Expression of CXCR4 and Its Down-Regulation by IFN-γ in Head and Neck Squamous Cell Carcinoma

Akihiro Katayama, Takeshi Ogino, Nobuyuki Bandoh, Satoshi Nonaka, and Yasuaki Harabuchi

Abstract

Purpose: The functional expression of CXCR4, which plays roles in cell migration and proliferation in response to its unique ligand stromal cell – derived factor-1 (SDF-1), has been reported in variety of carcinomas. However, CXCR4 expression and its functional role in head and neck squamous cell carcinomas (HNSCC) remain unclear. In this study, we investigated CXCR4 expression and analyzed its functions in HNSCC cell lines. We also attempted to regulate CXCR4 expression using cytokines, such as interleukin-1β, tumor necrosis factor-α, and IFN-γ. Finally, we investigated correlation between CXCR4 expression and clinical features in patients with HNSCC.
Experimental Design: Six HNSCC cell lines were used in this study. Reverse transcription-PCR and flow cytometry analysis were shown for CXCR4 expressions with or without stimulations of cytokines. SDF-1-mediated cell migration was assayed in Matrigel-coated chemotaxis chamber. The SDF-1-mediated cell proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The SDF-1-mediated signaling pathways were analyzed by Western blot analysis. Biopsy specimens from 56 patients with HNSCC were used for immunohistologic analysis.
Results: The significant CXCR4 expression was found in HSQ-89, IMC-3, and Nakamura cells. The SDF-1-mediated cell migration and proliferation were observed in CXCR4-positive cells. SDF-1 also promoted rapid phosphorylation of extracellular signal-regulated kinase 1/2 and Akt signaling pathways in CXCR4-positive cells. The SDF-1-mediated cell migration and proliferation of CXCR4-positive cells were inhibited by neutralization of CXCR4. Among three cytokines tested, IFN-γ significantly reduced CXCR4 expression and SDF-1-induced cell migration and proliferation of CXCR4-positive cells. Immunohistologic analysis revealed that patients with advanced neck status and patients who developed distant metastases showed significantly higher CXCR4 expression, and the cause-specific survival of patients with CXCR4-expression was significantly shorter. Furthermore, multivariate analysis confirmed that CXCR4 positive was the independent factor for cause-specific death.
Conclusion: Our results may provide an insight into future therapeutic agent that inhibits tumor metastasis and progression via down-regulating CXCR4 expression in patients with HNSCC.

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Chemokines are small secreted peptides that control adhesion and transendothelial migration of leukocytes, especially during immune and inflammatory reactions. Chemokines have other functions, including promoting mitosis and modulation of apoptosis, survival, and angiogenesis (1). Stromal cell – derived factor-1 (SDF-1) has potent chemotactic activity for lymphocytes (2). Recently, the physiologic receptor of SDF-1, CXCR4, has been reported to be expressed in wide variety of carcinomas (3 – 11). In such carcinomas, the functional roles of CXCR4 in cell migration (3 – 6, 8 – 10) and/or cell proliferation (7, 11) in response to SDF-1 have been suggested. Furthermore, it is reported that the SDF-1/CXCR4 interaction associated cell migration and/or proliferation are evoked through the extracellular signal-regulated kinase 1/2 (ERK1/2) and/or Akt signaling pathways in several cancer cells (5, 9, 10, 12). However, little is known regarding CXCR4 expression and its functional roles and signaling systems in head and neck squamous cell carcinoma (HNSCC) cells.

The CXCR4 expression may be beneficial not only to cell migration and proliferation but also to metastases of cancer cells. The transplantation of CXCR4-expressing breast cancer cells in mice model leads to development of metastases in the organs that contain relatively high amounts of SDF-1, such as lung, liver, kidney, bone marrow, and lymph nodes (3). Although little is known regarding how cytokines regulate CXCR4 expression in cancer cells, the expression of CXCR4 in leukocytes and epithelial cells is regulated by several cytokines, such as IFN-γ, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β; refs. 13 – 18). However, it seems that the influence of these cytokines on CXCR4 expression may be not uniform.

Authors' Affiliation: Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical College, Asahikawa, Japan
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Requests for reprints: Yasuaki Harabuchi, Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical College, Midorigaoka-Higashi 2-1-1-1, Asahikawa, Hokkaido 078-8510, Japan. Phone: +81-166-68-2554; Fax: +81-166-68-2559; E-mail: hyasu@asahikawa-med.ac.jp.
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In this study, we investigated at first whether HNSCC cells express functional CXCR4 in relation to SDF-1-mediated tumor migration and proliferation *in vitro* and whether the SDF-1/CXCR4 interaction induce ERK1/2 or Akt signaling pathways. Next, we attempted to regulate the CXCR4 expression by cytokines, such as IL-1β, TNF-α, and IFN-γ, *in vitro*. Finally, we investigated expressions of CXCR4 and SDF-1 in biopsy tissues from 56 patients with HNSCC in relation to clinical features and patients' prognosis. The goal of this study is to confirm whether CXCR4 has roles in tumor metastasis and progression, whether the CXCR4 expression has prognostic value, and whether cytokines could be useful for down-regulation of CXCR4 in HNSCC.

Materials and Methods

Cell culture. Six human HNSCC cell lines and Jurkat cells were used in this study. SAS and Jurkat were obtained from the American Type Culture Collection (Manassas, VA). Ho-1-u-1, CA9-22, HSQ-89, IMC-3, and Nakamura cells were provided by Dr. Y. Nishimura (Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan). The cell lines were established from following sites: SAS (19) was from tongue SCC, Ho-1-u-1 (20) from oral floor SCC, CA9-22 (21) and HSQ-89 (22) from gingival SCC, and IMC-3 (23) and Nakamura (24) were from maxillary sinus SCC. The cells were cultured in RPMI 1640 (Life Technologies, Auckland, New Zealand) supplemented with 10% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin (Life Technologies, Inc., Gaithersburg, MD) at 37°C with 5% CO_2.

Tissue samples. HNSCC tissue samples were obtained at pretreatment periods from 56 Japanese patients (43 males and 13 females) with a median age of 58 years (range, 31-79 years) who were treated in Department of Otolaryngology, Asahikawa Medical College (Asahikawa, Japan), between 1983 and 2003. All patients signed informed consent for therapy and tissue studies that had received prior approval by the institutional review board. Clinical and pathologic features of the patients are listed in Table 1. The primary site is the tongue in 43 (77%) patients and oral floor in 13 (23%) patients. According to the 1997 International Union Against Cancer tumor-node-metastasis staging systems (identical to 1997 American Joint Committee on Cancer classification), stage I was in 14 (25%) patients, stage II was in 16 (29%) patients, stage III in 20 (35%) patients, and stage IV in 6 (11%) patients. Nineteen (34%) patients had lymph node metastasis (N_1 in 13 patients, N_{2b} in 5 patients, and N_{2c} in 1 patient) at diagnosis. None of the patients had distant metastasis at diagnosis. The classification of tumor differentiation was well-differentiated SCC type in 34 (42%) patients, moderately differentiated SCC in 18 (34%), and poorly differentiated SCC in 4 (24%). Of 56 patients, 39 (70%) patients were treated with combined therapy that was composed of preoperative radiochemotherapy followed by surgical resection. Thirteen (23%) patients were treated with surgery alone. The remaining 4 (7%) patients were treated with radiotherapy alone. All patients experienced disease-free period. During follow-up periods, 15 (27%) patients developed local recurrence, 12 (21%) developed neck lymph node metastasis, and 12 (21%) patients developed distant metastasis. Twenty-four (43%) patients died during follow-up, 15 (27%) patients died of tumor, and 9 (16%) patients were tumor free and died of intercurrent diseases. Follow-up periods ranged from 3 to 222 months with a median of 60 months.

