수의학석사 학위논문

개 유선종양에서의 CXCL10/CXCR3의 종양 촉진 효과

The Protumor Effect of CXCL10/CXCR3 Axis in Canine Mammary Gland Tumor

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수의학과 임상수의학 전공

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ABSTRACT

The Protumor Effect of CXCL10/CXCR3 Axis in Canine Mammary Gland Tumor

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Chemokines and chemokine receptors play critical roles in cancer progression. CXCR3 is a chemokine receptor expressed in T cells, which mediates the anti-tumor effect. In contrast, CXCR3 in malignant cells promotes tumor proliferation and metastasis in human breast cancer. Little is known about the function of CXCR3 in canine mammary gland tumor (cMGT) cells. This study investigated the function of CXCR3 and its ligand, CXCL10, in cMGT cells. Two cMGT

cell lines, CIPp (primary) and CIPm (metastatic) were used for the study. To determine the cellular effect of interaction between CXCL10 and CXCR3, we assessed cell proliferation and migration potential. Moreover, we investigated the underlying molecular mechanism of the CXCL10/CXCR3 axis. Compared to CIPp cells, the CXCR3 expression level was significantly higher in CIPm cells. Similar to other G protein–coupled receptor mechanisms, CXCL10 induced CXCR3 internalization in both cell lines. Treatment with CXCL10 resulted in enhanced proliferation and migration and increased phosphorylated AKT1 and ERK levels. This study revealed that ligand binding caused CXCR3 internalization and activation of the CXCL10/CXCR3 axis, promoting proliferation and migration in cMGT cells. The data suggest that CXCR3 could be a potential therapeutic target to regulate cMGT progression.

Keywords: CXCR3, CXCL10, canine mammary gland tumor, cancer

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Table of Contents

ABSTRACT i

CONTENTS iii

ABBREVIATION iv

LITERATURE REVIEW 1

Chemokine and chemokine receptor 1

CXCR3 & its ligands: CXCL9, CXCL10, CXCL11 2

CXCL9, −10, −11/CXCR3 in cancer 5

CXCR3 in veterinary medicine 6

Introduction 8

Materials and methods 11

Results 17

Discussion 29

Conclusion 34

Reference 35

국문초록 43

ABBREVIATIONS

cMGT: canine mammary gland tumor

CXCR3: C−X−C motif chemokine receptor 3

CXCL9: C−X−C motif chemokine ligand 9

CXCL10: C−X−C motif chemokine ligand 10

CXCL11: C−X−C motif chemokine ligand 11

GPCR: G protein−coupled receptor

RT−PCR: Reverse transcription polymerase chain reaction

qPCR: Quantitative polymerase chain reaction

GAPDH: Glyceraldehyde 3−phosphate dehydrogenase

Na^+−K^+ ATPase: Sodium−potassium adenosine triphosphatase

PBS: phosphate−buffered saline

IFN−γ : Interferon− γ

TME: Tumor microenvironment

LITERATURE REVIEW

Chemokine and chemokine receptor

Chemokines are a broad family of small chemotactic cytokines (8 to 12 kDa) with a conserved tetra cysteine motif (Miller and Mayo, 2017). They help cell-cell communication via autocrine and paracrine pathways, specifically facilitating cell trafficking. Chemokines are commonly categorized into four sub-families based on the placement of the first two of four highly conserved cysteine residues: C, CC, CXC, and CXC3C. Among them, The CXC family is further subdivided into two groups based on the presence of the glutamic acid-leucine-arginine (ELR) motif, which is critical for receptor binding and selectivity (Strang et al., 2020). Generally, CXC chemokines with the ELR motif stimulate angiogenesis, while those without the motif exhibit angiostatic characteristics (Ma et al., 2015).

Chemokines can interact with chemokine receptors, G protein-coupled receptor (GPCR) superfamily seven-transmembrane proteins (Miller and Mayo, 2017). Although specific chemokine-chemokine receptor interactions are selective, many chemokine receptors bind multiple chemokines, resulting in chemotactic redundancy and plasticity (Allen et al., 2007). The activation of

chemokine receptors by their ligands causes conformational changes, which activate an intracellular signaling cascade. When chemokine receptors are activated, signaling is strictly regulated by desensitization, internalization, and lysosomal sorting (Patwardhan et al., 2021).

CXCR3 & its ligands: CXCL9, CXCL10, CXCL11

CXCR3 is a chemokine receptor which expressed on various cell types, including monocytes, T cells, dendritic cells, NK cells, and cancer cells. CXCR3 binds with the ELR−negative CXC chemokine subfamily, including CXCL9, CXCL10, and CXCL11 (Kuo et al., 2018). There are three CXCR3 isoforms with distinct characteristics; CXCR3A, CXC3B, and CXCR3−alt. CXCR3A plays classic CXCR3 functions such as chemotaxis and cell proliferation in interferon− γ (IFN− γ) induced immune responses. Conversely, CXCR3B, spliced at a 52 amino acid extension of the N terminus, promotes cell apoptosis and inhibits cell migration. CXCR3−alt, a 101−aminoacid−truncated version via exon skipping, mediates the functions of CXCL11 (Kuo et al., 2018; Tokunaga et al., 2018). However, there are very few studies on CXCR3−alt compared to other CXCR3 isoforms. CXCR3A and CXCR3B mediate distinct signaling cascades that depend on specific G protein coupling and different binding

affinity of the ligands and cell types. CXCR3A couples with $G\alpha_{i/q}$ and results in downstream activation of Ras/Raf/ERK and PI3K/AKT signaling leading to cell proliferation, survival, and migration/invasion. CXCR3B signals through $G\alpha_{s}$ result in adenyl cyclase and PKA and p38 and p21 activation, leading to the sensitization of cells to stress and apoptotic signals (Kuo et al., 2018).

