Biological Role and Potential Therapeutic Targeting of the Chemokine Receptor CXCR4 in Undifferentiated Thyroid Cancer

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Abstract

Anaplastic thyroid carcinoma (ATC) is a rare thyroid cancer type with an extremely poor prognosis. Despite appropriate treatment, which includes surgery, radiotherapy, and chemotherapy, this cancer is invariably fatal. CXCR4 is the receptor for the stromal cell–derived factor-1 (SDF-1)/CXCL12 chemokine and it is expressed in a variety of solid tumors, including papillary thyroid carcinoma. Here, we show that ATC cell lines overexpress CXCR4, both at the level of mRNA and protein. Furthermore, we found that CXCR4 was overexpressed in ATC clinical samples, with respect to normal thyroid tissues by real-time PCR and immunohistochemistry. Treatment of ATC cells with SDF-1 induced proliferation and increase in phosphorylation of extracellular signal–regulated kinases and protein kinase B/AKT. These effects were blocked by the specific CXCR4 antagonist AMD3100 and by CXCR4 RNA interference. Moreover, AMD3100 effectively reduced tumor growth in nude mice inoculated with different ATC cells. Thus, we suggest that CXCR4 targeting is a novel potential strategy in the treatment of human ATC. [Cancer Res 2007;67(24):11821–9]

Introduction

Thyroid cancer accounts for the majority of endocrine neoplasms worldwide (1). Malignant tumors derived from the thyroid gland include well-differentiated thyroid carcinomas (WDTC; papillary and follicular) and undifferentiated or anaplastic thyroid carcinomas (ATC). Another group of cancers falls between these two types, the so-called poorly differentiated thyroid carcinomas (PDC). WDTC represents >90% of all thyroid cancers, whereas ATC accounts for approximately 2% to 5% of them (2–4). WDTC management requires surgery and adjuvant radioactive iodine (5, 6). Whereas most of the patients with WDTC have an excellent prognosis, those that present with PDC or ATC have a poor prognosis. PDC displays intermediate biological and clinical features between WDTC and ATC. Indeed, these tumors display high propensity to recur and metastasize. Furthermore, they tend to a progressive dedifferentiation, which leads to the decrease in the levels of the sodium iodide symporter. As a consequence of this, these tumors are unable to concentrate iodine and become resistant to radiometabolic therapy (4, 7). ATC is the most malignant thyroid tumor and one of the more fatal human malignancies with a median survival from the time of diagnosis of only 4 to 12 months (8, 9). ATC is more frequent in iodine-deficient areas and can be associated with other thyroid disorders. These tumors arise at a mean age of 55 to 65 years, are more common in women, and present usually as a rapidly growing mass, localized in the anterior neck area, which rapidly metastasizes at lungs, bone, and brain. Treatment of ATC with surgery, radiotherapy, and chemotherapy, alone or in combination, shows little or no effect on patient’s survival (10). For these reasons, novel treatment strategies are urgently needed. Unlike the WDTC, the molecular mechanisms underlying the development of human ATC are largely unknown. Genetic rearrangements of the RET and TRKA tyrosine kinase receptors, point mutations of the BRAF serine-threonine kinase or, less frequently, Ras mutations, are typically found in papillary thyroid carcinoma (PTC). Rearrangements of PPARγ or Ras point mutations are instead found in human FTC (11, 12). Among these genetic alterations, Ras or BRAF point mutations are detected at low frequency in ATC, suggesting that some ATC may arise from a preexisting WDTC, whereas others arise de novo (12, 13). Inactivating point mutations of the p53 tumor suppressor and activating point mutations of the β-catenin or the PIK3CA are also found in ATC (13–15).