Reverse transcription-PCR. Total cellular RNA from HNSCC cell lines was extracted with SV Total RNA Isolation System (Promega Corp., Madison, WI). The first-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Gene Hunter, Inc., Nashville, TN) with oligo(dT) primers (Applied Biosystems, Foster City, CA) for 60 minutes at 37°C according to manufacturer's instructions. Oligomer primers were synthesized for *CXCR4* (sense 5′-GGCAGCAGGTAGCAAAGTGA-3′ and antisense 5′-TGATGACAAA-GAGGAGGTCGG-3′), *SDF-1* (sense 5′-TGATCGTCTGACTGGTGTTA-3′ and antisense 5′-CTTAGGGGATTTGGAAGTTT-3′), *β-actin* (sense 5′-ATGGGTCAGAAGGATTCCTATGT-3′ and antisense 5′-TCAGGAGGAG-CAATGATCTTGA-3′), and *IFN-γ receptor 1* (sense 5′-ACGCAGAAGGAA-GATGATTGTGACG-3′ and antisense 5′-TCTATTGGAGTCAGATGG-CTGCCC-3′; Sigma Genosis Japan, Hokkaido, Japan). PCR was done in a 10 μL reaction mixture containing 4.95 μL H_2O, 1 μL PCR buffer (contains 15 mmol/L $MgCl_2$, 10-fold concentration), 1 μL deoxynucleotide triphosphates (2 mmol/L), 1 μL forward primer (2 mmol/L), 1 μL reverse primer 2 (2 mmol/L), 0.05 μL Taq DNA polymerase (5 units/μL, AmpliTaq Gold, Applied Biosystems), and 1 μL cDNA (100 ng/μL). Thirty cycles of denaturation (94°C, 1 minute), annealing (58°C, 1 minute), and extension (72°C, 1 minute) were carried out in a DNA thermal cycler (Programmable Thermal Controller PTC-100, MJ Research, Inc., Waltham, MA). Jurkat cells were used as a positive control for *CXCR4* and human neck lymph node tissue as a positive control for *SDF-1*.

For cytokine stimulation studies, HNSCC cells were preincubated with IFN-γ (0, 100, 250, 1,000, and 2,000 IU/mL, Shionogi & Co., Ltd., Osaka, Japan), TNF-α (0, 50, 100, and 200 ng/mL, Dainihon Pharmaceutical, Osaka, Japan), or IL-1β (0, 1, 10, and 100 ng/mL, PeproTech, London, United Kingdom) for 48 hours; then, reverse transcription-PCR was done.

Table 1. Clinical and histologic features in 56 patients with HNSCC

Characteristics	Cases (%)
Gender	
Female	13 (23)
Male	43 (77)
Age (y)	
Median (range)	58 (31-79)
31-65	42 (75)
65-79	14 (25)
Primary site	
Tongue	43 (77)
Floor of mouth	13 (23)
Clinical stage	
I	14 (25)
II	16 (29)
III	20 (35)
IV	6 (11)
N status	
N_0	37 (66)
N_1	13 (23)
N_{2b}	5 (9)
N_{2c}	1 (2)
Tumor differentiation	
Well	34 (61)
Moderate	18 (32)
Poor	4 (7)
Initial therapy	
Surgery alone	13 (23)
Radiotherapy alone	4 (7)
Combined therapy	39 (70)

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Flow cytometry. Cell suspensions of HNSCC cells were prepared by trypsin EDTA treatment and washed thrice. Cells (1×10^6) were incubated with 100 μL of 10 μg/mL mouse anti-human CXCR4 monoclonal antibody (12G5, R&D Systems, Minneapolis, MN) or 100 μL of 10 μg/mL mouse anti-human IFN-γ receptor 1 monoclonal antibody (92101, R&D Systems) for 60 minutes at 4°C. Mouse IgG2a (Biodesign International, Saco, ME) was used as isotype control. The cells were washed thrice and incubated with phycoerythrin-goat anti-mouse immunoglobulin antibody (R480, DAKO, Glostrup, Denmark) at 1:100 dilution for 30 minutes at 4°C. After another wash, the cells were analyzed on a Coulter EPICS Elite flow cytometer with IMMUNO-4 software (Coulter Corp., Hialeah, FL). All dilutions and washings were done in ice-cold PBS containing 0.1% sodium azide and 0.5% bovine serum albumin. Jurkat cells were used as a positive control for CXCR4.

For cytokine stimulation studies, HNSCC cells lines were preincubated with 500 IU/mL IFN-γ, 100 ng/mL TNF-α, or 10 ng/mL IL-1β for 24, 48, and 72 hours; then, flow cytometry was done.

Migration assay. *In vitro* cell migration was assayed in 24-well cell culture plates using inserts with 8 μm pore membranes (Kurabo, Osaka, Japan; ref. 3). Membrane was precoated with 20 μL per insert of Matrigel (BD PharMingen, Bedford, MA) diluted 1:1 by RPMI 1640. HNSCC cells (1×10^4) were resuspended in 500 μL migration buffer (RPMI 1640/0.5% bovine serum albumin) per well and loaded into the upper compartment of the chamber. The lower compartment of the chamber was loaded with or without 50 ng/mL recombinant human SDF-1 (R&D Systems) in 500 μL migration buffer. After incubation at 37°C for 24 hours, the cells on the lower surface of membrane were fixed in 70% ethanol, stained with Lillie-Mayer's hematoxylin, and counted under microscope (Olympus, Tokyo, Japan; original magnification, ×100). Each value was expressed as a mean number of 10 different fields. Five experiments were done independently and the results were compared.

For neutralization studies, the cells were resuspended in 500 μL migration buffer containing 20 μg/mL anti-CXCR4 monoclonal antibody (12G5) or 20 μg/mL mouse IgG2a isotype control before loading into the upper compartment of the chamber. For IFN-γ stimulation studies, cells were resuspended in 500 μL migration buffer containing 500 IU/mL IFN-γ before loading into the upper compartment of the chamber.

Cell proliferation assay. HNSCC cells were planted 2×10^3 cells per well in 96-well plate for the 24, 48, and 72 hours in 100 μL RPMI 1640 with 0.5% bovine serum albumin (100 μL). Each well was then stimulated with 50 ng/mL SDF-1 for the indicated periods or not stimulated. To determine the number of viable cells, we used the Cell Titer One solution Cell Proliferation Assay (Promega). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (20 μL) was added to each well, and after 2-hour incubation at 37°C, the absorbance at 490 nm was measured by ELISA plate reader (NJ-2300, Nalge Nunc International, Tokyo, Japan). The assay was done 10 times.

For neutralization studies, the cells were incubated with 20 μg/mL anti-CXCR4 neutralizing monoclonal antibody (12G5) or 20 μg/mL mouse IgG2a isotype control. For IFN-γ stimulation studies, the cells were incubated with 500 IU/mL IFN-γ.

