Introduction

Cancers of the larynx and hypopharynx are common head and neck malignancies, constituting ~20% of all tumors. Among them, squamous cell carcinomas (SCCs) are the most common, accounting for over 90% of cancers in this region (1). Lymph node (LN) metastasis is the lungs and LNs. There is growing evidence that CXCR4 plays a crucial role in promoting metastasis of cancer cells to tissues that strongly express CXCL12 (5,9,15–18). LNs strongly express CXCL12, protein and use chemokines to metastasize in specific target organs, such as the hematopoietic cells (5,8–13).

These authors contributed equally to this work.

Abbreviations: AP, activator protein; ERK, extracellular signal-regulated kinase (ERK)1/2 pathway and its downstream transcription factor, activator protein (AP)-1.

Results indicate that LHSCCs strongly express CXCR4. CXCL12 treatment enhances LHSCC cell migration and chemoinvasion. Reverse transcription–polymerase chain reaction (PCR) and western blot analysis show matrix metalloproteinase (MMP)-13 to be a downstream effector gene of CXCL12/CXCR4 signaling. In addition, MMP-13 upregulation is mediated by the extracellular signal-regulated kinase (ERK)1/2 pathway and its downstream transcriptional factor, activator protein (AP)-1.

Materials and methods

Cell culture

HEp-2 (ATCC CCL-23, laryngeal SCC) and FaDu (ATCC HTB-43, hypopharyngeal SCC) cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown and maintained in Eagle’s minimal essential medium with Eagle’s basic salt solution and 2 mM L-glutamine (Eagle’s minimal essential medium) supplemented 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator with 5% CO2.

Immunohistochemistry

Paraffin sections were dewaxed and pretreated in 0.01 M sodium citrate buffer (pH 6.0) for 20 min at 95°C to unmask tissue antigens. These sections were then incubated with 1% hydrogen peroxide in methanol for 15 min at room temperature to block endogenous peroxidase; they were then incubated with phosphate-buffered saline containing 5% normal goat serum for 30 min to block any non-specific reactions. Immunostaining was performed with anti-CXCR4 (1:100 dilution; clones 12G5, R&D Systems) or anti-CXCL12 antibodies (100 ng/ml; R&D Systems) in a humidified incubator with 5% CO2.

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CXC12/CXCR4 promotes laryngeal and hypopharyngeal squamous cell carcinoma metastasis through MMP-13-dependent invasion via the ERK1/2/AP-1 pathway

Ching-Ting Tan1, Chia-Yu Chu2,3, Ying-Chang Lu1,3, Cheng-Chi Chang3, Been-Ren Lin3, Hsiao-Hui Wu1, Hsin-Ling Liu1, Shih-Ting Cha3, Ekambaranellore Prakash3, Jenq-Yuh Ko1,5, and Min-Liang Kuo1,3

1Department of Otolaryngology, 2Department of Dermatology, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei 100, Taiwan and 3Laboratory of Molecular and Cellular Toxicology, Institute of Toxicology, College of Medicine and Angiogenesis Research Center, National Taiwan University, Taipei 100, Taiwan

To whom correspondence should be addressed. Tel: +886 2 23123456 ext. 5222; Fax: +886 2 2341 0905; Email: jyko@ntu.edu.tw

Correspondence may also be addressed to Min-Liang Kuo.

Email: kuominliang@ntu.edu.tw

Cancers of the larynx and hypopharynx are common head and neck cancers with a high propensity for lymph node (LN) and lung metastasis. Here, we report that LHSCCs express high levels of functional CXCR4 receptors, native for chemokine stromal cell-derived factor-1 (SDF-1/CXCL12). Primary tumor immunohistochemistry from LHSCC patients has revealed significant expression of CXCL12 and CXCR4 in LHSCC cell lines. Greater expression of CXCR4 but not that of CXCL12 is strongly expressed in lymphatic vessels and vascularized LN and distant metastasis. Reverse transcription–polymerase chain reaction and western blots have demonstrated that CXCR4 messenger RNA (mRNA) and protein were always detected in LHSCC cell lines as well, but failed to detect CXCL12 mRNA expression. CXCL12 treatment increased extracellular signal-regulated kinase (ERK) pathway activation and the motility/invasiveness of LHSCC cell lines, which were blocked by treatment with a CXCR4 antagonist (AMD3100) and a specific MEK inhibitor (U0126). Results show that the mRNA and protein levels of matrix metalloproteinase (MMP)-13, but not MMP-2 or MMP-9, were elevated in HEP-2 cells in response to CXCL12. Again, U0126 almost inhibited the induction of MMP-13 in HEP-2 cells by stimulating CXCL12. The transcriptional factor, c-Jun, a downstream component of the ERK pathway, was found to be readily phosphorylated and translocated to the nucleus after 10 min of exposure to CXCL12. Blockage of c-Jun activity by transfection with c-Jun antisense oligodeoxynucleotide significantly decreased CXCL12-induced MMP-13 expression and cell invasion. CXCL12 seems to enhance LHSCC cell invasion through paracrine-activated CXCR4, which triggers ERK/c-Jun-dependent MMP-13 upregulation.

