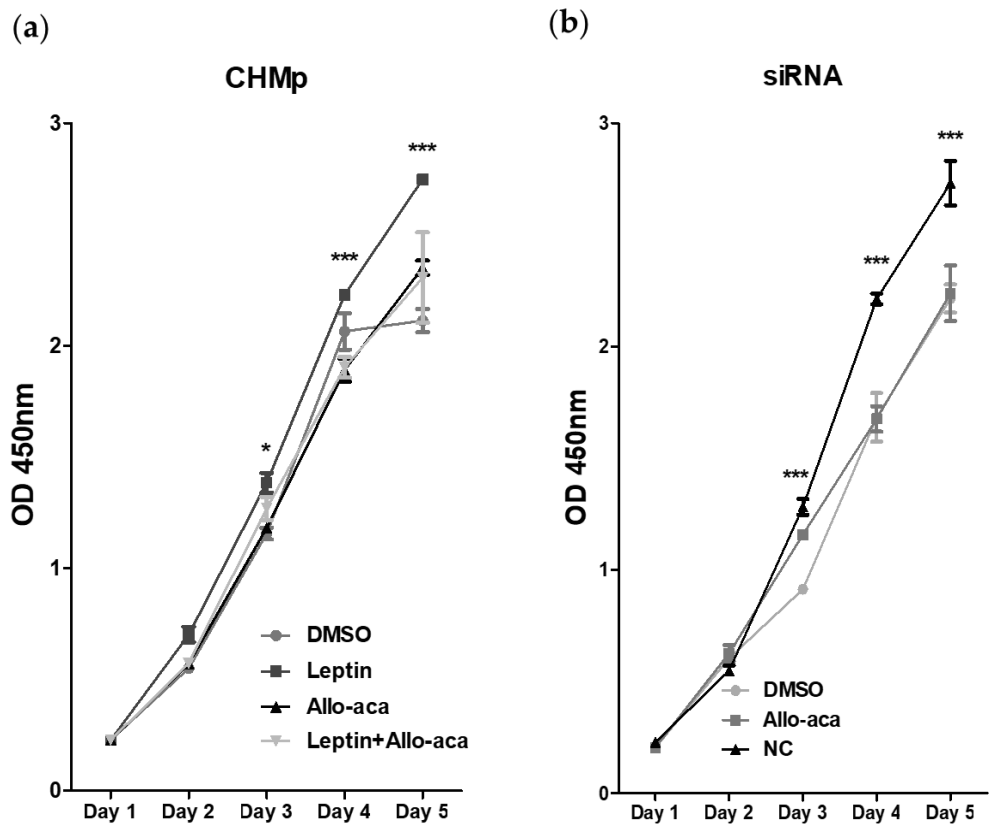


Figure 4. Leptin promotes cell proliferation in CHMp cells

Quantitative measurement was determined by MTT assay. 5,000 cells per well were seeded and cultured for 5 days in 37 ℃, 5% CO2 incubator. (a) Leptin significantly promotes cancer cell proliferation from day 3 whereas Allo-aca inhibits its effect in CHMp cells.;(b) Allo-aca treatment on CTSA-siRNA transfected cells did not affect cell proliferation. However, it seems that knockdown of CTSA decreases cell proliferation comparing with the negative control group. The results were expressed as mean ± SEM. * $p<0.05$, ***<0.001

3.3 Leptin stimulates epithelial-mesenchymal transition (EMT) in CHMp cells

To investigate the role of leptin in canine mammary adenocarcinoma progression, I introduced leptin and Allo-aca into CHMp cells. Matrigel invasion assay (Figure 5a) showed that the invasion capacities of CHMp cells were significantly increased by leptin treatment, while those of Allo-aca-treated cells (including the combination group) remained the same as the control group. In addition, siRNA-transfected cells showed significantly increased invasion capacities, but these were significantly reduced by treatment with Allo-aca.

