CXCR4 antagonist inhibits perineural invasion of adenoid cystic carcinoma

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Running head: CXCR4 Antagonist Inhibits Perineural Invasion

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ABSTRACT

Background
Perineural invasion is a characteristic feature of salivary adenoid cystic carcinoma (ACC). Expression of CXCR4 is also a characteristic feature of ACC which distinguishes it from other non-neurotropic head and neck cancers. Therefore, CXCR4 may play a role in the perineural invasion of ACC. In this study, we aimed to demonstrate CXCR4 expression in ACC, to identify its association with perineural invasion, and to investigate the impact of CXCR4 inhibitor in vitro and in a murine perineural invasion model.

Materials & Methods
Expression of CXCR4 was assessed in ACC cell lines and in human tissue. The effect of gene knockdown using siRNA and a specific blocker of CXCR4 (AMD3100) were evaluated in vitro. A preclinical perineural invasion model was developed using BALB/c nude mouse. The effect of AMD3100 was evaluated in vivo.

Results
CXCR4 was highly expressed in aggressive strains of ACC in vitro, in the tumor in the animal model, and in the tumor of human tissue. SDF-1 expression was also demonstrated in the nerve of murine and human tissue. Gene knockdown by siRNA and inhibition by a CXCR4 specific inhibitor AMD3100 effectively abrogated invasion but not proliferation of ACC in vitro. The rate of perineural invasion was significantly decreased with AMD3100 treatment in the animal model (71.4% vs 18.8%, P=.009).

Conclusions
CXCR4 is highly expressed in ACC and is associated with perineural invasion. AMD3100, which can effectively diminish perineural invasion of ACC in vitro and in vivo, may have an adjuvant role in the management of ACC.
INTRODUCTION

Adenoid cystic carcinoma (ACC) is the second most common malignancy of the salivary glands. ACC has unique features, characterized by frequent perineural invasion, inconspicuous hematogenous spread, and late recurrence. Whilst surgery is the only curative treatment for ACC, perineural invasion renders surgical control difficult. Hence, perineural invasion is regarded as an adverse feature and increases the risk of recurrence in vital neural structures. In a recent report, patients with perineural invasion and lymphovascular invasion were more likely to recur in head and neck ACC, which prompts the need for additional management to improve local control\(^1\). At the same time, the feature makes ACC an excellent candidate for investigation of perineural invasion.

Salivary ACC and head and neck squamous cell carcinoma (HNSCC) are representative carcinomas of the head and neck region, which has very distinct characteristics. HNSCC do not exhibit neurotropism, which, in contrast, is a main feature of ACC. However, there has not been any evidence addressing this difference. Recent literature demonstrates that CXCR4 is expressed highly in ACC cells, which is in sharp contrast to the HNSCC cells\(^2\). The striking difference in expression levels of CXCR4 between ACC and HNSCC could account for the distinct features regarding neurotropism. In this context, data support the role of CXCR4 in neural invasion in a number of recent elaborate studies\(^3,4\).

The aims of this study were three-fold. First, to confirm the expression of CXCR4 in ACC and identify its role in perineural invasion. Second, to develop a preclinical perineural invasion model in nude mouse. Third, to apply AMD3100, a specific inhibitor of CXCR4, \textit{in vitro} and \textit{in vivo} as a molecular targeted agent in perineural invasion of ACC.

MATERIALS AND METHODS

\textit{Cell lines and culture conditions}

Human ACC cell lines, ACC-2 and ACC-M, were a kind gift from Dr. Wei-Liu Qiu of Shanghai Second Medical University, Shanghai, China\(^5\). ACC-M is a subclone of ACC-2 which has aggressive trait compared to the mother strain. Cells were cultured in RPMI 1640 medium (Invitrogen,
Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x nonessential amino acids, and antibiotics in a 37 °C incubator with 5% CO₂.

**Compound**

AMD3100 octahydrochloride is a product of Sigma-Aldrich (St. Louis, MO). The compound was dissolved in distilled water, then diluted with RPMI 1640 to the desired concentration.

**Isolation of proteins**

Nuclear and cytoplasmic proteins were isolated as described in detail[^6][^7]. Briefly, cells were washed with cold phosphate-buffered saline (PBS) and suspended in 300 µL of lysis buffer [10 mM Hepes, pH 7.9/10 mM KCl/0.1 mM EDTA/0.1 mM EDTA/1 mM DTT/0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were allowed to swell on ice for 15 min, detached from the plate, and then the homogenate was centrifuged. Cytoplasmic proteins were collected from the supernatant. The pellet was resuspended in 30 µL of ice-cold nuclear extraction buffer [20 mM Hepes, pH 7.9/0.4 M NaCl/1 mM EDTA/1 mM EDTA/1 mM DTT/1 mM PMSF/10% Nonidet P-40], incubated on ice for 5 min, then centrifuged for 10 min at 4°C. Nuclear extract was obtained from the supernatant.