Reference

To whom correspondence should be addressed. Tel: +886 2 23123456 ext. 5222; Fax: +886 2 2341 0905; Email: jyko@ntu.edu.tw

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Email: kuominliang@ntu.edu.tw

Laryngeal and hypopharyngeal squamous cell carcinomas (LHSCCs) are common head and neck cancers with a high propensity for lymph node (LN) and lung metastasis. Here, we report that LHSCCs express high levels of functional CXCR4 receptors, native for chemokine stromal cell-derived factor-1 (SDF-1/CXCL12). Primary tumor immunohistochemistry from LHSCC patients has revealed significant expression of CXCL2 and CXCR4 in LHSCC cell lines. Greater expression of CXCR4 but not that of CXCL2 is correlated with LN and distant metastasis. Reverse transcription–polymerase chain reaction and western blots have demonstrated that CXCR4 messenger RNA (mRNA) and protein were expressed in LHSCC cell lines as well, but failed to detect CXCL2 mRNA expression. CXCL12 treatment enhanced extracellular signal-regulated kinase (ERK) pathway activation and the motility/invasiveness of LHSCC cell lines, which were blocked by treatment with a CXCR4 antagonist (AMD3100) and a specific MEK inhibitor (U0126). Results show that the mRNA and protein levels of matrix metalloproteinase (MMP)-13, but not MMP-2 or MMP-9, were elevated in HEP-2 cells in response to CXCL12. Again, U0126 almost inhibited the induction of MMP-13 in HEP-2 cells by stimulating CXCL12. The transcriptional factor, c-Jun, a downstream component of the ERK pathway, was found to be readily phosphorylated and translocated to the nucleus after 10 min of exposure to CXCL12. Blockage of c-Jun activity by transfection with c-Jun antisense oligodeoxynucleotide significantly decreased CXCL12-induced MMP-13 expression and cell invasion. CXCL12 seems to enhance LHSCC cell invasion through paracrine-activated CXCR4, which triggers ERK/c-Jun-dependent MMP-13 upregulation.

Reference
simultaneously performed by two investigators who were unaware of clinico-pathological features of the patients. Specimens were regarded as positive when staining intensity was either moderate (focal expression in 5–20% of tumor cells) or strong (diffuse expression in >20% of tumor cells) and negative when the intensity was negative or weak (focal expression in <5% of tumor cells).

Several serial specimens that are immunopositive for CXCR4 were stained with anti-CD34 antibody (R&D Systems) to identify vascular endothelial cells. We exposed several tissue specimens to non-specific mouse IgG primary antibody to confirm the specificity of the immunohistochemistry results.