The key chemokine ligands of CXCR3 are CXCL9, CXCL10, and CXCL11, which interact with the extracellular domains of CXCR3 (Ma et al., 2015). These ligands have a different affinity with the receptor. Human CXCL11 has the highest affinity for CXCR3, followed by CXCL10 and CXCL9 (Kuo et al., 2018). In homeostasis, the ligands are typically expressed at low levels, but cytokine stimulation increases their expression (Tokunaga et al., 2018). CXCL10 and CXCL11 can be induced by both IFN-γ and type I interferons, while IFN-γ primarily induces CXCL9 (Kuo et al., 2018). Many cell types, including endothelial cells, fibroblasts, monocytes, and cancer cells, can release CXCL9/10/11 in response to IFN-γ (Tokunaga et al., 2018).

The main functions of the CXCL9, CXCL10, and CXCL11/ CXCR3 axis include immune cell migration, differentiation, and activation. CXCR3 is expressed on all three variants on T cells for immune cell migration, where CXCL9, -10, and -11 concurrently stimulate the

144 loss of surface CXCR3 expression and elicit directed migratory
145 responses to the focal areas (Korniejewska et al., 2011). Chheda et
146 al. demonstrated a critical role of CXCR3 for cytotoxic T lymphocyte
147 (CTL) migration in a syngeneic murine model of B16 melanoma with
148 CXCR3 knock-out mice, which indicated significant tumor growth and
149 shortened survival time (Chheda et al., 2016).

150 For immune differentiation, some research showed that CXCL9, -
151 10, and -11 all lead to Th1 polarization via CXCR3 (Yang et al., 2011;
152 Zohar et al., 2014). CXCL10 enhanced T-bet and RORγ
153 transcription, leading to the polarization of Fox-p3$^-$ type 1
154 regulatory (Tr1) cells or T helper 17 (Th17) cells from naive T cells
155 via STAT1, STAT4 and STAT5 phosphorylation (Zohar et al., 2014).
156 Unlike CXCL10, CXCL11 reduced transcription of RORγ, causing
157 Tr1 or Tr2 cells to polarize from naive T cells via p70 kinase/mTOR
158 pathways (Apetoh et al., 2010). Also, the CXCL9, -10, -11/CXCR3
159 axis regulates tumor-associated macrophage (TAM) polarization,
160 modulating the tumor microenvironment(TME). In a mouse breast
161 cancer model, CXCR3-deficient mice had increased IL-4 production
162 and M2 polarization and reduced innate and immune cell-mediated
163 anti-tumor responses (Oghumu et al., 2014).

164 For immune cell activation, the CXCL9, -10, -11/CXCR3 axis
165 stimulated polarization and activation of Th1 cells, producing

cytokines including IFN-γ, -α, and IL-2. These cytokines stimulate CTLs, NK cells, NKT cells, and macrophages (Mosser and Edwards, 2008; Schoenborn and Wilson, 2007). In addition, IFN-γ dependent immune activation loop induces the secretion of CXCL9, -10, -11.

CXCL9, -10, -11/CXCR3 in cancer

Considering that CXCR3A plays a vital role in proliferation and metastasis, treatments targeting CXCR3A may be helpful in metastatic cancer (Li et al., 2019; C. Yang et al., 2016). Many studies on the anti-tumor effects of CXCL9, -10, -11/CXCR3 axis have been investigated because CXCR3 is primarily expressed on immune cells. However, In CXCR3 expressed cancer cells, autocrine CXCL9, -10, -11/CXCR3 signaling promotes cell proliferation, angiogenesis, and metastasis. Previous research has revealed that CXCR3-positive cancer cells tend to metastasize in vitro and in vivo due to autocrine signaling from the pre-metastatic niche (Cambien et al., 2009; Nagpal et al., 2006; Zhu et al., 2015).

Moreover, CXCR3 expression in clinical cancer samples is correlated to metastatic potential and poor prognosis (Kawada et al., 2007; Monteagudo et al., 2007). Therefore, this axis could be used

to predict treatment efficacy or as a prognostic marker. Although CXCL10/CXCR3 co-expression has been shown to have a vital role in boosting metastatic potential by Wightman et al. (2015), the correlation between the expression levels of the three ligands and metastasis or prognosis is still controversial. Some groups agree that the expression of CXCL9 (Mir et al., 2015) and CXCL10 (Liu et al., 2016) is associated with a poor prognosis or a response to therapy, whereas other groups claim that CXCL9 (Wu et al., 2016) and CXCL10 (Sato et al., 2016) are related to the opposite outcomes. These discrepancies in studies could be related to the intricate relationship that each ligand has with different cancer types.

CXCR3 in veterinary medicine

In veterinary medicine, CXCR3 expression was utilized to evaluate the polarization and differentiation state of antigen-specific T lymphocytes. To investigate the immune response to the inflammatory disease, several canine studies assessed the expression of CXCR3 on T cells (Guedes et al., 2010; Park et al., 2013; Vandamme et al., 2022). However, research on canine tumors related to CXCR3 is still limited, and only a few studies have been performed on canine mammary gland tumors (cMGTs). In

cMGTs, an increase in tumor development and spread has been linked to CXCR3 expression (Ariyarathna et al., 2020; Bujak et al., 2020). Nevertheless, only gene expression in tumor tissue was confirmed, and no research has been conducted on whether CXCR3 is actually expressed in mammary gland tumor cells or the role of CXCR3. So, further research in this field is needed.