Real-time PCR of EMT-related genes showed that both E-cadherin and vimentin were significantly altered by leptin treatment. Allo-aca significantly upregulated E-cadherin, a cell adhesion factor, in both CHMp cells and transfected cells. However, leptin and siRNA significantly upregulated vimentin, whereas its effect was inhibited by Allo-aca (Figure 5b).

To validate whether leptin alters EMT genes at the translational level, I also performed Western blot analysis (Figure 5c.). The overall tendencies were matched with mRNA expression, but leptin and knockdown of CTSA significantly upregulated MMP9 (matrix metalloproteinase 9), a biomarker of tumor invasion and metastasis [55]. In addition, Allo-aca downregulated vimentin and MMP9 and upregulated E-cadherin in both cell types. Taken together, these results suggest that leptin and subsequent CTSA downregulation stimulate tumor invasion and metastasis via activation of the EMT process.

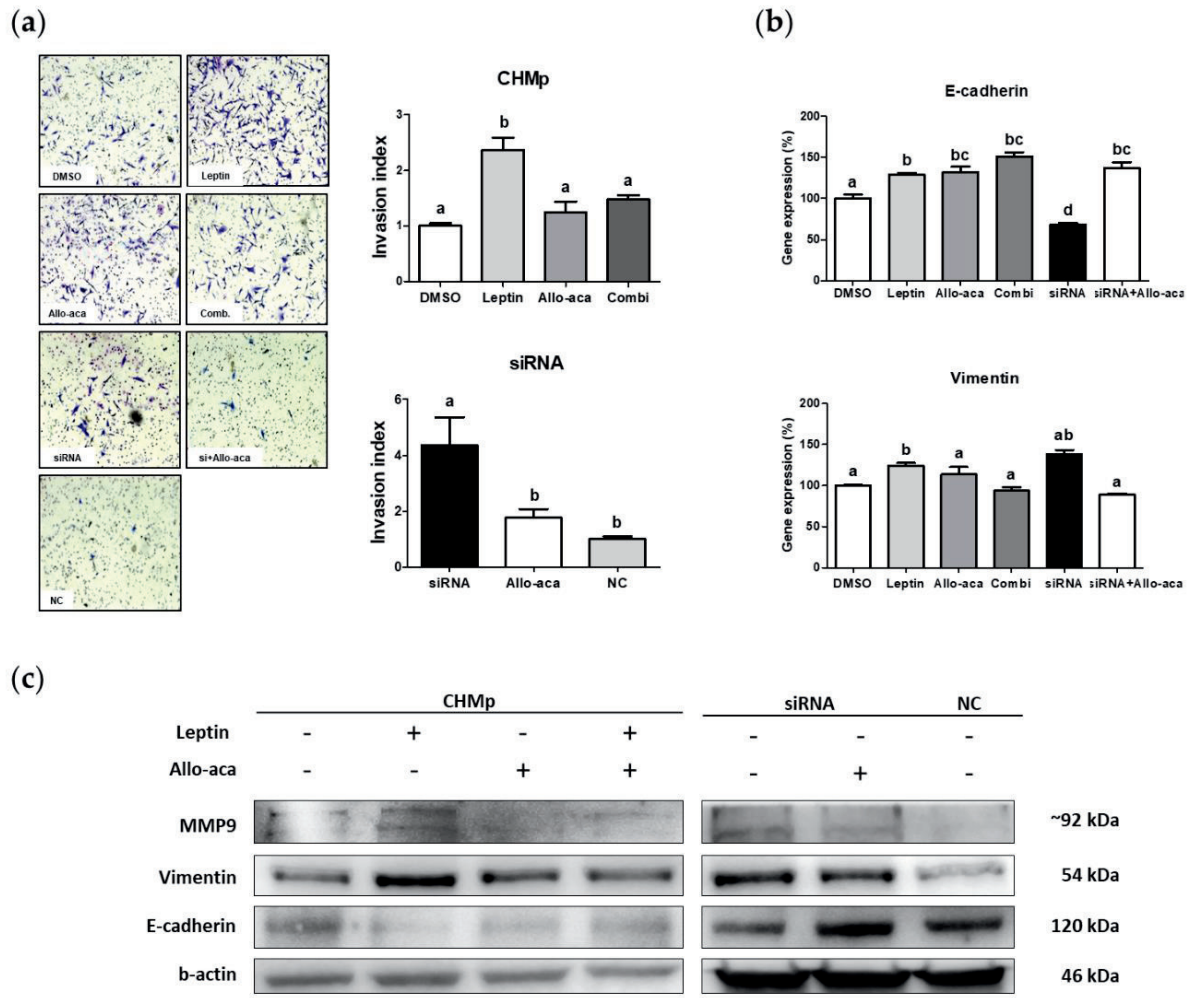

(a)
DMSO
Leptin
Allo-aca
Comb.
siRNA
si+Allo-aca
NC
CHMp
Invasion index
DMSO Leptin Allo-aca Combi
siRNA
Invasion index
siRNA Allo-aca NC
(b)
E-cadherin
Gene expression (%)
DMSO Leptin Allo-aca Combi siRNA siRNA+Allo-aca
Vimentin
Gene expression (%)
DMSO Leptin Allo-aca Combi siRNA siRNA+Allo-aca
(c)
CHMp
siRNA
NC
Leptin
Allo-aca
MMP9
Vimentin
E-cadherin
b-actin
~92 kDa
54 kDa
120 kDa
46 kDa