Reverse transcription–PCR
Total RNA extraction from homogenized pieces of fresh, frozen cancer tissue specimens and cultured cells was performed with Trizol (Life Technologies, Eggenstein, Germany) following the acid guanidium thiocyanate-phenol-chloroform method. The cDNA was synthesized through random priming from 1 mg of total RNA with the aid of a First-Strand cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer’s instructions. For the PCR, 2 µl of cDNA solution was mixed with 2 µl of a specific primer (20 µM each), 5 µl of 103 reaction buffer, 10 µl of 1 µM deoxyribonucleotide triphosphate mix, 0.5 µl of Taq DNA polymerase and 28.5 µl of double distilled water for a total volume of 50 µl. PCR was performed in a Perkin-Elmer Thermal Cycler (Norwalk, CT); primers used for the amplification of CXCL12, CXCR4 and MMPs are specified below (20,21). Amplification consisted of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s (33 cycles). Ten microliter of PCR products were separated on 2.5% w/v agarose gels and stained with ethidium bromide. The messenger RNA (mRNA) levels were quantified by densitometry and corrected for the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA in the same RNA samples. Sense primers: CXCR4, 5′–CGCCGCTCTGCTAGGAGGAGGAA–3′; MMP-2, 5′–CTG- CTTAGCCCATCTTACTGC–3′; MMP-9, 5′–TGGCAGGACAGTGAA–3′; CXCL12, 5′–CGCCGCTCTGCTAGGAGGAGGAA–3′; MMP-2, 5′–CTG- CTTAGCCCATCTTACTGC–3′; MMP-9, 5′–TGGCAGGACAGTGAA–3′; CXCL12, 5′–GGCCGCTCTGCTAGGAGGAGGAA–3′; MMP-9, 5′–TGGCAGGACAGTGAA–3′; CXCL12

Quantitative real-time PCR
Real-time quantitative PCR was performed using the Real-Time PCR System 7000 (Applied Biosystems, Warrington, UK). Briefly, the PCR amplification reaction mixtures (20 µl) contained cDNA, primer pairs which labeled with dual-labeled fluorogenic probe and TaqMan Universal PCR Master Mix (Applied Biosystems). The thermal cycle conditions included maintaining the reaction at 94°C for 2 min and at 95°C for 10 min and then alternating for 50 cycles between 95°C for 15 s and 66°C for 1 min. The CXCR4 and CXCL12 probe and primer pairs were obtained from Applied Biosystems. The relative gene expression for each sample was determined using the formula 2−(ΔCt/ΔCt) (target), which reflected the target gene expression normalized to actin levels.

Western blot analysis
The expression of CXCL12, CXCR4 and MMP-13 by LHSCC cells was determined by western blot analysis. Equal aliquots of the conditioned cell media were fractioned on 10% sodium dodecyl sulfate ± polyacrylamide gel electrophoresis and transferred to Hybond ECL filter (Amersham, Little Chalfont, UK). The amounts of CXCL12, CXCR4 and MMP-13 were determined using specific primary antibodies with peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (Amersham). The band densities were standardized by band intensities of β-actin or α-tubulin in each experiment as indicated.

Results
Expression of CXCR4 and CXCL12 proteins in LHSCC tissue was examined by immunohistochemical analysis of 30 LHSCC tissue samples. Positive staining of CXCR4 and CXCL12 was observed in 16 (53.5%) and 12 (40.0%) samples, respectively. Cancerous and non-cancerous LHSCC tissue regions positively stained with CXCR4 and CXCL12 are shown in Figure 1A. CXCR4 proteins were located in the cytoplasm and/or cell membranes of cancer cells, but not in the normal stromal cells of non-cancerous LHSCC tissue (Figure 1A, 1 and II). Negative or weak CXCR4 protein staining was observed in the majority of infiltrating inflammatory cells, whereas strong CXCL12 protein staining was identified not only in the cytoplasm of stromal cells and lymphocytes adjacent to cancer cells.
CXCL12/CXCR4 promotes LHSCC metastasis

Table I. Relationship between CXCL12/CXCR4 expression and clinicopathological features

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>CXCR4 positive (%)</th>
<th>P-value</th>
<th>CXCL12 positive (%)</th>
<th>P-value</th>
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<tbody>
<tr>
<td>LN metastases</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>11</td>
<td>2 (18.2)</td>
<td>0.003</td>
<td>2 (18.2)</td>
<td>0.06</td>
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<tr>
<td>Positive</td>
<td>19</td>
<td>14 (73.7)</td>
<td></td>
<td>10 (52.6)</td>
<td></td>
</tr>
<tr>
<td>Distant metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>24</td>
<td>10 (41.7)</td>
<td>0.01</td>
<td>8 (33.3)</td>
<td>0.14</td>
</tr>
<tr>
<td>Positive</td>
<td>6</td>
<td>6 (100)</td>
<td></td>
<td>4 (66.7)</td>
<td></td>
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<tr>
<td>Recurrence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>22</td>
<td>12 (54.5)</td>
<td></td>
<td>9 (40.9)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>4 (50.0)</td>
<td>0.82</td>
<td>3 (37.5)</td>
<td>0.87</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>16 (53.5)</td>
<td></td>
<td>12 (40.0)</td>
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</tbody>
</table>