**Immunoblot analysis**

Cells were plated at 5×10⁶ cells per well in six-well plates. After growth, cells were washed with cold PBS and lysed in culture dishes using Pro-prep protein extraction solution (Intron Biotechnology, Seongnam, Korea). Total protein concentration was quantified using BCA protein assay (Pierce Biotechnology, Rockford, IL). Twenty micrograms of protein from control and treated cell lysates were loaded on 4–15% gradient SDS-PAGE gels, electrophoresed under reducing conditions and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Blots were probed with antibodies and then blots were developed with enhanced chemiluminescence reagent (Amersham, Piscataway, NJ). The following antibodies were used; anti-CXCR4, anti-SDF-1, and anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).
**Quantitative RT-PCR**

Total RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA). Single-strand cDNA was synthesized with oligo-dT primer for 50 min at 50 °C using reagents provided in the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA). RT-PCR was performed using SYBR Green PCR Master Mix on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). Comparative CT method was used to compare expression, as described previously\(^8\). The sequences of the CXCR4 primers were forward (5'-GTA CTT GTC CGT CAT GCT TCT-3') and reverse (5'-CCA CCA TCT ACT CCA TCA TCT TC-3'). Human GAPDH was used as endogenous control.

**Flow cytometry analysis**

ACC cells were plated in six-well dishes. The cells were then washed with PBS and detached with trypsin at 37 °C. After rinsing in PBS, cells were incubated with PE mouse anti-human antibody against CXCR4 (1:100, BD Biosciences, San Jose, CA) for 20 min at 4 °C in the dark. Cells were then washed with PBS, fixed in 2% formaldehyde, then analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA).

**Immunocytochemistry**

ACC-M cells were seeded into LAB-TEK II chamber slide (Nalge Nunc International, Naperville, IL) at 3x10^4 cells per well. After 24 h, the cells were fixed with alcohol and nonspecific signals were blocked using peroxidase-blocking solution (Dako, Glostrup, Denmark). Cells were then incubated with a monoclonal antibody against CXCR4 (1:100; Abcam, Cambridge, UK) overnight at room temperature. After washing, coverslips were incubated with EnVision HRP (Dako, Glostrup, Denmark), then diaminobenzidine (Dako, Glostrup, Denmark), and then counterstained with hematoxylin. Cells were examined with a standard light microscope.

**Cell proliferation assay**

ACC-M cells (2x10^3) were plated in 96-well plate in triplicates and incubated for 3 days. AMD3100 was treated at concentrations of 1, 10, 50, 100, and 1,000ng/mL with control wells.
Cell proliferation assay was performed by WST-8 based colorimetric assay using Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD). For spectrophotometry, 100 µL of serum free media and 10 µL of CCK-8 solution were applied after removing the media. After 2 hours of incubation, absorbance was measured at 450 nm using a Synergy HT multidetection microplate reader (BioTek Instruments, Winooski, VT).

**Invasion assay**

The effect of AMD3100 on cell invasion of ACC-M cell line was evaluated in Boyden chambers fitted with Matrigel coated filters (Matrigel invasion chambers, BD Biocoat, San Jose, CA). Briefly, 1x10^5 cells were loaded in serum free media in the top wells of the Boyden chamber and normal media containing SDF-1 as a chemoattractant in the bottom well. In the top well, cells were treated with 0, 10, 100, and 1,000 ng/mL of AMD3100. After incubation for 22 hours, the cells invaded to the other side of the filter were fixed and stained with Harleco Hemacolor staining kit (EMD Chemicals, Gibbstown, NJ). The experiments were done in triplicates and the results reflect the outcome of at least three independent experiments. Cell number was counted at x200 at three different fields from each well and compared using unpaired t-test.

**Gene knockdown using siRNA**

CXCR4 siRNA (construct no. 1037826; sense sequence 5’-3’GAUAACUACACCGAGGAAA(dTdT); antisense sequence 5’-3’ UUUCUGCGUGUAGUAUC(dTdT)) and control siRNA were purchased from Bioneer (Daejeon, Korea). CXCR4 siRNA mediated gene knockdown was performed using DharmaFECT 4 (Dharmacon, Chicago, IL). The cells were harvested for experiment after 24 h of incubation with siRNA.