In the attempt to better characterize human ATC at the molecular level, we aimed to study the involvement of chemokine and chemokine receptors in these tumors. Chemokines are small secretory proteins that were initially reported to control the secretory proteins that were initially reported to control the regulation of tumor angiogenesis and leukocyte recruitment (17). These molecules exert their action through binding to a group of seven-transmembrane G protein–coupled receptors. All chemokine receptors initiate signal transduction by activating a member of the Gi family of G proteins which, on receptor activation, dissociates into α and βγ subunits. The Go subunit inhibits adenyl cyclase, whereas the Gβγ dimer activates the phospholipase Cβ and the phosphatidylinositol 3-kinase pathways, with the activation of downstream signaling. It has becoming clear recently that chemokines are also involved in cancer cell migration, survival, and growth (17). Not only chemokines regulate some important features of cancer cells but are also involved in the regulation of tumor angiogenesis and leukocyte recruitment (17). In particular, the chemokine receptor CXCR4 and its ligand stromal cell–derived factor-1 (SDF-1)/CXCL12 have been implicated in the metastatic spread of breast cancer cells (18). CXCR4 is one of the located in the antigen-presenting cells and recruit T lymphocytes. Chemokine receptors initiate signal transduction by activating a member of the Gi family of G proteins which, on receptor activation, dissociates into α and βγ subunits. The Go subunit inhibits adenyl cyclase, whereas the Gβγ dimer activates the phospholipase Cβ and the phosphatidylinositol 3-kinase pathways, with the activation of downstream signaling. It has becoming clear recently that chemokines are also involved in cancer cell migration, survival, and growth (17). Not only chemokines regulate some important features of cancer cells but are also involved in the regulation of tumor angiogenesis and leukocyte recruitment (17). In particular, the chemokine receptor CXCR4 and its ligand stromal cell–derived factor-1 (SDF-1)/CXCL12 have been implicated in the metastatic spread of breast cancer cells (18). CXCR4 is one of the...
most important chemokine receptors for cancer cells. Indeed, it is expressed in a great number of human solid and hematologic cancers, including breast, prostate, brain, colon, and lung cancer (19, 20). We and others previously reported the overexpression and functional activity of CXCR4 in thyroid cancer (21, 22). In this report, we show that human ATC cells express high levels of CXCR4 and that the CXCR4-SDF-1/CXCL12 axis sustains the growth of ATC cells. Finally, we provide evidences that targeting CXCR4 might be exploited as a novel anticancer therapy for human ATC.

Materials and Methods

Cell lines. Human primary cultures of normal thyroid and ATC cells were obtained from F. Curcio (Dipartimento di Patologia e Medicina Sperimentale e Clinica, University of Udine, Udine, Italy; P5, P5-2N, P5-3N, P5-4N, and HTU8) and H. Zitzelsberger (Institute of Molecular Radiobiology, GSF-National Research Center for Environment and Health GmbH, Neuherberg, Germany; S11T, S77T, and S14T) and cultured as described previously (23). Primary cultures of ATC were also a kind gift of H. Zitzelsberger. Of these, only the S11T displays a BRAF(V600E) mutation in heterozygosis.5 Human thyroid papillary cancer cell lines TPC1, FB2, and NIM have been described previously (24–26). TPC1 and FB2 cells harbor a RET/PTC1 rearrangement. NPA87 cells derive from a PDC and harbor a BRAF(V600E) mutation in homozygosis (23); and CAL62 cells express wild-type BRAF but mutant BRAF(V600E) mutation in heterozygosis; 8505C and FRO harbor a BRAF(V600E) mutation in heterozygosis; and CAL62 cells express wild-type BRAF but mutant BRAF(V600E) mutation in homozygosis (23). The anaplastic cells ARO, KAT4, BHT101, and FB1 cells harbor a BRAF(V600E) mutation in heterozygosis; 8505C and FRO harbor a BRAF(V600E) mutation in homozygosis (23); and CAL62 cells express wild-type BRAF but mutant NRAS allele. Continuous cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% glutamine.

RNA extraction and reverse transcription PCR. Total RNA was isolated by the RNeasy kit (Qiagen) and subjected to on-column DNase digestion with the RNase-free DNase set (Qiagen) according to the manufacturer’s instructions. The quality of RNA was verified by electrophoresis through 1% agarose gel and visualized with ethidium bromide. RNA (1 μg) from each sample was reverse transcribed with the QuantiTect Reverse Transcription (Qiagen) using an optimized blend of oligo(dT) and random primers according to the manufacturer’s instructions. To design a quantitative reverse transcription-PCR (RT-PCR) assay, we used the Human ProbLib library (Exagon). Briefly, Exagon provides 90 human prevalidated Taqman probes (8–9 nucleotides long) that recognize ≥ 99% of human transcripts in the RefSeq database at the National Center for Biotechnology Information. The ProbeFinder assay design software (available online)5 was used to design primer pairs and probes. All fluorogenic probes were dual labeled with FAM at 5′-end and with a black quencher at the 3′-end. Primer pairs and PCR conditions are available on request. Quantitative RT-PCR was performed in a Chromo 4 Detector (MJ Research) in 96-well plates using a final volume of 20 μL. For each PCR, 8 μL of 2.5× RealMasterMix Probe ROX (Eppendorf AG), 200 nmol/L of each primer, 100 nmol/L probe, and cDNA generated from 50 ng of total RNA were used. PCRs were performed in triplicate and fold changes were calculated with the following formula: 2ΔΔCT = (sample 1ΔCT − sample 2ΔCT), where ΔΔCT is the difference between the amplification fluorescent thresholds of the mRNA of interest and the mRNA of RNA polymerase 2 used as an internal reference.