CXCL12-enhanced migration and invasion of CXCR4-positive LHSCC cells

In compliance with the above data, we clearly detected CXCR4, but not its ligand, mRNA or protein in both LHSCC cell lines (HEp-2 and FaDu), either in serum-free or normal culture conditions (Figure 2A). We treated both the cell lines with CXCL12 and examined their changes in migration and invasiveness using modified Boyden chamber assay coating with Matrigel. CXCL12 treatment resulted in a dose-dependent increase in migration and extracellular matrix invasion of both LHSCC cell lines (Figure 2B and C). Pretreatment with a CXCR4 inhibitor, AMD3100, significantly blocked CXCL12-induced migration and invasion of LHSCC cells (Figure 2D and E). To exclude the possibility that CXCL12-enhanced migration and invasion stemmed from increased cell proliferation, we counted the number of viable cells at different times during CXCL12 incubation (up to 72 h). Figure 2F shows that treatment with exogenous CXCL12 did not influence the proliferation rates of HEp-2 and FaDu cells.

The ERK1/2 pathway is essential to CXCL12/CXCR4 functions in LHSCC

Mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase/Akt are involved in CXCL12 signaling in SCC cells (25–29). To explore the signaling pathway involved in CXCL12-induced enhancement of migration/invasion in LHSCC cells, we examined the activation of ERK1/2 and Akt by western blot analysis using antibodies specific for their phosphorylated forms. Exposure of HEp-2 cells to CXCL12 (100 ng/ml) rapidly and transiently activated ERK1/2, with a peak at 5 min (Figure 3A). Western blots showed ERK1/2 to be extensively phosphorylated in FaDu cells after 5 min of CXCL12 exposure (Figure 3A). ERK1/2 levels returned to baselines after 60 min of CXCL12 treatment in both cell lines. Said activation was effectively inhibited by treatment with U0126, a specific inhibitor of MEK1 (30). (Figure 3B) However, phosphorylated AKT levels did not change during CXCL12 treatment. U0126, but not LY294002, effectively blocked CXCL12-induced migration and invasion of LHSCC cells (Figure 3C and D), indicating that ERK1/2 activation by CXCL12 is functionally involved in increased cell migration and invasion of LHSCC cells.

CXCL-involved upregulation of MMP-13 in the invasion of LHSCC cells

SCC tumor cells in the tongue express MMP-2, while MMP-13 mRNA has been observed in 57% of laryngeal SCC tissues (31,32).
CXCL12 stimulates an increase in active MMP-9 but not MMP-2 secretion of HNSCC cells (33). Since MMPs have a vital link to extracellular matrix protein degradation and invasion, we determined whether or not these enzymes are crucial to CXCL12/CXCR4-mediated invasion of LHSCCs (34). CXCL12 enhanced the expression levels of both MMP-13 mRNA (Figure 4A) and protein (Figure 4B) in HEp-2 cells, whereas that of MMP-2 and MMP-9 were unaffected. Enzyme-linked immunosorbent assay confirmed that CXCL12-stimulated HEp-2 cells produce significantly more MMP-13 than untreated cells (Figure 4C). CXCL12-stimulated invasion was effectively reduced by a selective MMP-13 inhibitor (CL82198, 10, 25 μM) (Figure 4D). Results indicate that MMP-13 is a downstream effector of CXCL12/CXCR4 axis-mediated effects in LHSCCs.
ERK1/2/AP-1 kinase pathway involvement in CXCL12 modulation of MMP-13-mediated LHSCC cell invasion

Because the MMP-13 promoter region contains a conserved AP-1 transcription factor-binding site, activation of MAPK pathways results in downstream activation of c-Fos (ERK) and c-Jun (c-jun N-terminal kinase). We investigated the role of ERK1/2 in MMP-13 expression (35). To examine the necessity of ERK1/2 activity in CXCL12-elicited expression of MMP-13, we blocked the ERK1/2 pathway by adding U0126 to HEp-2 cells prior to CXCL12 treatment. As shown in Figure 5A, CXCL12 alone induced MMP-13 mRNA and protein expression, whereas U0126 blocked CXCL12-elicited expression of MMP-13 mRNA and protein, indicating that ERK1/2 activation is important to induction of MMP-13 expression by CXCL12. Pretreatment with U0126 (20 μM) for 1 h and analyzed with the CXCL12-induced migration and invasion abilities in both HEp-2 and FaDu cells. Error bars indicate SD. Asterisks indicate significant differences between the two groups (P < 0.05). Data were drawn from three independent experiments.