Western blot analysis. The cells used in this experiment were starved in serum-free RPMI 1640 overnight to diminish the influences of other cytokines that fetal bovine serum might contain and were stimulated with 50 ng/mL SDF-1 for 0, 5, 15, 30, and 60 minutes at 37°C with 5% CO_2. Cell lysates were prepared by adding 200 μL ice-cold lysis buffer [0.5% Triton X-100, 50 mmol/L Tris (pH 7.2), 140 mmol/L NaCl, 10 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L Na_3VO_4] containing the protease inhibitor cocktail Complete Mini (Roche, Mannheim, Germany). Proteins (20 μg) were run on 4% to 12% Bis-Tris SDS-PAGE gels (Invitrogen, San Diego, CA). Proteins were then blotted onto Immobilon-P (Millipore, Bedford, MA), blocked in 5% skim milk, and probed with rabbit anti-human phospho-ERK1/2 antibody (Thr^{202}/Tyr^{204}), rabbit anti-human ERK1/2 antibody, rabbit anti-human phospho-Akt antibody (Ser^{473}), and rabbit anti-human Akt antibody (Cell Signaling Laboratories, Beverly, MA) followed by sheep anti-rabbit IgG-horseradish peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Enhanced chemiluminescence system (Amersham Pharmacia Biotech) was used for detection.

Immunohistologic staining. Formalin-fixed and paraffin-embedded specimens were obtained from surgical biopsies at the pretreatment period. The specimens were cut in 5 μm sections. The slides were deparaffinized in xylene and ethanol. These sections were then incubated with 3% H_2O_2 for 30 minutes. The slides were placed in 10 mmol/L citric acid buffer containing 0.1% Tween 20 (pH 6.0) and underwent antigen retrieval for 20 minutes at 750 W and 95°C in a

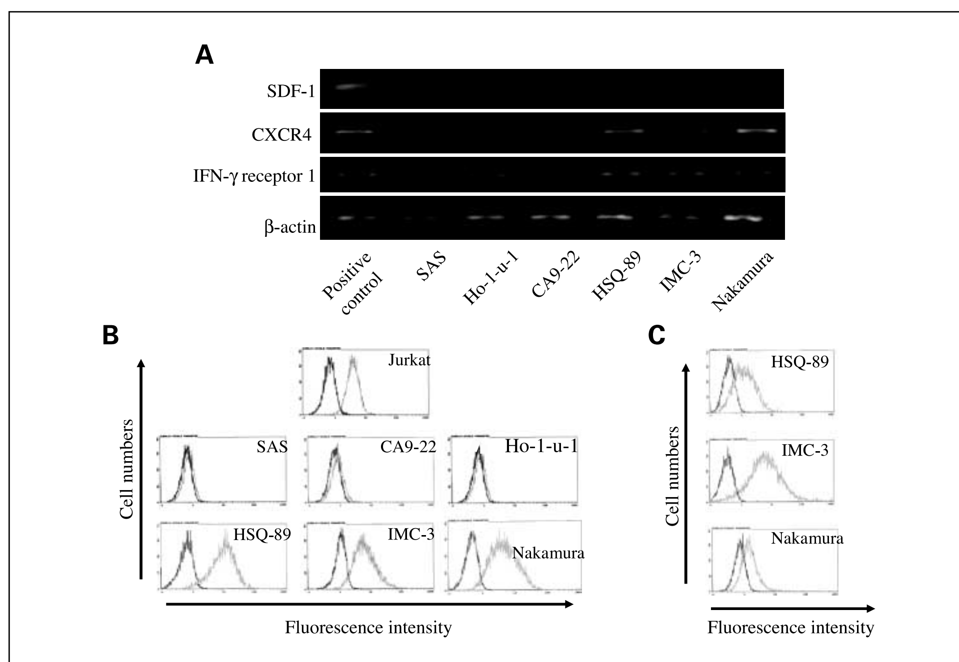

Fig. 1. *SDF-1*, *CXCR4*, and *IFN-γ receptor 1* mRNA expression in six human HNSCC cell lines (*A*). mRNA expression was measured by reverse transcription-PCR. *β-actin* was used as a housekeeping gene. Jurkat cell was used as positive control for *CXCR4* and *IFN-γ receptor 1*. Human neck lymph node tissue was used as positive control for *SDF-1*. Cell surface expression of CXCR4 protein in six human HNSCC cell lines (*gray line*; *B*). Cell surface expression of IFN-γ receptor 1 protein in CXCR4-positive human HNSCC cell lines (*gray line*; *C*). Cell surface expression of proteins was measured by flow cytometry. Cells were also incubated with mouse isotype control IgG2a (*black line*).

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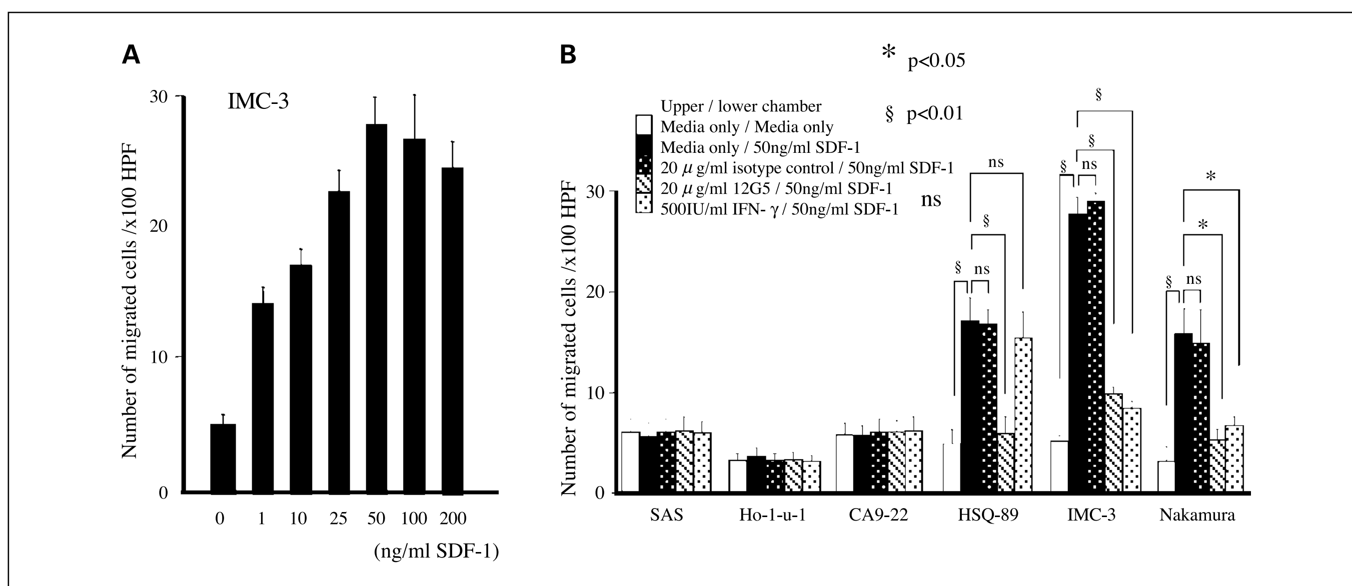