Figure 5. Leptin stimulates epithelial-mesenchymal transition (EMT) in CHMp cells

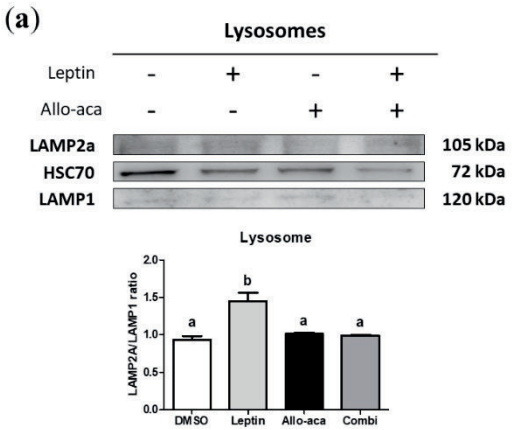
(**a**) Matrigel invasion assay and calculated invasion index of CHMp and siRNA transfected cells. Invaded cells were counted under a light microscopy (40X) using ImageJ software. (**b**) Comparison of EMT related gene (E-cadherin and Vimentin) expression using real-time PCR analysis among experimental groups. (**c**) Western blot analysis of tumor invasion-related genes. All graphs are visualized as mean ± SEM with at least 3 replicates. (Combi;Combination of leptin and Allo-aca, NC;Negative control siRNA)

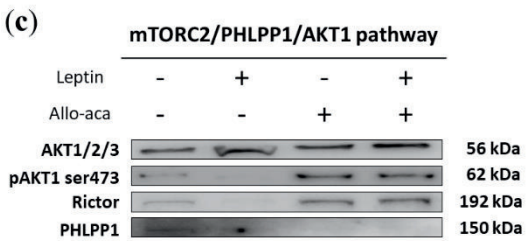
3.4 Leptin delays the degradation of LAMP2a through downregulation of CTSA.

To specifically investigate the alteration of lysosomal proteins, I isolated lysosome fractions from CHMp cells. I found that the LAMP2a/LAMP1 ratio increased with leptin treatment, and no significant differences were observed among the DMSO, Allo-aca, and combination groups (Figure 6a.). In addition, hsc70, LAMP2a, and LAMP1 colocalization tests analyzed with Mander's colocalization coefficient (MCC) demonstrated that leptin and knockdown of CTSA increased the ratio, while Allo-aca showed no agonistic effect and an antagonistic effect on leptin (Figure 6b.). Based on these results, I can assume that leptin and subsequent downregulation of CTSA are possibly correlated with higher CMA activity.