Discussion

Although local LHSCCs can be effectively controlled by surgical excision and radiotherapy, metastasis to the LNs and lungs significantly decreases the survival rate (2,36). CXCR4 may play an important role in promoting organ-selective metastasis by stimulating tumor metastasis and invasion. This study provides evidence that CXCL12/CXCR4 promotes LHSCC metastasis through the ERK1/2/AP-1 signaling pathway.
adhesion to microvascular endothelial cells and by enhancing the growth of tumor cells under stress (37). Recently, it has been proposed that chemokine receptors are critical in determining the metastatic destination of tumor cells (5,9,16,18,38,39). Uchida et al. demonstrated a possible role for CXCR4 in mediating the dissemination of oral SCCs to the LNs and the ability of CXCL12 for inducing oral SCC migration and stimulating multiple intracellular signaling pathways. CXCR4 was also found to be expressed on the surfaces of soft palatal cancer cells (33).

LHSCC is characterized by an easily detectable LN and lung metastasis and the latter is responsible for the poor survival of LHSCC patients. This study indicates that functional CXCR4 chemokine receptors are expressed in a significant number of LHSCC tissues and that this expression is strongly associated with increased LN and distant metastasis of tumors. To our knowledge, this is the first report to indicate that CXCR4 might be critical to LHSCC metastasis. The data show that CXCR4 expression on the primary tumor is significantly correlated with the incidence of LN and distant metastasis. Patients prone to metastasis can be easily identified by biopsy staining with a CXCR4 antibody. Combining the anti-CXCR4 regimen (such as AMD3100) with cytotoxic anticancer drugs may prolong the survival of the said patients.

Our results show that stimulation of LHSCC cells by CXCL12 triggers migration and invasion, suggesting that the CXCL12/CXCR4 axis is an important mediator of the migration and chemoinvasion of LHSCC cells. AMD3100, a bicyclam, is a novel agent that uniquely inhibits the entry of human immunodeficiency virus type 1 into CD4 T cells by selectively blocking the chemokine CXCR-4 receptor (40). The CXCL12-induced effect can be antagonized by AMD3100. Although previous observations have shown that CXCL12 enhances proliferation of some cells in culture, we did not detect any changes in LHSCC cell proliferation when incubated with CXCL12 (up to 72 h) (20–22,41,42). CXCL12 has a specific effect on migration and chemoinvasion of LHSCC cells that does not stem from enhanced proliferation.

In vivo, CXCL12 is secreted by stromal cells within destination tissues for LHSCC cell metastasis, such as the lung and LNs (5,19). Therefore, stromal cell-derived CXCL12 can activate CXCR4 chemokine receptors on LHSCC cells. Interestingly, this study shows that 40% of our tumor cells were also positively stained by anti-CXCL12 antibodies. Unlike oral SCC cells, which do not express CXCR12, expression of CXCL12 in LHSCC tumor cells suggests that there are not only paracrine but also possible autocrine effects by CXCL12 on the LHSCC metastasis.

This study found CXCL12 treatment to induce a robust, transient phosphorylation of ERK1/2/MAPK. Inhibition of ERK1/2 phosphorylation reduces CXCL12-induced migration and chemoinvasion, suggesting that activation of ERK1/2 is an important step in the signal that leads to increased migration and chemoinvasion. However, unlike the oral squamous cell carcinoma results, the phosphorylated form of Akt/PKB was not upregulated by CXCL12 in LHSCC cells. Moreover, this study shows that PI3K is probably not involved in the CXCL12-mediated effects on migration and invasion of LHSCC cells.

MMPs have been implicated to facilitate cancer cell invasion and metastasis through degradation of surrounding ECM proteins. Expression of MMPs and other extracellular proteinases has been shown to positively correlate with the progression of cancer in patients (23,43).