Fig. 2. CXCR4-positive IMC-3 cells migrated to SDF-1 thorough Matrigel-coated chemotaxis membrane in a dose-dependent manner ($P < 0.01$ for each SDF-1 concentration versus migration to control medium; *A*). Optimal migration of IMC-3 cells was induced by 50 to 100 ng/mL SDF-1. SDF-1 promotes migration of HSQ-89, IMC-3, and Nakamura cells thorough Matrigel-coated chemotaxis membrane (*B*). HSQ-89, IMC-3, and Nakamura cells showed significant migration toward 50 ng/mL SDF-1 ($P < 0.01$). This migration was significantly inhibited by treatment of the cells with 20 μg/mL anti-CXCR4 neutralizing monoclonal antibody (12G5) in the upper chamber ($P < 0.01$, $P < 0.01$, and $P = 0.015$, respectively). Mouse isotype control IgG2a did not influence the SDF-1-mediated cell migration. Incubation with 500 IU/mL IFN-γ in upper chamber significantly inhibited migration toward SDF-1 in IMC-3 and Nakamura cells ($P < 0.01$ and $P = 0.025$, respectively). Representative of five experiments. Columns, mean of 10 determinations; bars, SD. Mann-Whitney *U* test was used to determine the *P*s.

microwave oven. Goat anti-human CXCR4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution and mouse anti-human SDF-1 antibody (25 μg/mL, GT, Minneapolis, MN) were used. These primary antibodies were incubated overnight at 4°C followed by incubation with peroxidase-labeled dextran polymers, Simple Stain MAX (Nichirei, Tokyo, Japan) for CXCR4 stain and EnVision+ (DAKO A/S, Carpinteria, CA) for SDF-1 stain, for 30 minutes at room temperature (25). They were visualized by immersing the slides in freshly prepared 0.02% 3,3′-diaminobenzidine solution for 10 minutes. The sections were finally counterstained with Lillie-Mayer's hematoxylin and mounted. The sections were examined microscopically by three of the authors (A.K., T.O., and N.B.) without knowledge of clinicopathologic features. The expression of CXCR4 was categorized in four grades according to staining intensities comparing with that of interstitial infiltrates (26): score 3 (strong), staining intensity more than interstitial infiltrates; score 2 (moderate), staining intensity with equal to interstitial infiltrates; score 1 (mild), staining intensity less than interstitial infiltrates; and score 0 (negative), no staining intensity. We also categorized CXCR4 expression according to CXCR4 expression scores: score 1 to 3, CXCR4 positive; score 0, CXCR4 negative.

Statistical analysis. Two groups were compared using Mann-Whitney *U* test and were summarized with their appropriate *P*. Time was defined as the period until cause-specific death or last follow-up. The probabilities of cause-specific survival were calculated using Kaplan-Meier method and compared using log-rank test. For determination of factors related to cause-specific survival, a Cox proportional hazards model was used. The final results of these analyses are hazard ratios, 95% confidence intervals, and *P*. *P*s < 0.05 were considered to be statistically significant.

Results

Expression of CXCR4 and SDF-1 in head and neck squamous cell carcinoma cells. Six different HNSCC cell lines (SAS, Ho-1-u-1, CA9-22, HSQ-89, IMC-3, and Nakamura cells) were evaluated for mRNA expressions of *SDF-1* and *CXCR4* by reverse transcription-PCR. Expression of *SDF-1* mRNA was never found in any HNSCC cell line (Fig. 1A). Expression of *CXCR4* mRNA was found clearly in three HNSCC cell lines (HSQ-89, IMC-3, and Nakamura cells) but not in SAS, Ho-1-u-1, and CA9-22 cells (Fig. 1A).

After identifying *CXCR4* mRNA in three HNSCC cell lines, we did flow cytometry analysis for cell surface expression of CXCR4 protein. Cell surface expression of CXCR4 protein was found in HSQ-89, IMC-3, and Nakamura cells but not in SAS, Ho-1-u-1, and CA9-22 cells (Fig. 1B).

CXCR4 has a role in the SDF-1-mediated migration of head and neck squamous cell carcinoma cells. To investigate the SDF-1-mediated migration of HNSCC cells, we did migration assay. The CXCR4-positive IMC-3 cells migrated to SDF-1 in a dose-dependent manner ($P < 0.01$ for each SDF-1 concentration versus migration to control medium; Fig. 2A). Optimal migration of IMC-3 cells was induced by 50 to 100 ng/mL SDF-1 (Fig. 2A). The other CXCR4-positive HSQ-89 and Nakamura cells also migrated to 50 ng/mL SDF-1 ($P < 0.01$ each), but the CXCR4-negative SAS, Ho-1-u-1, CA9-22 cells did not (Fig. 2B). Pretreatment with anti-CXCR4 antibody significantly inhibited the SDF-1-mediated migration in CXCR4-positive HSQ-89, IMC-3, and Nakamura cells ($P < 0.01$, $P < 0.01$, and $P = 0.015$, respectively) but did not affect CXCR4-negative SAS, Ho-1-u-1, and CA9-22 cells (Fig. 2B). Mouse isotype control IgG2a did not influence the SDF-1-mediated cell migration (Fig. 2B).

CXCR4 has a functional role in proliferation of head and neck squamous cell carcinoma cells. To investigate the SDF-1-mediated proliferation of HNSCC cells, we did proliferation assay under culture condition with SDF-1. The proliferation of the CXCR4-positive IMC-3 cells was significantly enhanced

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by SDF-1 in a dose- and culture time–dependent manner ($P < 0.01$ each; Fig. 3A). Optimal proliferation of IMC-3 cells was induced by 25 to 100 ng/mL SDF-1 after 72 hours of culture (Fig. 3A). Proliferation of the other CXCR4-positive cell line HSQ-89 was also enhanced by 50 μg/mL SDF-1 ($P < 0.01$ each), but the proliferation of CXCR4-negative SAS cell was not affected (Fig. 3B). Mouse isotype control IgG2a did not influence the SDF-1-mediated cell proliferation (Fig. 3B). Cocultivation with anti-CXCR4 antibody significantly inhibited the SDF-1-mediated proliferation in CXCR4-positive cell lines HSQ-89 and IMC-3 at 72 hours ($P < 0.01$, each) but did not affect the CXCR4-negative cell line SAS (Fig. 3B).

CXCR4 acts as a signaling receptor of extracellular signal-regulated kinase 1/2 and Akt pathways in head and neck squamous cell carcinoma cells. To investigate the signaling pathways activated by CXCR4 in response to SDF-1 treatment, we treated CXCR4-positive HNSCC cells HSQ-89 and IMC-3 with SDF-1 and determined phosphorylated forms of ERK1/2 and Akt by Western blot (Fig. 4). The phosphorylated forms of ERK1/2 were detected at a very low level in HSQ-89 and IMC-3 cells without SDF-1 and increased after SDF-1 stimulation quickly in 5 to 60 minutes. Although the phosphorylated form of Akt was not detected in HSQ-89 and IMC-3 cells without SDF-1, phosphorylation was induced by SDF-1 stimulation in 5 to 60 minutes.