3.5 Leptin may promote lysosomal LAMP2a multimerization through mTORC2/PHLPP1/AKT1 pathway.

To further investigate the role of leptin in the regulation of CMA, I analyzed the lysosomal kinase/phosphatase complex. I found that leptin downregulates rictor, a subunit of the mTORC2 complex, and upregulates PHLPP1, a serine/threonine phosphatase, inhibiting the action of AKT1 via phosphorylation of pAKT1(ser473). Conversely, similar to the previous results, Allo-aca antagonized these effects regardless of the presence of leptin (Figure 6c). Thus, I can suggest that leptin promotes the multimerization of LAMP2a in lysosomal membranes through the activation of the mTORC2/PHLPP1/AKT1 pathway and that Allo-aca can prevent the formation of LAMP2a multimers.


(a)
Lysosomes
Leptin - + - +
Allo-aca - - + +
LAMP2a 105 kDa
HSC70 72 kDa
LAMP1 120 kDa
Lysosome
LAMP2A/LAMP1 ratio
2.0
1.5
1.0
0.5
0.0
a
b
a
a
DMSO
Leptin
Allo-aca
Combi


(c)
mTORC2/PHLPP1/AKT1 pathway
Leptin - + - +
Allo-aca - - + +
AKT1/2/3 56 kDa
pAKT1 ser473 62 kDa
Rictor 192 kDa
PHLPP1 150 kDa

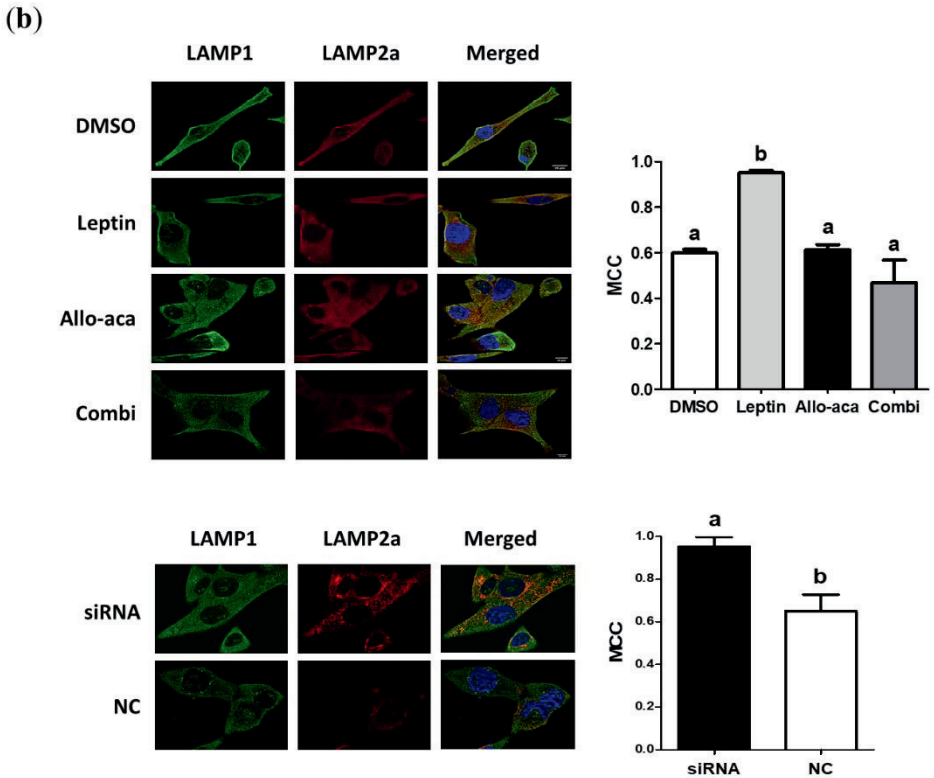

(b)
LAMP1
LAMP2a
Merged
DMSO
Leptin
Allo-aca
Combi
MCC
1.0
0.8
0.6
0.4
0.2
0.0
a
b
a
a
DMSO
Leptin
Allo-aca
Combi
LAMP1
LAMP2a
Merged
siRNA
NC
MCC
a
b
siRNA
NC