Introduction

The chemokine receptor is seven transmembrane proteins belonging to the G-protein coupled protein group (Kuo et al., 2018). The chemokine is composed of low molecular weight cytokines with an essential role in inflammation and immunity. (Mollica Poeta et al., 2019). Chemokines are classified into four groups by the pattern of cysteine residues: CXC, CC, C, and CX3C. Although the primary function of chemokine receptor signaling is leukocyte trafficking, they participate in various pathophysiological conditions, including human cancers (Mollica Poeta et al., 2019). The role of chemokines in cancer extends beyond recruiting immune cells into tumor sites including regulation of tumor angiogenesis and tumor cell dissemination. (Mantovani et al., 2010)

Among the several chemokine receptors associated with cancer, recent studies have focused on the CXCR3 and its ligands (Karin, 2020; Kuo et al., 2018; Tokunaga et al., 2018). CXCR3 is a chemokine receptor that interacts with CXC group chemokines: CXCL9, CXCL10, and CXCL11. CXCR3 expressed on immune cells promotes chemotaxis, differentiation, and activation by interacting with its ligands (Strang et al., 2020). In the tumor environment, this paracrine axis stimulates the recruitment of tumor-specific immune cells into

tumor sites, resulting in tumor suppression. Interestingly, in specific situations where the tumor cells express the CXCR3 receptor, the autocrine axis of CXCR3 and its ligands impact growth, progression, and metastasis. Through pathways, including activation of MAPK and PI3K/AKT signaling, CXCR3 activation promotes the invasion and migration of cancer cells (Cannon et al., 2021). So, CXCR3-targeted therapy has been proposed as a treatment option and prognostic marker for various cancers, especially breast cancer (Tokunaga et al., 2018; Zhang et al., 2018).

Mammary gland tumors are one of the most frequent cancers in dogs, particularly in intact females, and are still one of the leading causes of mortality in dogs (Benavente et al., 2016; Salas et al., 2015; Sorenmo, 2003). Human breast cancers and canine mammary gland tumors (cMGTs) share numerous characteristics, including a hormonal influence on development, histopathologic characteristics, expression patterns of several molecular markers, and an unpredictable clinical outcome (Abdelmegeed and Mohammed, 2018; Gray et al., 2020). Only a few studies have evaluated the expression of CXCR3 in canine tumor tissues. In these studies, CXCR3 expression is highly associated with the malignancy of cMGT (Ariyarathna et al., 2020; Bujak et al., 2020). However, there was simply the identification of gene expression in cMGT tissue and no

research on whether CXCR3 was expressed in cMGT cells or the role of CXCR3.

The objectives of this study were to assess CXCR3 expression in cMGT cell lines; to examine the effects of CXCR3 expressed in cMGT cells.

Materials and methods

Cell lines and culture

The present study used two cell lines (CIPp and CIPm), which originate from one dog. The CIPp cell line was collected from a primary lesion in the mammary gland, and the CIPm cell line was collected from an enlarged regional lymph node (Uyama et al., 2006). CIPp and CIPm were kindly provided by the Department of Veterinary Pharmacology, Seoul National University (SNU). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Cytiva, Marlborough, MA, USA) at 37℃ with 5% CO_2. The culture medium was supplemented with 10% fetal bovine serum (GE Healthcare), 10 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA), 2.0 g/L sodium bicarbonate (Sigma-Aldrich), 1 mM sodium pyruvate (Thermo Fisher, San Diego, CA, USA), and 100U/100 μg/mL penicillin-streptomycin (Thermo Fisher)

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was isolated from CIPp and CIPm cells using Trizol (Invitrogen; Thermo Fisher), and quantitation was performed using a

BioTek Epoch Microplate Spectrophotometer (Izasa, Barcelona, Spain). Total RNA was subjected to cDNA synthesis using a QuntiTect Reverse Transcription Kit (Enzynomics, Seoul, South Korea). An SYBR Green RT-PCR Kit (Enzynomics) was used for gene expression analysis. The primers for the target genes are listed in Table 1. The relative changes in gene expression levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western blot assay

Western blot assay was perfomed to determine the changes in related proteins. The cells were lysed using EzRIPA buffer (ATTO, Tokyo, Japan). The lysate protein concentration was quantified by a Bradford assay (BioRad, Hercules, CA, USA) and measured using a BioTek Epoch Microplate Reader. Thirty micrograms of protein were subjected to 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, GE Healthcare, Barcelona, Spain) using the electrophoretic method. The membrane was blocked by a phosphate buffered saline-tween (PBS-T) solution which contained 5% skim milk for 60 minutes at room temperature. Primary antibodies, including CXCR3 (Bioss Antibodies, Woburn, MA, USA), phospho-

AKT1 (Thermo-Fisher), AKT (Cell Signaling Technology, Danvers, MA, USA), p-ERK (Cell Signaling Technology), ERK (Cell Signaling Technology), Sodium-potassium adenosine triphosphatase ($Na^+ - K^+$ ATPase) (Abcam, Cambridge, MA, USA), and β-actin (Cell Signaling Technology), were diluted 1:1,000 in immunoreaction enhancer solution (TOYOBO, Japan) and incubated overnight at 4℃. The secondary HRP-conjugated anti-rabbit (Santa Cruz Biotechnology, Dallas, TX, USA) and anti-mouse (Santa Cruz Biotechnology) antibodies were used at a dilution of 1:4,000 in a blocking solution (PBS-T with 4% BSA) for 2 hrs. Protein expression was detected by a chemiluminescence imaging system (ATTO) after spreading the Luminata Forte Western HRP Substrate (Merck Millipore, Burlington, MA, USA).

Flow cytometric analysis

To evaluate the expression of CXCR3 on the cMGT cell membrane, we cultured CIPp and CIPm cells within the 6-well plates and exposed them to 10 ng/mL of CXCL10 recombinant protein (AssayGenie, Dublin, Ireland) for 0, 5, 15, 30, 45 and 60 min. After cell digestion and collection, cells were incubated for 1hr at 4℃ with CXCR3 (Invitrogen) in a 100:1 ratio. After washing with PBS, we immediately analyzed using FACSVerse (Becton Dickinson

Biosciences).

Membrane fractionation assay

Subcellular fractionation of the cytosol and membrane was achieved using a Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo-Fisher) according to the manufacturer's recommendations. Na^+-K^+ ATPase (Abcam) was used as endogenous control marker for the membrane.