Immunohistochemistry. Retrospectively collected archival thyroid tissue samples from patients affected by ATCs were retrieved from the files of the Pathology Department of the University of Pisa on informed consent. Sections (4 μm thick) of paraffin-embedded samples were stained with H&E for histologic examination to ensure that the samples fulfilled the diagnostic criteria required for the identification of ATC. Normal thyroid tissue samples were also retrieved from the files of the Pathology Department of the University of Pisa.

For immunohistochemistry, paraffin sections (3–5 μm) were dewaxed in xylene, dehydrated through graded alcohols, and blocked with 5% nonimmune mouse serum in PBS with 0.05% sodium azide for 5 min. Mouse monoclonal antibody against CXCR4 (clone 12G5; R&D Systems) was added at 1:1.000 dilution for 15 min. After incubation with biotinylated anti-mouse secondary antibody for 15 min followed by streptavidin-biotin complex for 15 min (Catalyzed Signal Amplification System, DAKO), sections were developed for 5 min with 0.05% 3,3-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 mol/L Tris-HCl buffer (pH 7.6), counterstained with hematoxylin, dehydrated, and mounted.

Protein studies. Immunoblotting experiments were performed according to standard procedures. Briefly, cells were harvested in lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/L EGTA, 1.5 mmol/L MgCl2, 10 mmol/L NaA, 10 mmol/L sodium pyrophosphate, 1 mmol/L Na2VO4, 10 μg/mL aprotinin, 10 μg/mL leupeptin] and clarified by centrifugation at 10,000 × g for 40 min. Protein extraction from human tissues, snap-frozen samples were immediately homogenized in lysis buffer by using the Mixer Mill apparatus (Qiagen). Protein concentration was estimated with a modified Bradford assay (BioRad). Antigens were revealed by an enhanced chemiluminescence detection kit (Amersham). Anti-CXCR4 antibodies were from Abcam Ltd. For the evaluation of mitogen-activated protein kinase (MAPK) and AKT activity on SDF-1α triggering, BHT101 and S11T cells were serum deprived for 12 h and then stimulated with human recombinant SDF-1α (R&D Systems) for the indicated time. Anti-phosphorylated p44/42 MAPK, anti-p44/42 MAPK, anti-phosphorylated AKT, and anti-AKT antibodies were from New England Biolabs. Anti-tubulin monoclonal antibody was from Sigma Chemical. Secondary anti-mouse and anti-rabbit antibodies coupled to horseradish peroxidase were from Bio-Rad.

Flow cytometric analysis. Subconfluent cells were detached from culture dishes with a solution of 0.5 mmol/L EDTA and then washed thrice in PBS buffer. After saturation with 1 μg of human IgG1/IgG2, cells were incubated for 20 min on ice with phycoerythrin (PE)-labeled antibodies specific for human CXCR4 (R&D Systems) or isotype control antibody. After incubation, unreacted antibody was removed by washing cells twice in PBS buffer. Cells resuspended in PBS were analyzed on a FACS caliber cytofluorometric using the CellQuest software (Becton Dickinson). Analyses were performed in triplicate. In each analysis, a total of 106 events were calculated.

Cell proliferation. 5-phase entry was evaluated by bromodeoxyuridine (BrdUrd) incorporation and indirect immunofluorescence. Cells were grown on coverslips, kept in 2% serum for 24 h, and then treated with recombinant SDF-1α (100 ng/mL) for 48 h. BrdUrd was added at a concentration of 10 μmol/L for the last 1 h. Subsequently, cells were fixed in 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. BrdUrd-positive cells were revealed with Texas red-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Cell nuclei were identified by Hoechst staining. Fluorescence was visualized with a Zeiss 140 epifluorescent microscope.

For growth curves, cells were plated at a density of 0.5 × 104 in low-serum conditions (2.5%) and counted at the indicated time points.

RNA interference. Small inhibitor duplex RNAs targeting human CXCR4 have been described previously and were chemically synthesized by Proligo. Sense strand for human CXCR4 small interfering RNA (siRNA) targeting was the following: 5′-GAGGGGAUCAGCAGUAUAUAC-3′. Small duplex RNAs containing the same nucleotides, but in scrambled fashion (siRNA SCR), were used as a negative control. For siRNA transfection, ATC cells were grown under standard conditions. The day before transfection, cells were plated in six-well dishes at 50% to 60% confluency. Transfection was performed using 5 to 15 μg of duplex RNA and 6 μL of Oligofectamine reagent (Invitrogen). Cells were harvested at 48 and 72 h after transfection and analyzed for protein expression and biological activity.