Figure 6. Leptin induces higher LAMP2a/LAMP1 ratio in lysosomal membranes

(a) Western blot analysis of lysosomes isolated from CHMp cells targeting CMA associated lysosomal proteins. After the incubation with the chemicals, the cells were cultivated using non-enzymatic cell detachment buffer and directly subjected to lysosome isolation. The isolated lysosomes were dissolved in RIPA buffers and quantified using BCA assay. 5ug of dissolved lysosomes were loaded to 12% Mini-PROTEAN® TGX™ Precast Gels. (b) Western blot analysis of lysosomal mTORC2/PHLPP1/AKT1 pathway in lysosomes isolated from CHMp cells. The isolated lysosomes were lysed in RIPA buffer supplemented with protein inhibitor cocktail and phosphatase inhibitor. Then the samples were subjected to SDS-PAGE. (c) Immunocytochemistry visualized with confocal microscopy. CHMp cells and CTSA-siRNA transfected cells were treated with 12 nM leptin and/or 100 nM Allo-aca for 6 hours in a 37 ℃, 5% CO2 incubator. Rabbit monoclonal anti-LAMP1 antibody was probed with Alexa Fluor® 488 secondary antibody and rabbit anti-LAMP2a antibody was conjugated with Alexa Fluor® 647 conjugation kit (Abcam) 1 hour before use. The colocalization ratio was measured by Fiji software developed by Schindelin and their colleagues [30] and analyzed based on Mander's colocalization coefficient (MCC). All graphs are visualized as mean ± SEM with at least 3 replicates. (Combi;Combination of leptin and Allo-aca, NC;Negative control siRNA)

IV. Discussion

In this study, I investigated the concurrent effects of leptin on the lysosomal enzyme CTSA and the following effects on tumor cell proliferation and invasion capacity of CHMp, a canine mammary adenocarcinoma cell line. In our preliminary study, I found that CTSA gene expression was significantly downregulated and enhanced cell viability with 12 nM leptin treatment, which was reversed with the OBR antagonist, Allo-aca. Interestingly, Allo-aca itself could also inhibit EMT and metastasis in both cell types. Moreover, the increased expression of MMP9 indicates a higher chance of metastasis to other organs [56]. To determine the effect of leptin on CTSA, I applied CTSA-siRNA to knockdown its expression in CHMp cells. In addition, I also found that leptin promotes tumor cell growth, which is significant in other control and experimental groups in the time-course cell proliferation assay. Interestingly, the knockdown of CTSA did not promote tumor cell proliferation but rather inhibited it. As patients with galactosialidosis typically show lower body weight, I suspect that higher LAMP2a activity due to delayed degradation is responsible for a slower proliferation rate [49]. Moreover, Allo-aca inhibited leptin-induced cell proliferation in the co-treatment group but did not inhibit the growth itself, and no alteration was observed in transfected cells. Therefore, I focused on utilizing leptin and Allo-aca to investigate the role of CTSA in CHMp cells.

I subsequently investigated leptin as a therapeutic target for preventing mammary cancer metastasis in dogs and identified it as an endogenous LAMP2a activator by modulating its stability and promoting multimerization. An *in vitro* invasion assay showed that leptin and knockdown of CTSA promoted CHMp cell invasion by upregulating vimentin and

metallo-proteinase 9 (MMP9), and downregulating E-cadherin. In addition, supplementation of Allo-aca antagonized the effect of leptin on EMT even without leptin co-treatment. E-cadherin, a transmembrane epithelial cell marker, is considered a prognostic factor in both human and dog breast cancer [57, 58]. Its decreased expression represents upregulated cancer invasion ability, poor prognosis, and causes EMT progression together with increased expression of vimentin, a mesenchymal cell marker. The radical knockdown of CTSA also induced EMT, similar to leptin treatment. Based on the ability to induce EMT, leptin and its ability to downregulate CTSA were found to promote the migration and invasiveness of CHMp cells and upregulate metastasis-related genes, which show poor prognosis for breast cancer patients. In addition, I also found that Allo-aca antagonized leptin in CHMp cells but also reversed the consequences of the knockdown of CTSA and further showed anti-EMT effects. Therefore, these results indicate that leptin may also induce metastasis in canine mammary adenocarcinoma cells and that Allo-aca seems to have anti-metastatic properties retrieving CTSA expression, which prolongs the half-life of LAMP2a.