Cell proliferation assay

Cell proliferation was determined using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) according to the manufacturer's instructions. Cells were treated with CXCL10 (AssayGenie) (10 ng/mL) and AMG487 (MedChemExpress, USA) (1 μM) for 24 hrs. The cells were seeded at 5 x 10^3 cells per well in 96-well plates. Cell viability was quantified by measuring photometric absorbance at 450nm using Epoch Microplate Spectrophotometer (Bio Tek Instruments) and expressed as a percentage relative to the viability of untreated control cells.

Scratch wound-healing assay

To investigate mobility, the CIPp and CIPm cells (1×10^5 per well) were cultured overnight to reach at least 80% confluence and scratched with a 200 uL pipette tip to create wound areas. The cells were then treated with or without CXCL10 (10 ng/mL) or AMG487 (1 μM) for incubation time (0, 2, 4, 6 hrs). The wound gap was observed, and cells were photographed using phase-contrast microscopy. The images were then analyzed using Image J software 1.53 s version (National Institutes of Health, Bethesda, Md, USA) to measure the scratch area.

Statistical analysis

All experiments were repeated at least 3 times, and the data were presented as the means ± standard error of the mean (SEM). GraphPad Prism 9 software (GraphPad Software) was used to perform statistical analysis, including an unpaired Student's t-test and one-way analysis of variance (ANOVA), and to generate all graphs. Statistical significance was determined at $P < 0.05$, $P < 0.01$, or $P < 0.001$.

Table 1. Primer sequences of the genes used for qRT-PCR

Gene	Direction	Primer sequences (5' → 3')
CXCR3	Forward	TTCTTTGCCATCCCAGATTTC
	Reverse	ATGCATGGCATTTAGGCG
GAPDH	Forward	GGAGAAAGCTGCCAAATATG
	Reverse	ACCAGGAAATGAGCTTGACA

Results

The expression of CXCR3 in cMGT cell lines

CXCR3 has been reported to show higher expression in metastatic canine mammary gland tumor tissue than in primary malignant mammary gland tumor tissue (Ariyarathna et al., 2020). To investigate the expression of CXCR3 in the cMGT cell line, we used the CIPp and CIPm cell lines. We first performed RT-qPCR to compare the expression of CXCR3 in these cell lines. CXCR3 expression was significantly higher in CIPm than CIPp (Figure 1 A). The results were further validated with western blot analysis of CXCR3 in CIPp and CIPm (Figure 1 B). We then confirmed the expression of CXCR3 proteins using flow cytometry. Consistent with the RT-qPCR and the Western-blot data, the CIPm expressed CXCR3 significantly higher than the CIPp (Figure 1 C).

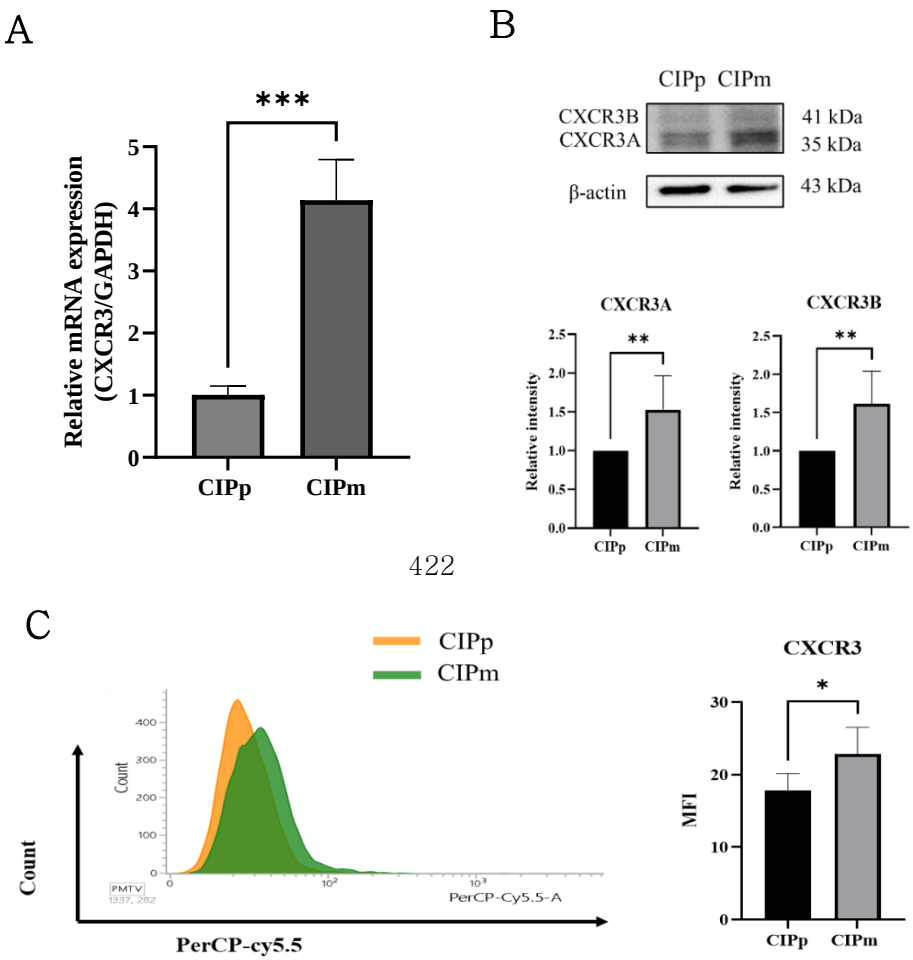


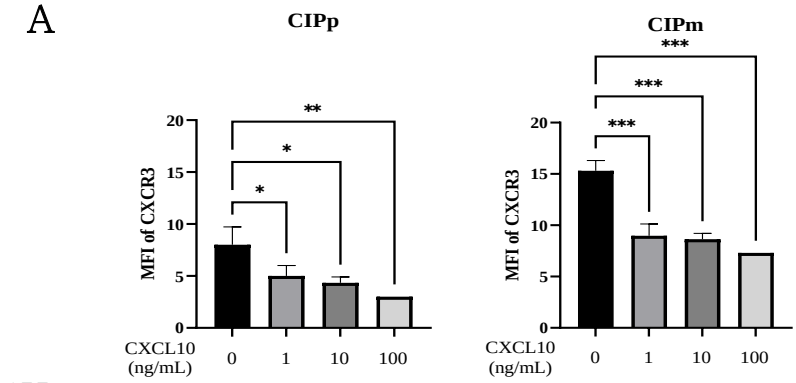
Figure 1. The CXCR3 expression in two cMGT cell lines.