Xenografts in nude mice. Mice were housed in barrier facilities and 12-h light-dark cycles and received food and water ad libitum at the Dipartimento di Biologia e Patologia Cellulare e Molecolare (University of Naples “Federico II”, Naples, Italy). This study was conducted in accordance with Italian regulations for experimentation on animals. All manipulations were performed while mice were under isoflurane gas anesthesia. No mouse...
showed signs of wasting or other signs of toxicity. BHT101, ARO, or KAT4 cells (5 × 10^6 per mouse) were inoculated s.c. into the right dorsal portion of 4-week-old male BALB/c nu/nu mice (The Jackson Laboratory). When tumors measured 40 mm³, mice were randomized to receive AMD3100 (n = 10; 1.25 mg/kg/twice daily) or vehicle alone (n = 10; PBS) by i.p. injection for 5 consecutive days per week for 3 to 4 weeks. Tumor diameters were measured at regular intervals with calipers. Tumor volumes (V) were calculated with the following formula: \( V = \frac{A \times B^2}{2} \) (A = axial diameter; B = rotational diameter). Tumors were excised and fixed overnight in neutral buffered formalin and processed by routine methods.

**Statistical analysis.** To compare CXCR4 mRNA levels in normal thyroid tissues versus ATC samples, we used the Mann-Whitney nonparametric test and the GraphPad Instat software, v.3.0b. To compare ATC xenograft growth in AMD3100-treated versus untreated animals, we used the unpaired Student’s \( t \) test (normal distributions and equal variances) and the GraphPad Instat software, v.3.0b. All \( P \) values were two sided, and differences were considered statistically significant at \( P < 0.05 \).

**Results**

**CXCR4 is overexpressed in surgical samples of human ATC.** We compared CXCR4 mRNA levels in a set of ATC samples (\( n = 13 \)) versus different samples of normal thyroid tissue (\( n = 6 \)). As shown in Fig. 1A, CXCR4 mRNA was found to be up-regulated in most of the tumor samples (10 of 13). When we performed statistical analysis, the differences in the expression levels of CXCR4 between tumors and normal thyroid tissues were statistically significant (\( P = 0.0084 \); Fig. 1A).

To verify whether CXCR4 mRNA overexpression resulted in an increase in the protein levels, we used protein extracts from a different set of ATC samples and three normal thyroid tissues in an immunoblot experiment with CXCR4-specific antibodies. As shown in Fig. 1B, CXCR4 protein levels were higher in ATC samples than in normal thyroid. As a positive control for CXCR4 expression, the ATC cell line ARO was used.

Finally, CXCR4 antibodies were used in immunohistochemical experiments. We evaluated CXCR4 expression in normal thyroid tissues and a set of ATC samples (\( n = 33 \)). Whereas no CXCR4 expression was detected in normal thyroid tissues, 13 (39%) of the ATC samples scored positive for CXCR4. A representative CXCR4 immunostaining is shown in Fig. 1C. These data indicate that a significant fraction of human ATCs, similarly to other epithelial

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**Figure 1.** A, expression levels of CXCR4 in human ATC samples versus six normal thyroid tissues by real-time RT-PCR. CXCR4 expression levels of tumors (Y axis) are calculated relative to the mean CXCR4 level of normal human thyroid tissues (NT). All experiments have been performed in triplicate, and the average value of the results was plotted on the diagram. \( P \) value was calculated with the two-tailed, nonparametric Mann-Whitney test. B, protein lysates (100 μg) extracted from the indicated samples underwent Western blotting with anti-CXCR4-specific antibodies. Immunocomplexes were revealed by enhanced chemiluminescence. Equal protein loading was ascertained by anti-tubulin immunoblot. C, immunohistochemical staining for CXCR4 of formalin-fixed, paraffin-embedded ATCs. Tissue samples from normal thyroid or ATC were incubated with a mouse monoclonal anti-CXCR4 antibody. ATCs show a strong immunoreactivity for CXCR4, whereas normal thyroid tissue is negative. Representative pictures of normal and pathologic positive samples are shown. Isotype control was also performed (data not shown). D, the expression levels of CXCR4 protein were analyzed in thyroid tumor samples from transgenic mice models. Tumor tissues were snap frozen and immediately homogenized by using the Mixer Mill apparatus in lysis buffer. Equal amounts of proteins were immunoblotted and stained with anti-CXCR4 polyclonal antibodies (Abcam). ATC-like samples displayed a more intense immunoreactivity for CXCR4. As a control for equal loading, the anti-α-tubulin monoclonal antibody was used.
cancers, features high expression levels of the CXCR4 receptor. Furthermore, they suggest that the increase in CXCR4 levels occurs at the transcriptional level.