IFN-γ down-regulates expression of CXCR4 gene and protein in head and neck squamous cell carcinoma cells. To investigate whether cytokines affect CXCR4 expression in HNSCC cells, we cultured HNSCC cell lines with IFN-γ, TNF-α, or IL-1β and did reverse transcription-PCR analysis for mRNA expressions of *CXCR4*. We have confirmed mRNA expressions of cytokine receptors for IFN-γ (Fig. 1A), TNF-α, and IL-1β (data not shown) on all six HNSCC cell lines (SAS, Ho-1-u-1, CA9-22, HSQ-89, IMC-3, and Nakamura). The cell surface expressions of IFN-γ receptor 1 were detected in both CXCR4-negative cells (data not shown) and CXCR4-positive cells (Fig. 1C). TNF-α and IL-1β did not affect *CXCR4* gene expression at any concentration in all cell lines. IFN-γ did not induce *CXCR4* expression in CXCR4-negative cell lines SAS, Ho-1-u-1, and CA9-22. IFN-γ significantly reduced *CXCR4* gene expression in a dose-dependent manner in CXCR4-positive IMC-3 and Nakamura cells but not in CXCR4-positive HSQ-89 cells at any concentration (Fig. 5A).

Next, we did flow cytometry analysis for change of cell surface CXCR4 expression in IMC-3 and Nakamura stimulated with 500 IU/mL IFN-γ. IFN-γ significantly reduced cell surface CXCR4 expression in a time-dependent manner in both IMC-3 and Nakamura cells (Fig. 5B-E).

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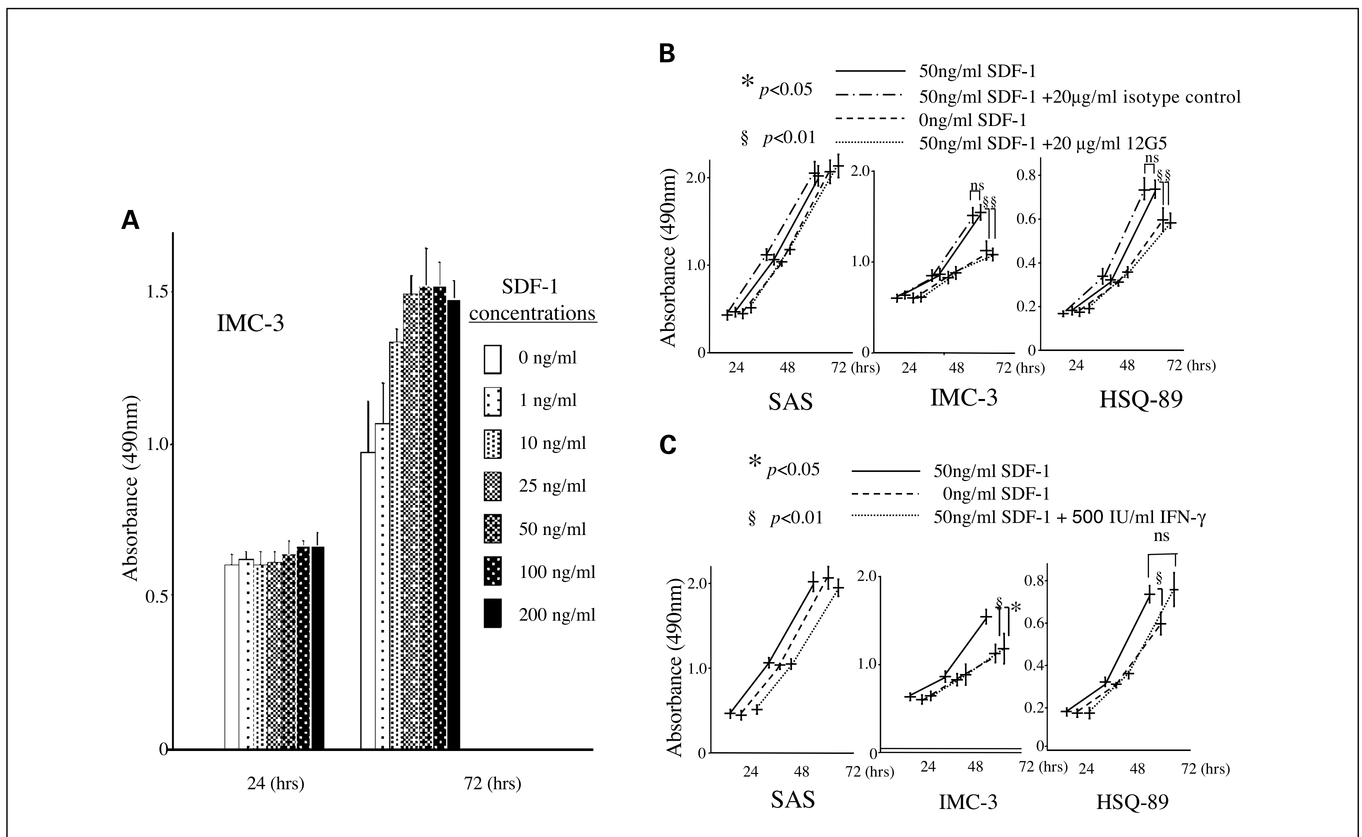

Fig. 3. CXCR4-positive IMC-3 cells significantly proliferated under culture condition with SDF-1 in a dose- and culture time–dependent manner ($P < 0.01$ each; *A*). Optimal proliferation of IMC-3 cells was induced by 25 to 100 ng/mL SDF-1 at 72-hour culture. Columns, mean of 10 experiments; bars, SD. Mann-Whitney *U* test was used to determine the *P*s. Proliferation of SAS, IMC-3, and HSQ-89 cells with or without 50 ng/mL SDF-1 stimulation. SDF-1 stimulation significantly promoted proliferation of IMC-3 and HSQ-89 but not that of SAS cells ($P < 0.01$; *B*). This proliferation was significantly inhibited with addition of anti-CXCR4 neutralizing antibody (12G5; $P < 0.01$). Mouse isotype control IgG2a did not influence the SDF-1-mediated proliferation. IFN-γ induced suppression of proliferation in response to SDF-1 in IMC-3 cell (*C*). The proliferation in response to 50 ng/mL SDF-1 in IMC-3 cell was significantly inhibited by incubation for 72 hours with 500 IU/mL IFN-γ ($P < 0.01$). Points, mean of 10 experiments; bars, SD. Mann-Whitney *U* test was used to determine the *P*s.

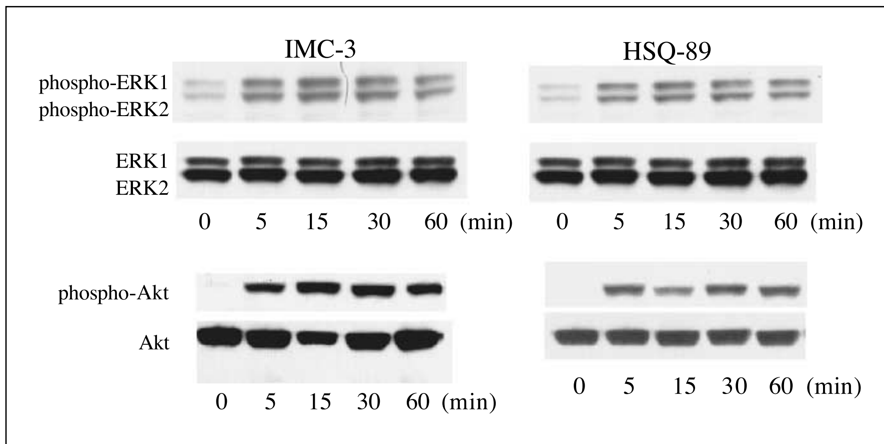

Fig. 4. Signaling effect of SDF-1 in IMC-3 and HSQ-89 cells. Total cell lysates were prepared at various time points after stimulation with 50 ng/mL SDF-1. Western blots were done with 20 μg total protein from IMC-3 and HSQ-89 cells. Membranes were probed for ERK1/2 and its active form phospho-ERK1/2 or probed for Akt and its active form phospho-Akt.