**Preclinical murine perineural invasion model**

All animal studies involving mice were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC No. BA0901-036-002-01). Preclinical murine perineural invasion model was developed in 8- to 12-week-old female nude mice. In brief, the skin overlying the left thigh was incised and the muscle was exposed. From
the posterior margin, the muscle was reflected anteriorly and the sciatic nerve was exposed. 1x10^6 ACC-M cells in 10 µL volumes were injected around the sciatic nerve. Mice were randomly allocated to control and treatment group. From day 5 after transplantation, 5mg/kg of AMD3100 or normal saline of the same volume was injected intraperitoneally once a day for 2 weeks. At 4 weeks, the mouse was sacrificed and the tumor was harvested together with the sciatic nerve. Standard H&E and immunohistochemistry staining was performed for CXCR4 and SDF-1. One pathologist reviewed the slides to evaluate perineural invasion.

**Immunohistochemistry**

A human ACC tumor and mouse specimens were stained for CXCR4 and SDF-1. The tissue sections on the microslides were deparaffinized with xylene, hydrated in serial dilutions of alcohol and then immersed in 3% H₂O₂. For antigen retrieval, the sections were then microwaved for 15 min in Tris-EDTA buffer (pH 9.0) supplemented with distilled water. Sections were incubated for 12 hours at ambient temperature with monoclonal anti-CXCR4 primary antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-SDF-1 primary antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). After rinsing with washing buffer, further incubation was performed using biotin-free polymeric HRP-linker antibody conjugate system (LabVision, Fremont, CA) for 30 min at ambient temperature. The slides were washed and the AEC chromogen was developed for 10 min. The slides were counterstained with Meyer's hematoxylin, then mounted with Immuno-mount for examination. The control slides were stained without primary antibody and secondary antibody to verify direct staining by secondary antibody and endogenous peroxidase activity and/or autofluorescence, respectively.

**Statistical analysis**

SPSS 16.0 software (SPSS Inc. Chicago, IL) was used. Fisher's exact test and Student's t-test were used to compare the groups.

**RESULTS**

*CXCR4 and SDF-1 expression in human ACC*
Close examination of areas of perineural invasion showed CXCR4 expression in the cytoplasm of tumors cells (Fig. 1). SDF-1 was found to be expressed in the nerve tissue, which was in sharp contrast with no stain within the tumor nests.

**CXCR4 expression in ACC**

Immunoblot assay revealed CXCR4 expression mainly in the cytoplasm of two ACC cell lines, ACC-M and ACC-2. The ACC-M cell line, which is more aggressive compared to its mother strain ACC-2, demonstrated an intense band in the cytoplasm (Fig. 2A). Cell surface expression of CXCR4 was demonstrated using flow cytometry (Fig. 2B). Quantitative RT-PCR showed that CXCR4 was 1.53 times more expressed in ACC-M cells compared to ACC-2 cells (Fig. 2C). Immunocytochemistry also showed expression of CXCR4 in the cytoplasm (Fig. 2D). ACC-M cells exhibited stronger expression of CXCR4 in the cytoplasm compared to that of ACC-2 cells.

**siRNA knockdown of CXCR4 in vitro**

In ACC-M cells, expression of CXCR4 significantly decreased after knockdown with CXCR4 siRNA, which was not observed in ACC-2 cells (Fig. 3A). A 0.56 fold decrease of CXCR4 expression was observed in ACC-M cells after siRNA knockdown compared to that of the control. Specific inhibition of CXCR4 did not affect proliferation of ACC cells on WST-8 assay (Fig. 3B). However, invasion abilities of ACC cells were significantly compromised after knockdown of CXCR4 (P=.0007, Fig. 3C-D).

**Development of preclinical murine perineural invasion model**

The preclinical murine perineural invasion model was developed using the sciatic nerve in BALB/c nude mouse (Fig. 4A-B). Tumor growing adjacent to the sciatic nerve was harvested, and areas of tumor invasion were closely observed (Fig. 4C-D). Immunohistochemistry revealed strong CXCR4 expression in the tumor, and SDF-1 expression in the nerve in the histology section of the animal model (Fig. 4E-F).

**CXCR4 blockade using AMD3100 in vitro**
The growth curve of ACC-M cell line showed no difference with treatment of AMD3100 (Fig. 5A). Blocking CXCR4 with AMD3100 did not suppress cell proliferation in this cell line from 1 ng/mL to 1,000 ng/mL concentration. In contrast, AMD3100 suppressed invasion \textit{in vitro} in a dose dependent manner (Fig. 5B-C).