**CXCR4 is highly expressed in animal models of ATC.** Several transgenic mice model of thyroid cancer have been developed by using various oncogenes under the transcriptional control of the thyroid-specific thyroglobulin bovine promoter. Depending on the specific transgene, these mice develop carcinomas that resemble, for cytologic and histologic features, human PTC, FTC, or ATC. In particular, mice expressing either RET/PTC3 (TGPTC3) or TRK/T1 (TGTRK) oncogene develop PTC-like tumors (27, 28). NRAS transgene expression results in follicular tumors that progress to poorly differentiated carcinomas (TGNRAS; ref. 29). Finally, animals expressing the SV40 large T antigen (TGSV) present aggressive thyroid cancer with features similar to human ATC (30).

To evaluate the expression of CXCR4 in these animal models, we extracted proteins from different tumor samples of the different transgenic lines and performed Western blot analysis with CXCR4 antibodies. Histologic diagnosis of the thyroid lesions was verified before processing of the samples. As shown in Fig. 1D, CXCR4 levels were higher in ATC models than in normal mouse thyroid tissue. PTC samples displayed intermediate levels of CXCR4. These data, together with previously published data (21, 22), suggest that CXCR4 up-regulation is a frequent event in thyroid tumorigenesis and that it correlates with the malignancy of the disease.

**CXCR4 is a functional receptor in human ATC cells.** To study the role of CXCR4 in human ATC, we first needed to identify a suitable cell model. To this aim, various normal thyroid and ATC-derived primary cultures and continuous cell lines were tested for CXCR4 expression by Western blot analysis. As shown in Fig. 2A and B, whereas normal thyroid cultures displayed low or undetectable CXCR4 expression levels, several ATC cell lines featured high levels of the CXCR4 receptor. In particular, of 10 ATC cell lines, 7 displayed high expression levels of CXCR4. In the case of ATC cells, the increased levels of CXCR4 proteins were associated to an increase in CXCR4 mRNA levels as assessed by quantitative PCR analysis (Fig. 2C). We then asked whether this receptor was expressed on the cell surface. To this aim, we performed flow cytometry experiments using a PE-conjugated mouse monoclonal anti-CXCR4 antibody. The percentage of CXCR4-positive cells was

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**Figure 2.** A, CXCR4 up-regulation in cell lines derived from human thyroid carcinomas was evaluated by immunoblot with a polyclonal anti-CXCR4 antibody. The expression levels of CXCR4 protein were analyzed in the P5 human primary thyroid cells, and in the indicated cell lines derived from human PTCs (NIM, TPC-1, FB-2, and NPA) or from human ATCs (8505C, ARO, CAL62, BHT101, FRO, and FB1). B, ATC-derived (S11T, S77T, S14T, HTU8, and ARO) and normal thyroid primary cultures (P5) were screened for CXCR4 expression by Western blot analysis with the polyclonal anti-CXCR4 antibody. As a control for equal loading, the anti-α-tubulin monoclonal antibody was used. C, expression levels of CXCR4 in human ATC cells versus the P5 normal thyroid culture were evaluated by real-time RT-PCR analysis. CXCR4 expression levels of ATC cell lines (Y axis) are calculated relative to the expression level in the normal human cell culture P5. All experiments were performed in triplicate, and the average value of the results was plotted on the diagram. SDs were smaller than 25% in all cases (data not shown). D, flow cytometry analysis (fluorescence-activated cell sorting) of surface-expressed CXCR4 in ATC cells. Subconfluent cells were detached from culture dishes and incubated with PE-labeled antibodies specific for human CXCR4.
determined. As shown in Fig. 2D, CXCR4 was expressed in almost all the ATC cell lines tested, with the exception of the FB1 cells. The ARO cells, which in a previous report were shown to feature high CXCR4 levels (21), were included as a positive control. In contrast, normal thyroid cells did not express CXCR4 (data not shown). SDF-1, the CXCR4 ligand, was not expressed by ATC cells as assessed by quantitative PCR or ELISA assay (data not shown).