Fig. 4. The role of MMPs in CXCL12-induced invasion of LHSCC cells. HEp-2 cells were incubated in serum-free medium for 24 h and treated with rhCXCL12 (100 ng/ml) for the indicated periods of time. (A and B) Expression of MMP-13 mRNA and protein in HEp-2 cells stimulated by rhCXCL12. MMP-2 and MMP-9 were also detected. (C) Secreted MMP-13 was assessed using an enzyme-linked immunosorbent assay kit (R&D Systems) according to the manufacturer’s protocols and increased by rhCXCL12 (100 ng/ml) in HEp-2 cells. (D) Invasiveness of HEp-2 cells in Matrigel toward serum-free medium, containing either a control mouse IgG1 (10 μg/ml) or a selective MMP-13 inhibitor (CL82198, 10, 25 μM), was determined in a 24 h invasion assay. MMP-13 inhibitor blocked the CXCL12-induced cell invasion in HEp-2 cells. Error bars indicate SD. Asterisks indicate significant differences between the two groups (P < 0.05). Similar results were observed in three independent experiments.
Incubation of oropharyngeal SCC cells with CXCL12 stimulates an increase in active MMP-9 secretion (33). Kawamata et al. (44) reported that a large amount of matrix degrading enzymes, produced by oral SCC cells, contributes to tumor invasion and that the net activity of matrix metalloproteinase (MMP-2) (active MMP-2/tissue inhibitor of MMP-2) contributes to LN metastasis in a nude mouse orthotopic inoculation model. Bogusiewicz et al. (45) found that MMP-2 and MMP-9 may be involved in the expansion of laryngeal cancer, whereas MMP-2 may also play an important role in the lymphatic spread of some laryngeal tumors. MMP-13 has also been found in laryngeal SCC and its expression is correlated with local invasiveness (32).

Although LHSCC and all oral and oropharyngeal SCC are all HNSCCs, they have different MMP expression responses to CXCL12 stimulation. Our results demonstrate that MMP-13, but not MMP-2 or MMP-9, is activated in CXCL12-induced migration and chemoinvasion of HEp-2 cells. (A) Nuclear and cytosolic extracts of CXCL12-treated LHSCC cells were extracted as described in Materials and Methods. Phospho-c-Jun (p-c-Jun), SP-1 and α-tubulin were detected of the nuclei and cytosolic extract in CXCL12-treated cells. (C) HEp-2 cell line was pretreated with U0126 for 30 min and stimulated with CXCL12 (100 ng/ml) for the indicated time (10 min) and nuclear extracts were collected and analyzed with the indicated antibodies. (D) c-jun antisense oligonucleotide blocked the CXCL12-enhanced MMP-13 mRNA and protein production. (E) c-jun antisense oligonucleotide was transfected in HEp-2 cells and detected the CXCL12-induced chemoinvasion abilities. Data are representative of three independent experiments.
CXCL12, indicating that the ERK1/2 MAPK pathway is important in MMP-13 expression. MMP-13 is expressed by HNSCC tumor cells and its expression correlates with their invasion capacity (32,48–51). Our results suggest that CXCL12-induced, ERK1/2-mediated MMP-13 expression is involved in LHSCC cellular invasiveness. The interesting observation is the fact that the primary LHSCC tumor cells expressed CXCL12, which is indicative of locally induced CXCL12-mediated MMP-13 expression and its importance in both local invasiveness and metastasis. Previous studies have revealed that the triggering of CXCL12 signaling results in activation of AP-1 through phosphorylation of Ser-727 by ERK1/2 (52); our data demonstrate that CXCL12 activates ERK1/2 in LHSCC cells. Because our previous study of basal cell carcinomas confirmed the involvement of the AP-1 site in CXCL12-upregulated MMP-13 transcription (52), we checked the role of c-Jun in the CXCL12-induced chemoinvasion and found that antisense treatment of c-Jun results in reduction of CXCL12-induced MMP-13 expression and chemoinvasion. Our results indicate that CXCL12/CXCR4 pathway is important in the metastasis of LHSCC. Signals from CXCR4 receptors may induce MMP-13 activation, resulting in chemoinvasion of LHSCC cells. CXCR4 antagonist AMD3100 blocks the CXCL12-induced migration and chemoinvasion. CXCR4 antagonists and MMP-13 blockers seem promising new therapeutic tools in LHSCC treatment.

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Conflict of Interest Statement
None declared.

References

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