IFN-γ reduces SDF-1-mediated cell migration and proliferation via down-regulation of CXCR4 in head and neck squamous cell carcinoma cells. Because IFN-γ down-regulated CXCR4 expression in IMC-3 and Nakamura cells, we finally investigated whether IFN-γ affects the SDF-1-mediated migration and proliferation in HNSCC cells. In migration assay, cocultivation with 500 IU/mL IFN-γ for 24 hours significantly inhibited the SDF-1-mediated migration in CXCR4-positive IMC-3 and Nakamura cells ($P < 0.01$ and $P = 0.025$, respectively) but did not affect CXCR4-negative cell lines (SAS, Ho-1-u-1, and CA9-22) and CXCR4-positive HSQ-89 cells in which CXCR4 expression was not altered by IFN-γ (Fig. 2B). In proliferation assay, cocultivation with 500 IU/mL IFN-γ for 72 hours significantly inhibited the SDF-1-mediated proliferation of CXCR4-positive IMC-3 cells ($P < 0.05$) but did not affect either CXCR4-negative SAS cells or CXCR4-positive HSQ-89 cells in which CXCR4 expression was not altered by IFN-γ (Fig. 3C).

CXCR4 expression was detected in head and neck squamous cell carcinoma tissues. The CXCR4 expression was not detected

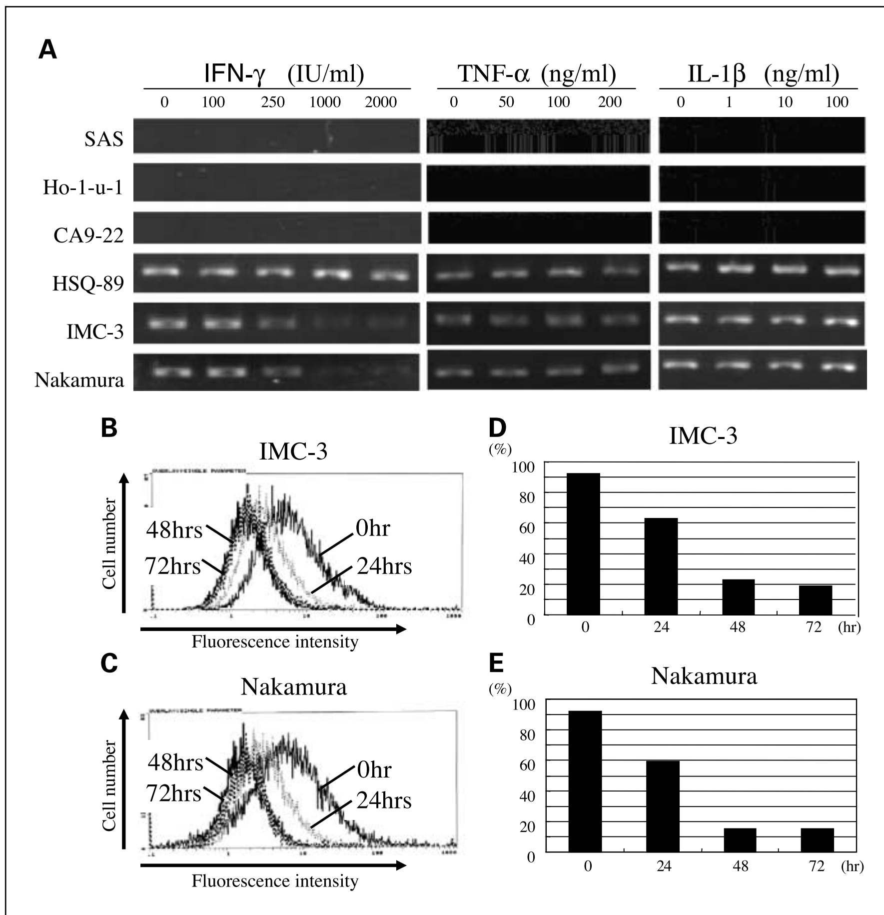

Fig. 5. Alteration of *CXCR4* mRNA expression induced by cytokine stimulation in six human HNSCC cell lines (*A*). Cells were preincubated for 48 hours with IFN-γ, TNF-α, or IL-1β at indicated concentrations before mRNA extraction. *CXCR4* mRNA expression was measured by reverse transcription-PCR. IFN-γ down-regulated *CXCR4* gene expression in IMC-3 and Nakamura cells at >250 IU/mL. Completeness of mRNA was confirmed by using *β-actin* primers (data not shown). Suppression of surface expression of CXCR4 protein induced by IFN-γ in IMC-3 and Nakamura cells (*B-E*). Surface expression of CXCR4 protein was measured on IMC-3 and Nakamura cells cultured in the presence of 50 ng/mL SDF-1. Cells were analyzed using a Coulter EPICS Elite flow cytometer. Histograms of IMC-3 cells (*B*) and Nakamura cells (*C*) in various time points. Percentages of CXCR4-positive IMC-3 cells (*D*) and Nakamura cells (*E*) were also measured in various time points compared with cells treated with IgG2a isotype control. IFN-γ down-regulated CXCR4 protein expression on IMC-3 and Nakamura cells in a time-dependent manner.

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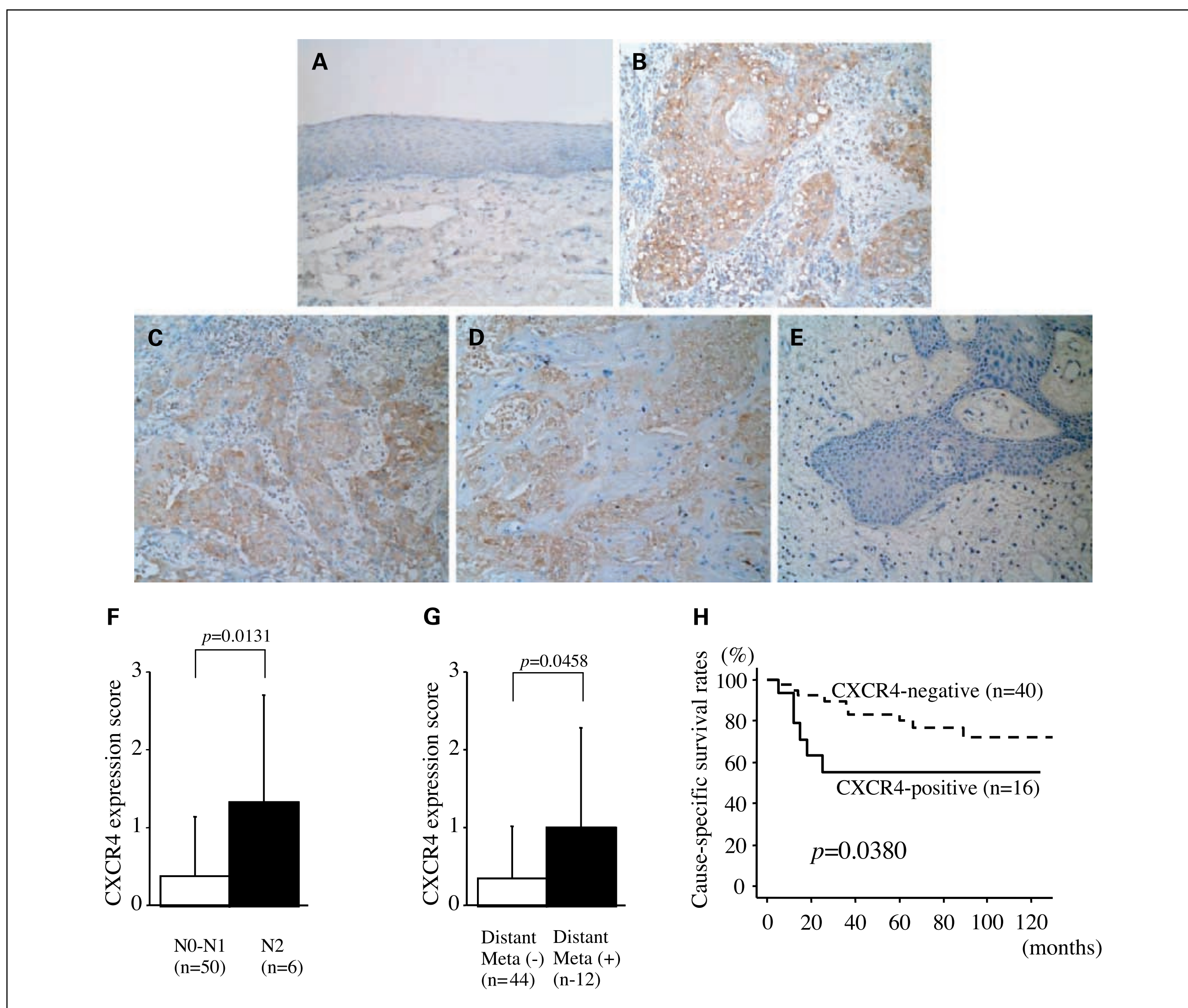