It has been suggested that the knockdown of LAMP2a, which induces lower CMA, reduces the metastatic capacity of lung cancer cells [23]. In other words, the level of CMA in cancer cells is associated with cancer metastasis. In agreement with the previous study, the upregulation of LAMP2a through knockdown or leptin treatment in CHMp cells resulted in enhanced invasion and metastasis. These alterations were prevented with Allo-aca treatment and reduced LAMP2a ratio normalized by LAMP1 (LAMP2a/LAMP1 ratio) significantly. That is, the prolonged LAMP2a half-life results in a higher level of LAMP2a on the lysosomal membrane. Moreover, leptin stimulated PHLPP1 thus showed inhibitory

effects on AKT. PHLPP1 is known to inhibit CMA activity through AKT dephosphorylation. In addition, phosphorylated AKT1 ser473 [pAKT1(ser473)] is lower in CMA active lysosomes in starved animals [26]. Together with these findings, I conclude that leptin also promotes CMA activity through LAMP2a multimerization. Moreover, Allo-aca can offset its effects in CHMp cells by blocking OBR.

Higher levels of LAMP2a in cancers play important roles in cell survival and their microenvironment. Therefore, previous studies have focused on LAMP2a as a therapeutic target. However, specific manipulation of one specific gene or protein using RNAi (RNA interference) can result in unpredictable side effects such as RNAi off-target effects and carrier-mediated toxicity [59, 60]. Therefore, through Allo-aca treatment, which can offset the CMA-stimulating effect of leptin demonstrated in this study, I suggest the possibility of inhibiting metastasis with relatively few side effects in the treatment of cIMCs. However, the fact that Allo-aca itself can enter the blood-brain barrier must be improved because it can stimulate appetite and weight gain. In addition, the lack of direct confirmation of CMA activity as a substrate and the inability to quantitatively analyze LAMP2a multimerization using Blue-Native Page due to the absence of suitable antibodies and lack of additional established cell lines are limitations in this study.

V. Conclusions

From our findings, I suggest that leptin, which increases with age and obesity in dogs, can stimulate mammary gland cancer metastasis by elongating the half-life and multimerization of LAMP2a. I also demonstrated that Allo-aca can reverse the promotion of EMT and invasiveness of CHMp cells treated with leptin through selective pairing to OBR without any agonistic effects. These findings provide evidence that leptin receptor antagonists are a therapeutic and prophylactic option for cIMCs without unwanted side effects.