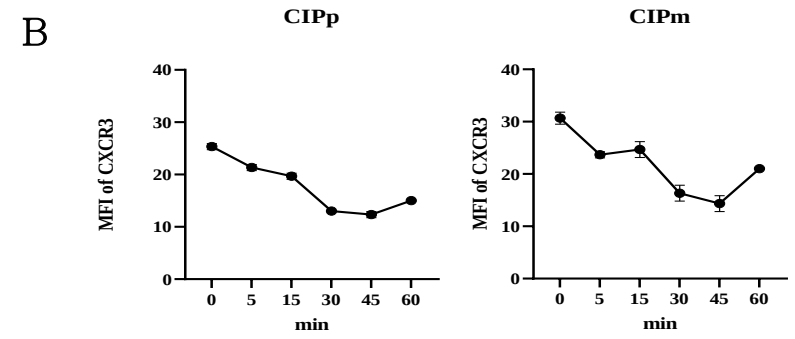
(A) The mRNA expression of CXCR3 was measured in CIPp and CIPm cells. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Each data represents the mean ± SEM (n = 4). (B) Western blot analysis of CXCR3 expression from both cell lines. The β-actin was used for normalization. (C) Analysis of the expression of CXCR3 by flow cytometry in both cell lines.

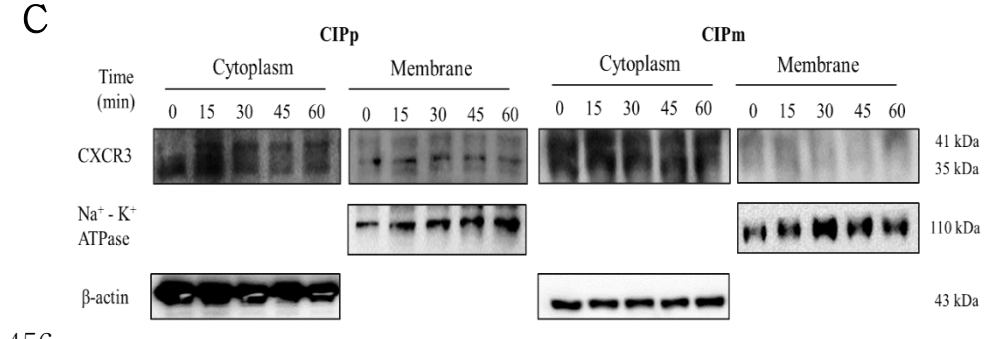
CXCL10 reduced CXCR3 expression on the surface of cMGT cells

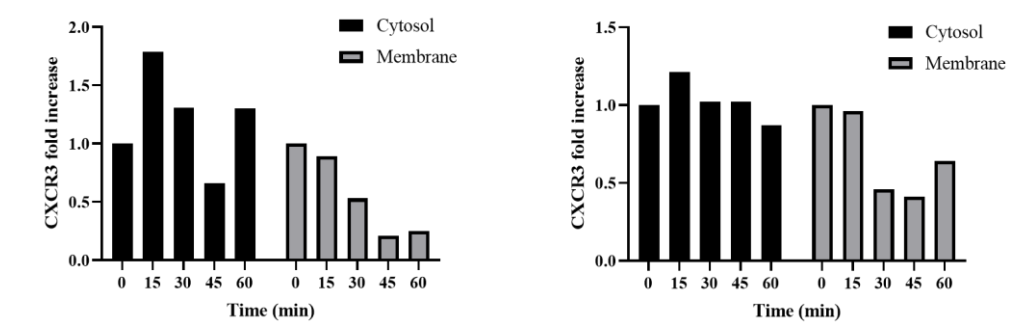
To determine the effect of CXCL10 on CXCR3 expression, the CIPp and CIPm cell lines were cultured in a conditioned medium containing CXCL10 ligand. Flow cytometry measured the surface expression of CXCR3 depending on CXCL10 concentration (0, 1, 10 and 100 ng/mL) and treatment time (0, 5, 15, 30, 45 and 60 min). Incubation with CXCL10 induced a dose-dependent loss of CXCR3 from the cell surface (Figure 2 A). Using 10 ng/mL concentrations of CXCL10, the expression of CXCR3 was subsequently examined. Loss of CXCR3 was observed by 45 minutes in both cell lines. The CXCR3 expression increased in 60 minutes in two cell lines (Figure 2 B). To further confirm whether CXCL10 could reduce CXCR3 expression on protein levels in cMGT cells, membrane and cytosolic proteins were fractionated using Mem-PER™ Plus Membrane Protein Extraction Kit and then detected using Western blotting. We found that CXCR3 was downregulated in membrane proteins in both cell lines after incubation with CXCL10. The membrane protein levels of CXCR3 were lowest at 45 minutes. In cytosolic fraction, the CXCR3 level was slightly increased at 15 min. In CIPp, it tended to fall to 45 minutes before increasing to 60 minutes, while it remained at a similar level

454 in CIPm. (Figure 2 C, D).