We selected two cell lines, S11T and BHT101, for further experiments. First, we tested the ability of recombinant SDF-1 to stimulate signal transduction in ATC cells. It has been previously reported that stimulation of CXCR4 induces the activation of several kinase cascades mainly through the activation of the G\(\text{i}\)/\(\text{G}\) subunit of the G\(\text{i}\) protein (31, 32). We therefore tested the phosphorylation of two downstream effectors, extracellular signal-regulated kinase (ERK) 1/2 and AKT, using phosphorylation-specific antibodies. To this aim, cells were serum starved for 12 h and then stimulated with SDF-1\(\alpha\) for different time points. As shown in Fig. 3A and B, SDF-1\(\alpha\) induced rapid and sustained activation of ERK1/2 in both cell lines. AKT activation was also achieved in BHT101 cells on SDF-1\(\alpha\) treatment, whereas it was less evident in S11T cells. Together, these data indicate that CXCR4 is functional in ATC cells. Activation of ERK1/2 and AKT was observed in virtually all the ATC cell lines expressing CXCR4, whereas normal thyroid cells, which do not express CXCR4, did not display these effects (data not shown).

**Biological activity of CXCR4 in ATC cells.** To further test the functional responsiveness of CXCR4 in ATC, we stimulated these cells with SDF-1\(\alpha\) and evaluated its ability to induce cell proliferation. To this aim, BHT101 and S11T cells were maintained in low-serum (2.5%) growth conditions for 24 h and then either left untreated or stimulated with SDF-1\(\alpha\) for 12 h. As a measure of DNA synthesis, we counted BrdUrd-positive cells on a 1-h BrdUrd pulse. As shown in Fig. 4, SDF-1\(\alpha\) consistently enhanced DNA synthesis in both BHT101 and S11T cells. We then used a specific CXCR4 inhibitor, AMD3100, to block this effect. AMD3100 is a competitive antagonist of SDF-1\(\alpha\), but it also displays partial agonist activity (33). Normal thyroid cells were insensitive to SDF-1\(\alpha\) stimulation and to the effect of AMD3100 (data not shown). As shown in Fig. 4A, AMD3100 inhibited SDF-1\(\alpha\)-mediated BrdUrd incorporation in ATC cells. The positive effect of SDF-1 on cell proliferation, measured as S-phase entry, was also observed in other ATC cell lines (Fig. 5C). To evaluate whether SDF-1\(\alpha\) could stimulate ATC cell growth, we also performed growth curves in low-serum (2.5%) conditions. As shown in Fig. 4B, the stimulation of BHT101 with SDF-1\(\alpha\) increased their proliferation rate, and AMD3100 reverted this effect. SDF-1\(\alpha\) was also able to increase the proliferation rate of three different ATC cell lines, KAT4, CAL62, and ARO, which express CXCR4, but was unable to do so on FB1 cells, which we previously reported to be devoid of CXCR4 (Fig. 4B). AMD3100 alone did not have any effect on ATC cells (data not shown).

To exclude off-target effects of AMD3100 and to directly determine the role of CXCR4 on ATC cell proliferation, we used small duplex RNA oligos to knock down CXCR4. CXCR4 RNA interference was verified by Western blot analysis in BHT101 cells (Fig. 5A). We then transfected CXCR4 siRNAs into BHT101, KAT4, CAL62, and 8505C cells. CXCR4 silencing substantially impaired SDF-1\(\alpha\)-induced S-phase entry in all the ATC cells but had no effect on BrdUrd incorporation in the absence of the chemokine, as shown in Fig. 5. When we used the control scrambled siRNA, this inhibitory effect was not observed. Furthermore, scrambled oligos had no effect on CXCR4 protein levels (Fig. 5A).

**AMD3100 inhibits ATC tumor formation in nude mice.** It has been previously shown that the CXCR4/SDF-1 axis plays an important role in the growth and in the metastatic ability of several epithelial cancers (20). Because we had shown that CXCR4 inhibition blocked SDF-1\(\alpha\)-mediated ATC cell growth in culture, and because it has been shown that this chemokine is secreted by stromal tumoral cells (34), we reasoned that SDF-1\(\alpha\)-CXCR4 axis blockade by AMD3100 might inhibit ATC tumor growth. To this aim, we selected BHT101, ARO, and KAT4 cells for their ability to respond to SDF-1\(\alpha\) and their ability to form tumors in vivo with high efficiency. Nude mice were injected s.c. with 5 \(\times\) 10\(^6\) cells. When tumors measured \(\sim 40\) mm\(^3\), mice (\(n = 20\) for each cell line) were randomized to receive AMD3100 (1.25 mg/kg/twice daily i.p.) or vehicle 5 days per week for 3 to 4 weeks. Tumor diameters were measured at regular intervals with caliper. After 21 days, the mean volume of BHT101 tumors in mice treated with AMD3100 was 48 mm\(^3\), whereas that of mice treated with vehicle was 620 mm\(^3\). Representative experiments are shown in Fig. 6A and B. Tumors induced by ARO and KAT4 reached the volume of 40 mm\(^3\) in only 1 week. In addition, in this case, AMD3100 was able to inhibit tumor growth, although to a lesser extent. In fact, ARO-induced tumor mean volume at the end of treatment with AMD3100 was 220 mm\(^3\), whereas that of mice treated with vehicle was 625 mm\(^3\). Similar results were also obtained when KAT4 cells were used. In this case, the difference between the mean volume of AMD3100-treated versus vehicle-treated tumors was not statistically significant after 3 weeks. However, when treatment
was extended for 1 additional week, AMD3100-treated tumor mean volume was 180 mm$^3$, whereas that of mice treated with vehicle was 690 mm$^3$, and the $P$ value was 0.039 (Fig. 6A). These data, taken together, show that treatment with AMD3100 strongly inhibits ATC tumor growth.