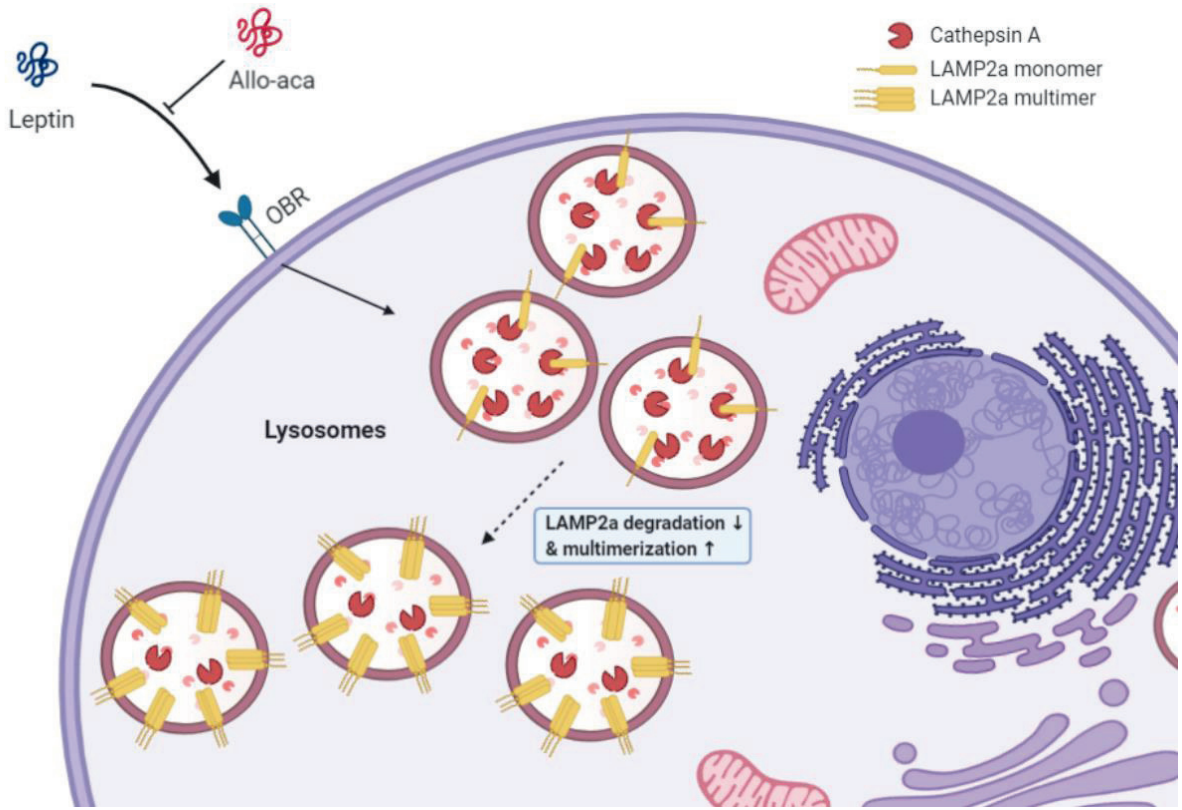


Figure 7. Schematic images describing summarized results in this study.

Leptin downregulates CTSA and following delayed LAMP2a degradation results in CMA activation and stimulates LAMP2a multimerization through mTORC2/PHLPP1/AKT1 pathway.

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국문 초록

렙틴의 라이소좀 내 Cathepsin A 효소 발현 저하를 통한 개의 악성 염증성 유선종양 전이 활성화 연구

김 진 욱

(지도교수 : 장 구)

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현재까지 연구된 바로는 chaperone mediated autophagy (CMA)가 암세포의 생존 및 전이에 중요한 역할을 하는 것으로 밝혀졌다. CMA는 아미노산 서열 내 KFERQ 모티프를 보유한 펩타이드를 표적으로 선택적 자가포식을 수행하는 기전으로, 인간의 유방암에서도 높은 수준으로 발현되는 것으로 알려져 있다. 세포 내 라이소좀의 효소 중 하나인 카텝신 A는 CMA의 속도 조절 인자인 LAMP2a의 반감기를 조절하는 것으로 알려져 있는데, 본 연구의 사전 실험에서 렙틴을 개 유선종양 세포주 (CHMp)에 처리하였을 때 유의적으로 발현이 감소하는 것을 확인하였다. 그러나 지금까지 개의 종양 세포에서 CMA와 카텝신 A 간의 상관 관계는 연구되지 않았다. 따라서 본 연구에서는 렙틴과 그 길항제인 Allo-aca를 적용하여 CMA의 활성 변화를 통해 종양 세포의 침윤 및 전이에 유의적인 변화가 나타나는지 평가하였다.