A
CIPp
CIPm
MFI of CXCR3
CXCL10 (ng/mL)
0
1
10
100
*
**
***


B
CIPp
CIPm
MFI of CXCR3
min
0
5
15
30
45
60


C
CIPp
CIPm
Time (min)
Cytoplasm
Membrane
CXCR3
Na+ - K+ ATPase
β-actin
41 kDa
35 kDa
110 kDa
43 kDa


D
Cytosol
Membrane
CXCR3 fold increase
Time (min)

461 **Figure 2. Downregulation of CXCR3 by CXCL10**

462 (A) CIPp and CIPm cells were cultured in a complete medium without or with the
463 increasing concentrations of CXCL10 for 24hrs. Surface-expressed CXCR3 was
464 detected by flow cytometry. (B) CIPp and CIPm cells were cultured in a complete
465 medium with 10 ng/mL CXCL10 for the times indicated. Surface-expressed CXCR3
466 was detected by flow cytometry. All error bars represent SEM with n = 3.
467 Significance: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (C, D) CIPm cells were stimulated
468 with 10 ng/mL CXCL10 at 15, 30, 45, and 60 min. Western blot analyses of CXCR3
469 expression from the membrane and cytosolic fractions. β-actin was used as a
470 cytosolic marker, whereas Na^{+}-K^{+} ATPase was used as a cell membrane marker.
471
472

CXCL10/CXCR3 axis promotes tumor cell proliferation in cMGT cells

Cell proliferation assay was performed to determine the cellular effect of the CXCL10/CXCR3 axis. The CIPp and CIPm cell lines were cultured for 24hrs without or with CXCL10 (10 ng/mL) or co-treated CXCL10 (10 ng/mL) and AMG487 (1 μM). The proliferation was significantly increased with only CXCL10 in both cell lines. In the co-treatment group, there was no difference in proliferation from the control group (Figure 3).

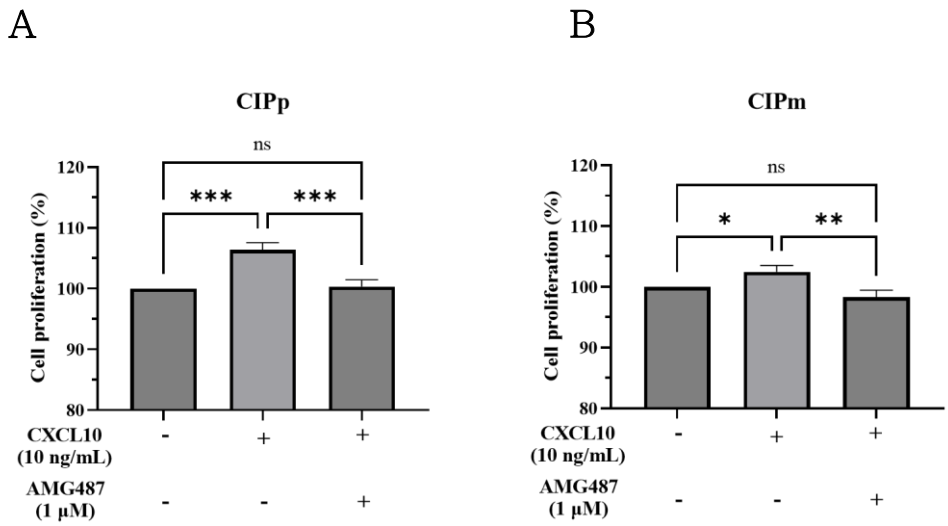


Figure 3. CXCL10/CXCR3 axis induces cell proliferation

The proliferation of CIPp (A) and CIPm (B) cells treated with only CXCL10 (10 ng/mL) or cotreated with CXCL10 (10 ng/mL) and AMG487 (1 μM) was analyzed using CCK-8 assay. All error bars represent SEM with n = 3. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and ns : Not Statistically Significant.

CXCL10/CXCR3 axis significantly increased the migration of cMGT cells

A scratch-wound-healing assay was then conducted to determine the effect of CXCL10 on cMGT cell migration. After CXCL10 (10 ng/mL) or co-treatment with CXCL10 (10 ng/mL) and AMG487 (1 μM), the gap area was measured at 0, 2, 4, and 6 hrs. The cMGT cells treated with CXCL10 showed significantly more migration capacity than control and cotreated cells (Figure 4).

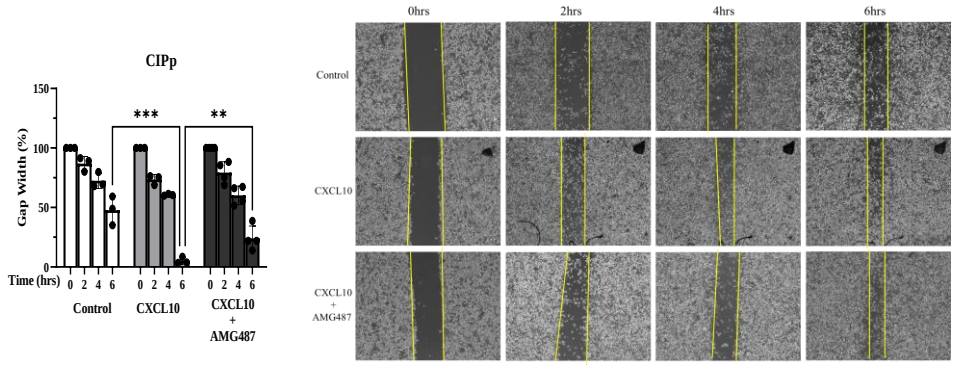


Figure 4. CXCL10/CXCR3 axis induces cMGT cells migration

Scratch wound-healing assay. CIPp (A) and CIPm (B) Cells were treated with either 10 ng/mL CXCL10 or co-treatment of 10 ng/mL CXCL10 and 1 μM AMG487 for 0, 2, 4, and 6hrs. Representative images from a scratch wound-healing assay are shown. Yellow outlines indicated the edge of the wound. The gap width percentage signifies the remnant gap size after making scratches, compared to the initial gap size. All error bars represent SEM with n = 3. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

CXCL10/CXCR3 axis stimulated the phosphorylation of AKT and ERK

To explore the possible signaling pathway by which CXCL10 binds CXCR3, several signal transduction pathways, including AKT and ERK, were assessed by western blot analysis. Exposure of CIPp and CIPm to CXCL10 for different periods resulted in increased phosphorylation of both ERK and AKT1. The addition of CXCL10 caused an increase in the phosphorylation of ERK and AKT1 (Figure 5).