Discussion

Despite ATC is a rare disease, it is one of the most aggressive human cancers (9). Although various therapeutic strategies have been exploited to slow down the growth of this tumor, none of these treatments improved survival (10). The molecular pathways implicated in this disease are poorly understood, and this hampers the application of novel rational therapeutic strategies. Genetic alterations found in ATC are inactivating mutations of the $p53$ tumor suppressor and activating mutations of $\beta$-catenin, RAS, BRAF, and PIK3CA (11). Recently, molecular genetic alterations of FHIT have been also detected in ATC (35). Among the genes involved in ATC, BRAF serine-threonine kinase has been exploited as a potential therapeutic target (23).

Most epithelial cancers feature high levels of expression of the chemokine receptor CXCR4 (20). This receptor has been widely

![Figure 4](image-url)
studied because its expression contributes to several phenotypes of cancer cells, such as the ability to grow, survive, and spread throughout the body. On the contrary, most epithelial cancers do not express SDF-1, the unique CXCR4 ligand, whereas SDF-1 is produced in high amounts in specific body districts. It has been suggested that the role of this chemokine in cancer is mainly to attract cancer cells to these districts (18). In support of this hypothesis, it has been shown that SDF-1 is produced in several metastatic sites. Recently, it has also been suggested that tumoral stroma secretes high amounts of SDF-1, supporting the concept that this chemokine is pivotal in sustaining local protumorigenic events, such as growth and survival of cancer cells (34). Furthermore, the expression of SDF-1 by stromal cancer cells directly recruits endothelial progenitors that are required for tumor angiogenesis (19). As most epithelial and hematopoietic malignancies, also thyroid cancer expresses high levels of CXCR4.

We previously reported functional expression of CXCR4 in human papillary thyroid cancer (22). Furthermore, Hwang et al. (21) showed that an anaplastic cell line, ARO, expressed high levels of functional CXCR4. In this report, we analyzed human ATC samples for CXCR4 expression. We also screened a large panel of human ATC established and primary cell cultures for CXCR4 expression. We show that, both at the mRNA and at the protein level, this receptor is overexpressed in ATC with respect to normal thyroid samples. In contrast, SDF-1 was not detected. The molecular mechanisms underlying CXCR4 up-regulation in ATC are currently unknown. Because we had previously shown that CXCR4 expression was under the control of the RET/PTC-RAS-BRAF-ERK pathway in PTCs (22), and because this pathway is also activated in ATC, we asked whether CXCR4 expression correlated with the BRAF status in ATC. The ATC cell lines that we used in this study had been previously characterized for BRAF mutations.