CHMp 세포주는 RPMI-1640에 10 % 소태아혈청 및 0.5% 겐타마이신을 항생제로서 첨가하여 배양하였다. 본 세포주를 이용하여 12 nM 렙틴, 100 nM Allo-aca를 처리하였으며, 사전실험에서 확인한 카텝신 A를 siRNA transfection을 이용해 Knockdown 하여 카텝신 A 유전자의 종양 전이 향상 평가도 수행하였다. 실험군의 배양은 24시간으로 설정하였고, 이후 시료들은 Real-time PCR, 웨스턴 블랏, 면역세포화학염색, 매트리겔 침윤 시험 그리고 라이소좀 분리에 적용하였다. 세포 증식 실험에서 렙틴은 유의적으로 증식이 촉진되는 효과를 보였으며, Allo-aca의 처리로 증식은 차단되었다. 반면, 카텝신 A

Knockdown 세포는 대조군과 비교하여 증식이 유의적으로 억제되었는데, 이는 특정 카텝신 A가 저하될 경우 갈락토시알리오도시스증 (라이소좀축적질환)을 유발함에 기인한 것으로 판단된다. 이에 더하여, Allo-aca의 단독 처리는 CHMp 및 Knockdown 군 모두에서 세포 증식에 영향을 미치지 않은 것을 확인하였다. 다음으로, 렙틴 처리군과 길항제 처리군을 분자수준에서 비교하였을 때, 렙틴 처리군에서 카텝신 A의 mRNA 및 단백질 수준 모두에서 발현 저하가 된 것을 확인하였다. 반면, Allo-aca의 처리군들(단독 및 공처리)은 렙틴의 공처리군에서도 대조군과 비교하였을 때 유의적인 차이를 보이지 않았다.

다음으로, 렙틴 처리군은 침윤 지표(Invasion index) 전이 평가를 위한 실험에서 렙틴은 대조군과 다른 실험군과 비교하였을 때 유의적으로 침윤 지표를 증가시켰다. 이에 더해, 카텝신 A KD 세포주에서 Allo-aca를 처리한 경우 침윤 지표가 감소한 것을 관찰하였다. 웨스턴 블랏 실험을 통한 단백체 분석에서도 종양 전이 관여 인자 중 MMP9, Vimentin의 발현 증가와 E-cadherin의 감소를 확인하였다. 특이하게도, 카텝신 A Knockdown 세포가 CHMp 보다 유의적으로 높은 MMP9 및 Vimentin의 발현 증가를 보였는데 Allo-aca를 처리하였을 때 유의적으로 발현이 감소함을 확인하였다. 즉, 렙틴의 카텝신 A 발현 저하 효과는 Allo-aca의 CTSA 발현 촉진 효과로 상쇄될 수 있음을 시사한다. 이를 분자생물학적으로 검증하기 위한 웨스턴 블랏 실험에서 렙틴은 전사체 (mRNA)와 번역체 (Protein) 수준 모두에서 통계적으로 유의미한 발현 저하를 확인하

였고, 이와 동시에 CMA의 속도 조절 인자인 LAMP2a는 카텝신 A 발현 감소에 이차적으로 반감기가 지연됨을 확인하였다. 뿐만 아니라, 렙틴은 라이소좀막의 mTORC2 / PHLPP1 / AKT 경로를 통해 PHLPP1의 발현 상승 및 pAKT1(ser473)의 생성 억제를 통해 LAMP2a의 중합화를 유도함을 확인하였다.

결론적으로, 본 연구에서는 비만 환자에서 상대적으로 혈중에 고농도로 분포하는 렙틴과 개의 유선종양 전이능 활성화 기전 연구를 위하여 분자생물학적 기전을 통해 검증하는 실험을 진행하였으며, 그 결과 렙틴이 선택적 자가포식의 중요한 조절인자인 카텝신 A 발현 조절을 통한 전이능 활성화를 확인하였다. 또한 렙틴 수용체를 표적으로 하는 Allo-aca의 전이능 완화 효과를 확인하여 앞으로 악성 유선종양에 이환된 반려견 환자들의 종양 연구 및 치료에 활용할 수 있다는 것이 의의가 있다.

주요어: 렙틴, 카텝신 A, 샘암종, 전이, 비만

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