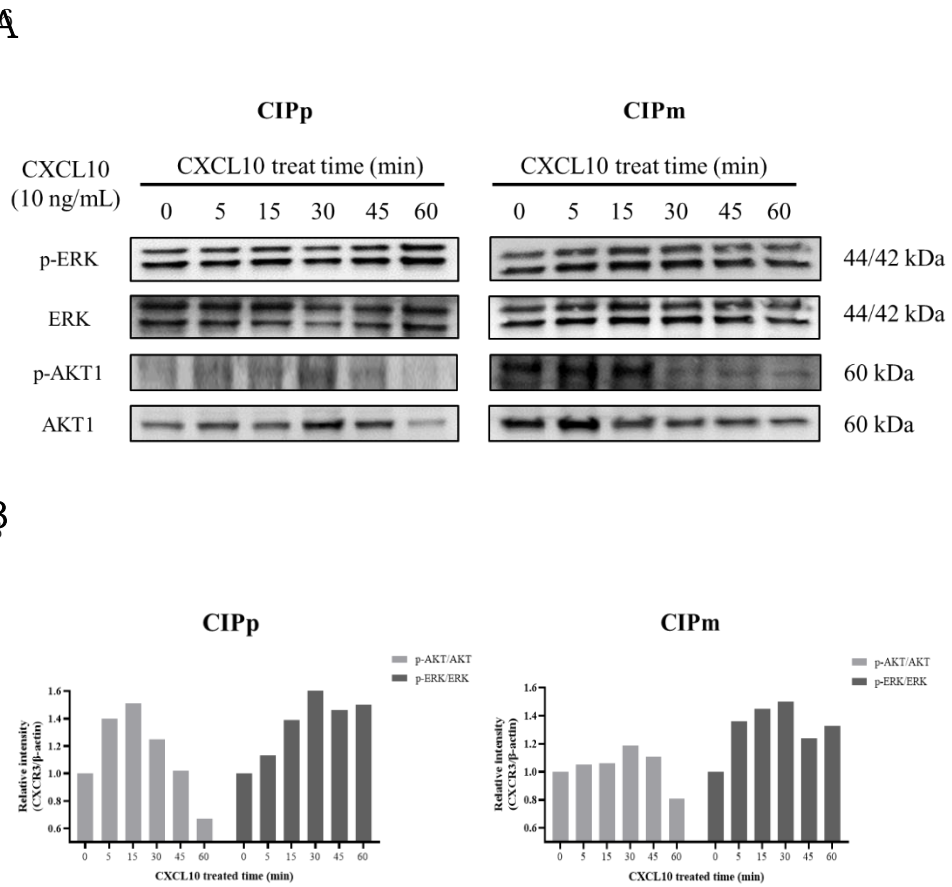


Figure 5. Western blot of ERK and AKT1 in cMGT cells

CIPp and CIPm cells were treated with 10 ng/mL of CXCL10 for 15, 30, 45, and 60 min. p-AKT1/AKT and p-ERK/ERK levels were detected by Western blot assay. (A) Protein expression of p-ERK, ERK, p-AKT1 and AKT1 in CIPp and CIPm. (B) Quantification of protein expression in CIPp and CIPm.

Discussion

CXCR3 and its ligands have been the main focus of immunity research because it primarily affects immune cell activation and differentiation. Recent research showed that they also play a tumorigenic involvement in some tumors by enhancing tumor cells proliferation and migration. For the first time, we focused on whether CXCR3 expresses on cMGT cells and how the CXCL10/CXCR3 axis affects tumor progression in cMGT.

Although studies on CXCR3 expression are few in veterinary medicine, in some studies performed from cMGT, CXCR3 showed higher gene expression in metastatic tissues than in normal, benign, and primary malignant tumors. To confirm this finding in cell lines, two cMGT cell lines originating from primary and metastatic lesions were chosen. Our data revealed that both cell lines expressed CXCR3 mRNA and protein and that CIPm had significantly higher expression of CXCR3 compared CIPp. These results demonstrated the correlation of CXCR3 level with the metastatic ability in cultured cell lines. Similarly, a previous study reported that CXCR3 expression was the highest in the 4T1 cell line, which is the most malignant in murine mammary gland tumor cell lines. (Zhu et al., 2015).

CXCR3 stimulates cellular downstream pathways by responding

with its ligands: CXCL9, CXCL10, and CXCL11. Among them, CXCL10 is well known to be significantly correlated with tumor grade and poor prognosis in numerous cancers, including human melanoma (Wightman et al., 2015), colorectal carcinoma (Toiyama et al., 2012), prostate cancer (Nagaya et al., 2020) and breast cancer (Clark et al., 2021). Furthermore, CXCL10 was the most abundant ligand in triple-negative human breast cancer disease (Clark et al., 2021). So, we focused on CXCL10/CXCR3 axis in this study. Concerning CXCR3 expression on tumor cells, prior studies in human cancers have demonstrated that CXCL10 can induce upregulation of CXCR3 (Goldberg-Bittman et al., 2004; Nagpal et al., 2006). Contrary to our expectation, our results showed that the cell surface level of CXCR3 was significantly decreased by CXCL10 with increasing concentration and time. In addition, we observed by cellular membrane fractionation studies that CXCL10 caused a reduction in the levels of CXCR3 at the tumor cell membrane. The reason for this is considered to be due to the internalization of CXCR3. CXCR3 is a GPCR that is typically internalized into cells following ligand binding. Once internalized, GPCR has multiple fates, including recycling, degradation, and endosomal signaling (Patwardhan et al., 2021). In the case of CXCR3, following internalization, CXCR3 is degraded, and de novo synthesis of the receptor replenishes it at the cell surface

(Meiser et al., 2008). According to this mechanism, the cytosolic protein is reduced after 15 minutes due to the degradation process that occurs following internalization.

