CXCR4 Overexpression Is Associated with Poor Outcome in Females Diagnosed with Stage IV Non-small Cell Lung Cancer

Shannon Otsuka, MSc, Alexander C. Klimowicz, PhD,* Karen Kopciuk, PhD,†‡ Stephanie K. Petrillo, MSc,* Mie Konno, BSc,* Desiree Hao, MD, FRCPC,‡ Huong Muzik, BSc,* Erin Stolte, BSc,* William Boland, BSc,* Don Morris, PhD, MD, FRCPC,*‡ Anthony M. Magliocco, MD, FRCPC,*‡ and D. Gwyn Bebb, MA, BMBCh, PhD, FRCPC*‡*

Background: It has been proposed that the chemokine receptor, CXCR4, and its ligand, stromal cell-derived factor-1 (SDF-1), play a critical role in organ-specific tumor metastasis. High CXCR4 expression in resected non-small cell lung cancer (NSCLC) tumors is associated with poorer outcome; however, its effect on patient outcome in advanced NSCLC has not been explored.

Methods: After institutional ethical approval was obtained, demographic details, clinical variables, and outcome data were collected on consecutive NSCLC patients diagnosed at the Tom Baker Cancer Centre from 2003 to 2006 (Glans-Look Lung Cancer Database). Formalin-fixed paraffin-embedded diagnostic biopsies from stage IV patients were obtained and tissue microarrays generated. CXCR4 expression within NSCLC cells was analyzed by quantitative fluorescent immunohistochemistry using the HistoRx PM-2000 platform and then correlated with clinical outcome.

Results: Of 832 patients, 170 had samples suitable for tissue microarray generation and analysis. Automated immunohistochemistry for CXCR4 was successfully completed on all 170 patients. High expressors had a significantly poorer median overall survival of 2.7 months versus 5.6 months for the low expressors ($p = 0.0468$). This difference is driven by high-expressing females who have a median overall survival of 1.6 months versus 6.4 months for the low expressors ($p = 0.006$).

Conclusions: CXCR4 is expressed in the majority of NSCLC tumors, and overexpression is associated with significantly poorer survival in stage IV NSCLC patients. Interestingly, this poor outcome is disproportionately represented in the female population. Our results suggest a gender-dependent difference in clinical outcome based on CXCR4 overexpression in stage IV NSCLC.

*Translational Research Laboratory, Tom Baker Cancer Centre and University of Calgary; †Population Health Research, Alberta Health Services-Cancer Care; and ‡Department of Oncology, Tom Baker Cancer Centre and University of Calgary, Calgary, Alberta, Canada.

Disclosure: The authors declare no conflicts of interest.

Address for correspondence: Gwyn Bebb, MA, BMBCh, PhD, FRCPC, Tom Baker Cancer Centre, 1331, 29th St NW, Calgary, Alberta, Canada T2N 4N2. E-mail: Gwyn.Bebb@albertahealthservices.ca

ISSN: 1556-0864/11/0607-1169

Key Words: CXCR4, Non-small cell lung cancer, Outcome, Females, Stage IV.

(*J Thorac Oncol.* 2011;6: 1169–1178)

Lung cancer is the leading cause of death from cancer worldwide; however, despite extensive research, only small incremental outcome improvements have been realized. Metastatic spread constitutes the primary source of morbidity and mortality in all cancers, and dissemination to lung, liver, bone, and brain is characteristic of non-small cell lung cancer (NSCLC). The majority of patients present with advanced or metastatic disease, and consequently overall 5-year survival is a disappointing 15%. The use of platinum-based doublet chemotherapy has pushed median overall survival in the metastatic setting from 4 to 6 months to 8 to 10 months,[1,2] but even with the advent of targeted therapy, metastatic NSCLC remains incurable.[3,4] Just as discouraging is the high rate of distant recurrence for resected stage I and II disease, despite adjuvant treatment.[5,6] Clearly, a thorough understanding of the metastatic process is crucial to developing effective new therapies for lung cancer.