\textbf{CXCR4 blockade using AMD3100 in preclinical murine model}

At 4 weeks, average mass size was smaller in the AMD3100 treatment group (291.0±127.9mm\(^3\)) than in the control group (586.2±423.8mm\(^3\)). However, statistical significance was not demonstrated (\(P=0.127\), Fig. 5F). On the other hand, perineural invasion was significantly reduced in the AMD3100 treatment group (18.8\%) compared to the control group (71.4\%), which was statistically significant (\(P=0.009\), Fig. 5G).

\textbf{DISCUSSION}

This study demonstrates that CXCR4 is expressed in ACC, especially in highly aggressive ones. Inhibition of CXCR4 by knockdown using siRNA and AMD3100 did not alter proliferation, but impaired invasiveness in a dose dependent manner \textit{in vitro}. AMD3100 was applied to the preclinical murine perineural invasion model, which was successfully developed in a nude mouse, in which strong inhibition of perineural invasion was demonstrated.

CXCR4 is an alpha-chemokine cell surface receptor which is widely expressed in various cancers. It has been demonstrated in many neurotropic cancers including melanoma\(^9-11\), prostate\(^12-15\), pancreatic carcinoma\(^16-18\), and ACC\(^19\). CXCR4 interacts with its ligand, SDF-1 (CXCL12), which activates various downstream pathways including the MAPK, PI3K-Akt, and PLC-PKC pathways. Together, the receptor-ligand interaction plays an important role in invasion, directional migration, locomotion, extravasation, homing, and cell survival\(^15,18,20\).

Muller and colleagues reported that CXCR4 was the only chemokine receptor highly expressed by ACC cell lines at the mRNA level\(^2\). They also demonstrated that its signaling led to the induction of directional tumor cell migration which supports a role of CXCR4 in tumor invasion. The finding is in concordance with ours, in which we have shown that ACC cell lines express high levels of CXCR4, not only at the RNA level, but also at the protein level and also by
immunocytochemistry and flow cytometry, which reflects the cytoplasmic/surface expression of the molecule. We also have shown that compared to ACC-2, CXCR4 was even more expressed in ACC-M, a more aggressive variant of the mother strain, ACC-2. This, in part, may support the claim of Muller et al. that CXCR4 has a role in tumor invasion and survival, since a more aggressive strain has shown higher levels of CXCR4 expression.

Chemokine SDF-1 (CXCL12) has been shown to be expressed in the peripheral nerve in various reports. Zhang and colleagues reported SDF-1 expression in the peripheral nerve. We were also able to observe SDF-1 expression in the peripheral nerve in human tissue and in the preclinical murine model. Furthermore, we were able to show perineural invasion on histology, and confirm decrease in the rate of invasion with treatment of AMD3100, whereas Zhang et al. described the tumor nerve relation as number of nerves near and far from the tumor.

AMD3100 is a highly specific CXCR4 antagonist. We observed that AMD3100 effectively inhibits invasion of ACC in in vitro studies. It is noteworthy, however, that CXCR4 blockade using siRNA and also AMD3100 did not affect proliferation in in vitro studies. The same was observed in the preclinical murine model in which the size of the tumor did not show statistically significant difference between the two groups. The finding is compatible with observation in other studies, in which AMD3100 has been shown to inhibit invasion but not proliferation in other neoplasia. Although CXCR4 has been associated with tumor growth, CXCR4/SDF-1 interaction has been more implicated with tumor locomotion, invasion, and metastasis, which in part can explain the results of our finding.

We have also successfully developed an in vivo perineural invasion model in athymic nude mouse. The sciatic nerve was chosen for the model development because it is a peripheral nerve, easy to access, and associated with less morbidity and mortality. The tumor could be resected en bloc with the nerve, and the perineurium could be readily identified under microscope after standard staining. As previously mentioned, perineural invasion is characteristic of ACC, but is not limited to ACC. Melanoma, prostate, and pancreas, and many other cancers have neurotropism as well, in which the preclinical murine perineural invasion model can be adopted. The current perineural invasion model may serve as a good in vivo model in these cancers.
Surgical treatment is the only curative option for ACC. Obtaining a sufficient clear resection margin is difficult for ACC, which in turn is related to the high recurrence rate. Furthermore, perineural invasion itself is associated with dismal prognosis. Targeted therapy directed at decreasing perineural invasion in an adjuvant setting perioperatively could improve surgery results and overall outcome. In this context, AMD3100 has an advantage over other compounds as it is a US FDA approved drug with a relatively mild toxicity profile, although the current approval is for other use. In this study, AMD3100 was well tolerated in the preclinical murine model, and demonstrated effective abrogation of perineural invasion, which is a step closer for clinical application.