After identifying the expression of CXCR3 on cMGT cells, the next aim was to determine how CXCR3 influenced the malignancy of cMGT cells by interacting with CXCL10. Our results showed that the proliferation of both cell lines was increased significantly by CXCL10 through the CCK-8 assay. Moreover, CXCR3 expression in cMGT cells enhanced the migratory ability of tumor cells in the presence of CXCL10 by scratch wound healing assay. In addition, the proliferation and migration of cMGT cells were inhibited significantly by CXCR3 inhibitor AMG487. These results demonstrated that the CXCL10/CXCR3 receptor-ligand interaction might promote cMGT cell progression by enhancing the proliferation and migration of tumor cells. Like other chemokine receptors, CXCR3 triggers several downstream pathways that affect cellular responses. In human medicine, after CXCL10 binding, CXCR3 changes its conformation and recruits the specific coupled G protein. This stimulation activates the MAPK/ERK and the PI3K/AKT pathways.

Similarly, our results demonstrated that the phosphorylation levels of AKT1 and ERK were increased with CXCL10 incubation. In other words, upregulating CXCL10 expression could activate the AKT and

602 ERK signaling pathways in cMGT cells. Therefore, the AKT and ERK
603 signaling pathways might be correlated with proliferation and
604 migration induced by the CXCL10/CXCR3 axis.
605 Although this study offers new information regarding the
606 expression and function of CXCR3 in cMGT cell lines, several
607 limitations should be addressed. First, since this study only identified
608 the interaction between exogenous CXCL10 and CXCR3, further
609 studies are needed on CXCL10 secreted by tumor cells to confirm
610 the CXCL10/CXCR3 autocrine axis. Second, we observed our in-
611 vitro findings for increased proliferation and migration in response to
612 exogenous CXCL10 in a controlled, artificial environment. This
613 investigation could not determine whether similar biologic responses
614 by cMGT cells occur within the natural tumor microenvironment.
615 Third, despite the finding that cMGT cells express CXCR3, this study
616 was not designed to look into the clinical significance of these results.
617 Given that CXCR3 expression affects disease progression and
618 prognosis in human breast cancer patients (Ma et al., 2009; Hilborn
619 et al., 2014). Therefore, future research into the clinical relevance
620 of CXCR3 expression as a prognostic marker for cMGT may be
621 warranted.
622 In conclusion, our study revealed the expression of CXCR3 and its
623 interaction with CXCL10 in cMGT cells. Our data also proved that

624 CXCR3 has a protumor effect in tumor cells. Despite some limitations,
625 this study suggests that CXCR3 could be a potential therapeutic
626 target for regulating tumor progression in cMGT.
627

Conclusion

The data in the present study make it clear that CXCR3 is expressed in cMGT cell lines and that the metastatic cell line has a higher expression of CXCR3 than the primary cell line. Additionally, when binding with CXCL10, the membrane protein level of CXCR3 decreased. These results are presumed to be through internalization. We found that the CXCL10/CXCR3 axis mediates the proliferation and migration of cMGT cells. Furthermore, we explored the signaling pathways that promote cMGT cell progression, including AKT and ERK. Although there are some limitations in the present study, these results indicate that CXCR3 is valuable as a potential therapeutic target for regulating cMGT progression.

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국문 초록

개 유선종양에서의 CXCL10/CXCR3의 종양 촉진 효과

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Chemokine 과 Chemokine receptor는 암의 진행에 중요한 역할을 한다. CXCR3은 주로 T 세포에서 발현하는 chemokine receptor로 항암작용에 주로 관여한다. 이와 달리 종양세포에서 발현하는 CXCR3는 사람 유방암을 포함한 다양한 암에서 종양 증식 및 전이를 촉진한다. 인의 연구에서는 이러한 작용에 초점을 맞추어 CXCR3을 타겟으로 한 항암요법에 관한 연구가 진행 중에 있다. 그러나, 수의에서는 개 유선종양 조직에서 종양 악성도에 따른 CXCR3의 유전자 발현을 확인한 것 외에 연구된 바가 없다. 이에 따라 본 연구는 실제 종양세포 자체에서 CXCR3의 발현 및 CXCR3와 ligand가 종양세포에 미치는 영향에 대해 연구하였다. 이 연구에서는 한 개체에서 각각 원발 병변, 전이병변에서 유래한 두가지 개 유선종양 세포주를 사용하였으며, ligand로 CXCL10를 사용하였다.

두 종양세포주에서 모두 CXCR3 발현이 확인되었으며, CIPp 보다 CIPm 에서 CXCR3의 발현이 유의미하게 더 높게 나타났다. 또한 다른 GPCR 의 기전과 유사하게, CXCL10를 농도 별, 시간 별로 처치 시 CXCR3가 내재화를 통해 표면 발현이 감소하는 것을 확인했다. 이러한 상호작용은 두 세포주 모두에서 종양 증식능 및 이동능을 향상시켰으며, 이때 AKT 와 ERK 세포 하부 시그널이 관여하는 것을 확인했다. 본 연구는 개 유선종양세포에서 발현하는 CXCR3이 CXCL10와 결합하여 내재화를 통해 CXCL10/CXCR3 활성화를 유발하여 종양세포에서 증식 및 이동을 촉진한다는 것을 보여주었다. 이는 CXCR3이 개 유선종양세포의 진행을 조절하는 잠재적인 치료 타겟으로써 가치가 있음을 시사한다.

핵심어: CXCR3, CXCL10, 개 유선종양, 암

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