Fig. 6. Representative immunohistologic features. *A*, normal head and neck epithelium did not express CXCR4. *B*, strong (score 3) CXCR4 expression on primary tumor cells. *C*, significant CXCR4 expression on metastatic tumor cells. *D*, strong SDF-1 expression on stromal tissue around tumor nest in metastatic lymph node. *E*, slight SDF-1 expression on stromal tissue around primary tumor. Original magnification, ×200. *F*, CXCR4 expression score of patients with N_2 neck status at diagnoses (*black columns*; $n = 6$; 1.333 ± 1.336) was significantly higher than that of patients with N_0-N_1 neck status (*white columns*; $n = 50$; 0.380 ± 0.753; $P = 0.0131$). *G*, CXCR4 expression score of patients who developed distant metastases (*black columns*; $n = 12$; 1.000 ± 1.279) was also significantly higher than that of patients who did not develop distant metastases (*white columns*; $n = 44$; 0.341 ± 0.680). Columns, mean; bars, SD. Mann-Whitney *U* test was used to determine the *P*s. *H*, cause-specific survival of CXCR4-positive patients was significantly shorter than that of CXCR4-negative patients ($P = 0.0380$). Probabilities of cause-specific survival were calculated using Kaplan-Meier method and compared using log-rank test.

Table 2. Expression score of CXCR4 in 56 patients with HNSCC

CXCR4 expression score	Score 0	Score 1	Score 2	Score 3
Cases (%)	40 (71.4)	8 (14.3)	5 (8.9)	3 (5.4)

NOTE: Intensity of the expressions in carcinoma cells was scored into four grades according to staining intensity compared with that of interstitial infiltrates: score 0, no staining; score 1, weak; score 2, moderate; and score 3, strong.

on normal head and neck epithelium (Fig. 6A). In HNSCC tissues, CXCR4 was mainly expressed on cell surface and cytoplasm of carcinoma cells (Fig. 6B). The CXCR4 expression was also detected on some interstitial infiltrates cells. Tumor cells that metastasized to lymph nodes also expressed CXCR4 strongly (Fig. 6C). SDF-1 expression was not detected on tumor cells but was present at a high level on stromal tissues together with CXCR4-expressing cancer nest in metastatic lymph nodes (Fig. 6D). On the other hand, SDF-1 expression in stromal tissues around primary cancer nests was not or weakly detected (Fig. 6E).

CXCR4 expression has a prognostic value in patients with head and neck squamous cell carcinoma. The CXCR4 expression

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Table 3. Univariate Cox proportional hazards analysis for cause-specific survival of variables in 56 patients with HNSCC

Variable	Hazard ratio	95% Confidence interval	P
Age (≥60 y)	0.975	0.459-2.294	0.9501
Gender (male)	0.985	0.282-3.646	0.9821
Primary site (floor of mouth)	1.36	0.423-4.370	0.6052
Tumor differentiation (poor/moderate)	2.723	0.953-7.781	0.0614
Clinical stage (III-VI)	4.098	0.076-0.786	0.0181
CXCR4 (positive)	3.611	0.093-0.820	0.0204

scores of carcinoma cells in 56 patients with primary HNSCC are shown in Table 2. The CXCR4 expression on primary carcinoma cells was detected at various levels in 16 (28.6%) patients. In three patients, the tumors showed a strong expression (score 3) of CXCR4, in 5 patients a moderate expression (score 2), and in 8 a mild expression (score 1). Gender, age, histology, tumor status at diagnosis, and initial therapies did not influence the CXCR4 expression. Patients with N_2 neck status at diagnosis showed significantly higher CXCR4 expression score than patients with N_0-N_1 neck status (1.333 ± 1.336 versus 0.380 ± 0.753; P = 0.0131; Fig. 6F). Patients who developed distant metastases after tumor-free periods showed significantly higher CXCR4 expression scores than patients without distant metastases during follow-up periods (1.000 ± 1.279 versus 0.341 ± 0.680; P = 0.0458; Fig. 6G). The cause-specific survival of CXCR4-positive patients was significantly worse than that of CXCR4-negative patients (P = 0.0380; Fig. 6H). Univariate analysis also showed that CXCR4 positive was a significant indicator for poor cause-specific survival (hazard ratio, 3.611; 95% confidence interval, 0.093-0.820; P = 0.0204; Table 3). Multivariate analysis confirmed that CXCR4 positive was the independent factor for cause-specific death (hazard ratio, 3.731; 95% confidence interval, 0.073-0.986; P = 0.0475; Table 4).

Discussion

In this study, we showed expressions of both *CXCR4* mRNA and cell surface CXCR4 protein in three (HSQ-89, IMC-3, and Nakamura) of six HNSCC cell lines tested. Exogenous SDF-1 promoted cell migration as well as proliferation in a dose-dependent manner in CXCR4-positive cells but never in CXCR4-negative cells. The SDF-1-mediated cell migration and proliferation of CXCR4-positive cells were inhibited by neutralizing anti-CXCR4 antibody. These results suggest that these three HNSCC cell lines have CXCR4, which acts as a receptor specific for SDF-1 and plays roles in cell migration and proliferation. In several types of carcinoma cell lines, the functional role of CXCR4 in cell migration in response to SDF-1 has been shown (3–6, 8–10). Although the mechanism how SDF-1/CXCR4 signal enhance cell migration is not fully evaluated, several investigators have shown that the SDF-1/CXCR4 interaction induced matrix metalloproteinases in megakaryocytes (27), rhabdomyosarcoma cells (28), and $CD34^+$ cells (29).