Figure 5. A, CXCR4 RNA interference was used to transiently suppress CXCR4 expression in BHT101 cells. BHT101 cells were transfected with siRNAs against CXCR4 (siRNA CXCR4) or control nonspecific small duplex RNA containing the same nucleotides, but in scrambled fashion (siRNA SCR), and harvested 48 and 72 h later. Protein lysates were subjected to immunoblotting with anti-CXCR4 and anti-tubulin antibodies. Control siRNA did not affect CXCR4 protein levels. B, CXCR4 RNA interference (siRNA CXCR4) in BHT101 cells inhibited SDF-1α–stimulated S-phase entry as evaluated by BrdUrd incorporation assay. Control siRNA (siRNA SCR) did not inhibit DNA synthesis. Unstimulated BHT101 cells were not affected by siRNA transfection. C, CXCR4 RNA interference (siRNA CXCR4) inhibited S-phase entry in SDF-1α–stimulated KAT4, CAL62, and 8505C. ATC cells were transfected with siRNAs against CXCR4 (siRNA CXCR4) or control siRNA (siRNA SCR) and harvested 48 h later. Control siRNA did not inhibit DNA synthesis. Unstimulated cells were not affected by siRNA transfection.
Furthermore, human ATC samples were screened for the presence of BRAF mutations. We found that most of the samples expressed CXCR4, and this expression was present in both the BRAF-positive and in the BRAF-negative tumors and cell lines. These data suggest that CXCR4 up-regulation in ATC is not necessarily linked to the BRAF pathway and that it can be possibly achieved through different mechanisms. The mechanisms of CXCR4 up-regulation in cancer thus far described are various and complex. It has been shown that nuclear factor-κB (NF-κB) positively regulates the expression of CXCR4 (36) in breast cancer cells. Interestingly, NF-κB is activated in human thyroid cancer cells (37, 38). Transduction of human thyroid cancer cells with the mutant BRAF(V600E) allele induced an increase in NF-κB DNA-binding activity (39). Thus, it is possible that CXCR4 expression in ATC is sustained by high NF-κB activity, which can be the result either of BRAF activation or of the activation of other still undiscovered pathways.

We also show that the CXCR4 expressed on the ATC cell surface is able to transduce biochemical signals into the cell. Indeed, stimulation of ATC cells with recombinant human SDF-1α activated ERK1/2 and, less consistently, AKT pathways in ATC cells. Moreover, we found that SDF-1α stimulated cell growth of different ATC cell cultures, which was inhibited by the small CXCR4 inhibitor AMD3100. Given the high rate of mortality of this cancer and the lack of effective therapies, we focused our efforts in the identification of novel potential therapeutic targets in ATC. We found that the treatment with AMD3100 significantly suppressed the development of tumors in different xenograft models of ATC cells in nude mice.

The more dramatic biological effects of CXCR4 inhibition observed in the animals with respect with those observed in cell culture could be explained by the fact that SDF-1 can act, in tumor microenvironment, at multiple levels. Indeed, tumoral stromal cells, such as fibroblasts and bone marrow–derived cells, express high levels of SDF-1 (34), which can directly enhance the growth of epithelial tumoral cells and can recruit endothelial progenitors, thus favoring angiogenesis. However, when we analyzed xenograft tumors for CD31-positive tumor capillaries, we found that there were no differences in vessel density of AMD3100-treated versus untreated tumors. Preliminary data suggest that AMD3100 activity in xenografts correlates better with a proapoptotic than with an antiproliferative activity. Our findings are in accord with previous reports about the use of CXCR4 inhibitors in brain tumor models (40, 41). Although treatment of ATC xenografts with AMD3100 did not induce a complete regression of tumors, we observed a strong reduction in growth rate, which was more dramatic in the case of BHT101 xenografts. It is conceivable that the combination of conventional anticancer therapies with CXCR4 targeting would display a stronger antineoplastic effect. Given the strong antitumor activity of AMD3100, newer-generation compounds have been developed, such as AMD3465. This compound differs from the bicyclam AMD3100 in that it is a monocyclam endowed with greater solubility in water, higher affinity for CXCR4, and a potent antitumor activity (41). Although these compounds are effective in inhibiting various cancers, long-term sustained dosing of

Figure 6. A, antitumorigenic effects of AMD3100 in ATC cell xenografts. BHT101, ARO, and KAT4 cells (5 x 10⁶ per mouse) were injected s.c. into the right dorsal portion of BALB/c athymic mice. When tumors measured 40 mm³, mice were randomized to two groups (10 mice per group) to receive AMD3100 or vehicle (PBS) by i.p. injection. Treatment was given for 5 consecutive days per week for 3 to 4 wk (day 1 is the treatment starting day). Tumor diameters were measured with calipers and tumor volumes were calculated. Unpaired Student’s t test (normal distributions and equal variances) was applied. All P values were two sided, and differences were statistically significant at P < 0.05. B, tumors were excised and photographed. Two representative examples of BHT101 xenografts are shown.

6 F. Basolo and P. Faviana, unpublished observations.
7 V. Guarino et al., unpublished observation.
AMD3100 displayed a certain toxicity (42). For this reason, further studies, aimed at understanding the effects of long-term administration of CXCR4 inhibitors, must be pursued. Despite these considerations, our data, together with several other reports, strongly indicate that the inhibition of this pathway should be actively evaluated as a novel anticancer therapy.

In conclusion, in this report, we identify CXCR4 as another potential target of ATC anticancer therapy and suggest that AMD3100, or other specific CXCR4 inhibitors, should be developed and tested for the therapy of human ATC.

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