One of the limitations of the current study is that the preclinical murine model used was not orthotopic. Orthotopic model for salivary ACC can be developed, but evaluation of perineural invasion may not be feasible because the nerve in the head and neck area surrounding the salivary glands are very small. An orthotopic model for salivary ACC perineural invasion can be better developed in rat or larger animals. Nevertheless, the sciatic nerve seems suitable as a preclinical model as the nerve bears the traits of the peripheral nerve that the ACC invades.

CONCLUSION

In conclusion, CXCR4, which is highly expressed in salivary ACC, can be effectively blocked by AMD3100, inhibiting invasiveness both in vitro and in preclinical murine perineural invasion model. The finding can be applied in perioperative setting for chemoprevention and adjuvant role in suppressing perineural invasion.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

None to declare.
REFERENCES


FIGURE LEGENDS

Figure 1: Human histology of adenoid cystic carcinoma with perineural invasion demonstrates expression of CXCR4 and SDF-1 (CXCL12) in the tumor and the nerve, respectively (x200). (A) A tumor nest of ACC (arrow) shows perineural invasion of the adjacent nerve (arrowhead). (B) Immunohistochemistry of CXCR4 shows dense staining of tumor cells (arrow). (C) The nerve shows speckled pattern of SDF-1 expression (arrowhead), which is in contrast to nearly absent staining in the tumor nest (arrow).
**Figure 2:** CXCR4 is expressed in adenoid cystic carcinoma, especially in highly invasive strain.  
(A) CXCR4 is mainly expressed in the cytoplasm of ACC-M cells in immunoblotting. (B) Flow cytometry exhibits cell surface expression of CXCR4 in both ACC-M and ACC-2 cells. (C) Quantitative RT-PCR shows a 1.53 fold higher RNA expression of CXCR4 in ACC-M cells compared to that of ACC-2. (D) Expression of CXCR4 is stronger in the cytoplasm of ACC-M cell lines compared to ACC-2 cells by immunocytochemistry (CXCR4-DAB, Hematoxylin, x200). Control panel was stained void of the primary antibody. Data shown are representative of three independent experiments.
**Figure 3:** Specific inhibition of CXCR4 using siRNA inhibits invasion but not proliferation of adenoid cystic carcinoma in vitro. (A) Quantitative RT-PCR shows decreased expression of CXCR4 by 0.56 fold after siRNA treatment compared to that of the control in ACC-M. (B) Proliferation of ACC-M is not effectively inhibited with specific inhibition of CXCR4 in WST assay. (C-D) Invasion assay using matrigel in Boyden chamber demonstrates significantly decreased number of cells in the lower chamber compared to the control (*P*=.0007, t-test).
Figure 4: Preclinical murine perineural invasion model using ACC-M cell line in nude mouse. (A) After fine dissection under microscope, ACC-M cells are transplanted by injecting the cells along the sciatic nerve in 8- to 12-week-old female BALB/c nude mouse. (B) ACC-M cell line forms tumor along the sciatic nerve. (C) The tumor is harvested and prepared for section. (D) Standard H&E histology of murine perineural invasion model shows an area of frank perineural invasion (x100). (E) Immunohistochemistry of a representative tissue for CXCR4 shows CXCR4 expression in the tumor nest on the upper and left side (x200). (F) Immunohistochemistry for SDF-1 (CXCL12) shows expression in the nerve (x400).
**Figure 5:** AMD3100 inhibits perineural invasion in vitro and in preclinical murine perineural invasion model. (A) Proliferation assay of ACC-M cells in various concentrations of AMD3100 fails to inhibit proliferation of ACC-M cells in vitro. (B) Invasion assay using Boyden chamber shows significantly less cells in the lower chamber after treatment of AMD3100 compared to control. (C) The degree of inhibition increases with increasing concentration of AMD3100. (D) Histology section of murine perineural invasion model shows intact perineurium in a mouse treated with AMD3100 (H&E, x100). (E) Histology section of murine model shows definite perineural invasion of the tumor in a control group mouse (H&E, x100). (F) Tumor volume seems somewhat decreased after treatment of AMD3100, but shows no statistical significance (*P=0.202). (G) The rate of perineural invasion was 71.4% for tumors in the control group compared to 18.6% for the AMD3100 treated group, which was statistically significant (*P=.009). Data regarding animal study are representative of two independent experiments.
**Figure 1:** Figure 1.tif

A. Nucleus Cytoplasm

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B. ACC-2 ACC-M

![Graphs showing fluorescence intensity](image)

C. Relative expression of CXCR4 mRNA

![Bar chart](image)

D. Control 100 µm ACC-2 100 µm ACC-M 100 µm

**Figure 2:** Figure 2.tif
Figure 3: Figure 3.tif
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