In contrast to the cell migration, the role of CXCR4 in cell proliferation is still controversial. Phillips et al. (5) reported that SDF-1 stimulation did not enhance cell proliferation in non–small cell lung cancer cell lines. Hwang et al. (9) also failed to find the SDF-1-mediated cell proliferation in anaplastic thyroid cancer cell lines. In contradiction, Kijima et al. (11) showed that SDF-1 stimulation increased cell proliferation in small cell lung carcinoma cell lines. Sun et al. (7) showed that neutralizing anti-SDF-1 diminished the proliferation of CXCR4-positive prostate cancer cell lines that autocrine SDF-1. We clearly showed that CXCR4 plays a role in cell proliferation in response to SDF-1. These contradictory findings suggest that cell proliferation role for CXCR4 may vary in tumor types and/or sites. It is possible that SDF-1/CXCR4 initiated different signal pathways for cell proliferation and migration. ERK1/2 (5, 9, 12) and Akt (11, 12) signaling pathways were reported to be activated by SDF-1/CXCR4 interaction in carcinoma cells, and its functions are still controversial. In our experiments with HNSCC cells, the SDF-1/CXCR4 stimulation induced both ERK1/2 and Akt phosphorylation together with cell migration and proliferation. Further studies will be needed to clarify the SDF-1/CXCR4 signaling pathway systems in cancer cells.

It is known that SDF-1 is constitutively expressed in almost all tissues at different levels, and *SDF-1* gene is often used as a housekeeping gene (30). The autocrine mechanism of SDF-1/

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Table 4. Multivariate Cox proportional hazards analysis for cause-specific survival of variables in 56 patients with HNSCC

Variable	Hazard ratio	95% Confidence interval	P
Age (≥60)	0.396	0.788-8.098	0.1189
Gender (male)	1.142	0.216-3.549	0.8525
Primary site (floor of mouth)	1.662	0.429-6.442	0.4624
Tumor differentiation (poor/moderate)	2.213	0.772-6.779	0.1643
Clinical stage (III-VI)	3.559	0.080-0.991	0.0483
CXCR4 (positive)	3.731	0.073-0.986	0.0475

CXCR system that might take part in cell migration and/or proliferation was reported in several types of carcinoma (7, 9, 31). However, we did not detect SDF-1 expression in six HNSCC cell lines tested and in biopsy samples from 56 patients with HNSCC. Begum et al. (32) also reported that *SDF-1* mRNA expressions were lost in premalignant colonic adenomas and 27 malignant human cell lines. Shibuta et al. also showed a loss of *SDF-1* mRNA expression in malignant tissues such as colon, esophageal, and gastric cancers (33). In our series, we found strong *SDF-1* expressions in stromal tissues surrounding CXCR4-expressing cancer nests in metastatic lymph nodes but hardly detected SDF-1 expression in stromal tissues surrounding primary cancer nests. The loss of SDF-1 expression in HNSCC may play an important role on lymph node and/or distant metastases to the organ with high SDF-1 expression as suggested previously in a mice model with transplanted breast cancer (3).

Although expression of CXCR4 is reported in a variety of human cancer tissues (3–5, 7, 8, 10), the correlation between CXCR4 expression and clinical features is still controversial. Kato et al. (26) reported that high expression of CXCR4 in cancer cells significantly correlated with the extent of lymph node metastases in invasive breast carcinoma. Staller et al. (10) reported that strong expression of CXCR4 correlated with poor tumor-specific survival and was the independent poor prognostic factor in renal cell carcinoma. Contradictory, Sun et al. (7) did not find difference on the expression levels of CXCR4 between localized and metastatic prostate cancer. Spano et al. (34) reported that nuclear staining of CXCR4 was associated with better survival in non–small cell lung cancer. Concerning HNSCC, Uchida et al. (35) reported that the level of *CXCR4* mRNA in metastatic oral cancer tissues was significantly higher than that in nonmetastatic tissues. In our series, we clearly showed that patients with advanced neck status and patients who developed distant metastases showed significantly higher CXCR4 expression. Furthermore, we found that the cause-specific survival of CXCR4-positive patients was significantly shorter than that of CXCR4-negative patients. Finally, we clearly showed that CXCR4 positive was the independent factor for poor prognosis in patients with HNSCC.

Several cytokines, such as IFN-γ, TNF-α, and IL-1β, have been reported to regulate CXCR4 expression in human blood cells (13–18). Concerning tumor cells, Oh et al. (36) showed that TNF-α and IL-1β up-regulated CXCR4 expression in astroglioma cells, but IFN-γ did not affect the expression. On the other hand, Han et al. (37) reported that CXCR4 expression in primary murine astrocytes was down-regulated by TNF-α or IL-1β. In our series of HNSCC cells, TNF-α and IL-1β did not affect CXCR4 expression in any HNSCC cell tested. On the other hand, IFN-γ down-regulated CXCR4 expression at both mRNA and surface protein levels in a dose- and time-dependent manner in CXCR4-positive IMC-3 and Nakamura cells. IFN-γ also inhibited the SDF-1-mediated cell migration and cell proliferation in these cells but not in the CXCR4-negative cell line and CXCR4-positive HSQ-89 cells in which CXCR4 expression was not altered by IFN-γ.

Although all CXCR4-positive cells expressed IFN-γ receptor 1, the CXCR4 expression of HSQ-89 cells did not respond to IFN-γ in contrast to IMC-3 and Nakamura cells. Although we cannot completely solve this contradiction, it may be caused by the differences of their origins. Both IMC-3 and Nakamura cells were established from maxillary sinus, whereas HSQ-89 cells were from oral gingival. Alternatively, it may be caused by the differences of the signal pathway systems induced by IFN/IFN receptor interaction.

Although we cannot completely explain how IFN-γ down-regulates CXCR4 expression, we hypothesize two possibilities. The first is that IFN/IFN receptor stimulation activates IFN regulatory factor-1 through the Janus-activated kinase/STAT signaling pathway resulting in repression of SP1 that is a one of the transcription factors binding to the CXCR4 promoter region (38). Recently, Xie et al. (39) reported that SP1-dependent transcriptional activation was repressed by IFN regulatory factor-1. IFN regulatory factor-1 is a member of IFN regulatory factor family that is activated by the Janus-activated kinase/STAT–mediated signaling pathway (40). The Janus-activated kinase/STAT signaling pathway is induced by IFN/IFN receptor response. The second is that IFN/IFN receptor stimulation inhibits the cyclic AMP–mediated intracellular element that acts on the CXCR4 promoter. CXCR4 promoter is reported to contain a cyclic AMP–responsible element that enhances CXCR4 expression in human T cells (41), human glial cells (42), and dendritic cells (43). Recently, Christian et al. (44) showed that IFN-γ markedly inhibited cyclic AMP–mediated gene expression in a dose-dependent manner in human endothelial cells.

In summary, we clearly showed functional expression of CXCR4 involved in cell migration and proliferation in certain HNSCC cell lines. Immunohistologic study of HNSCC tissues revealed that the CXCR4 expression was correlated with lymph node and distant metastases and poor prognosis as well as poor survival. We also showed that IFN-γ down-regulated both gene and cell surface protein expression of CXCR4 in certain HNSCC cells, resulting in suppression of cell migration and proliferation *in vitro*. Our results may provide an insight into future therapeutic agent that inhibits tumor metastasis and progression via down-regulating CXCR4 expression in patients with HNSCC.

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