A growing appreciation of the role of chemokines in cancer has generated insight into molecular pathways that may drive invasion and metastasis. Chemokines, a class of small (8–14 kDa) proinflammatory chemotactic cytokines,[7] play a predominant role in regulating the homing and trafficking of various leukocyte subpopulations, particularly during inflammation, tissue damage, and infection.[8,9] The stromal cell-derived factor (SDF-1)/CXCR4, chemokine/receptor axis, has attracted particular interest in this context. This chemokine/receptor axis normally plays a critical role in the homing and retention of hematopoietic stem cells and lymphocytes in the bone marrow[10,11] and the trafficking of these cells to sites of tissue inflammation and damage. It has been noted that the metastasis of tumor cells shares many similarities with the normal trafficking of hematopoietic stem cells, and CXCR4 activation can induce cytoskeletal rearrangement, adhesion to endothelial cells, polarized migration of cells to specific organs, and the

secretion of angiopoietic factors,[12–15] all important components of the metastatic process.

Preclinical and clinical studies support the suggestion that the CXCR4/SDF-1 axis plays a role in the metastasis of many types of tumors including breast,[16–19] ovarian,[20] colorectal,[21] head and neck,[22,23] and pancreatic carcinomas,[24,25] among others. Increasing evidence also suggests that the CXCR4/SDF-1 chemokine axis plays a pivotal role in the metastasis of lung cancer, particularly in NSCLC. It has been shown that many NSCLC cell lines express high levels of CXCR4 and that SDF-1-activated CXCR4 promotes migration and invasion of these cell lines in vitro.[26,27] In addition, preferential sites of lung cancer metastases in vivo have significantly higher levels of SDF-1 protein expression than the primary tumor or plasma levels, suggesting that a chemotactic gradient may be established between the site of the primary tumor and metastatic sites. Furthermore, neutralization of the CXCR4/SDF-1 axis is associated with a decrease in NSCLC metastases to several organs, including the adrenal glands, liver, lung, brain, and bone marrow in vivo.[28]

Importantly, several retrospective studies in stage I and II patients have also examined the role of CXCR4 in NSCLC by investigating the association between CXCR4 expression and clinical outcome. Spano et al. assessed the expression of CXCR4, by semiquantitative immunohistochemistry (IHC), in NSCLC tumors resected from patients with stage I disease. They found that CXCR4 was present in the cytoplasm of the tumor cells of all tissue specimens tested but was absent in normal lung tissue. They also found that there was strong nuclear staining in a significant number of tumor specimens and a positive correlation was seen between CXCR4 nuclear expression and better prognosis, but no association between cytoplasmic CXCR4 expression and outcome was seen.[29]

Minamiya et al.[30] reported that high CXCR4 expression, as assessed by semiquantitative real-time reverse transcription PCR, was associated with a better clinical outcome and longer 5-year disease-free survival in early-stage resected patients with adenocarcinoma tumor histology. Similarly, Wagner et al.[31] demonstrated that nuclear CXCR4 expression conferred a survival benefit in patients with adenocarcinoma and also found that high cytomembranous CXCR4 expression was an independent prognostic marker of worse survival. In contrast, other studies have demonstrated that high nuclear staining of CXCR4 was associated with lymph node metastasis,[32] and both cytoplasmic and nuclear staining was an indicator of a worse prognosis.[33]

On this basis, it can be suggested that the CXCR4/SDF-1 axis plays an important yet incompletely defined role in the development and metastasis of NSCLC. Although there seems to be a somewhat established role for CXCR4 in the metastasis of early-stage NSCLC, the impact of CXCR4 expression on outcome of stage IV NSCLC patients has not been explored. Herein, we describe investigations to determine whether CXCR4 is an independent prognostic biomarker of overall survival in NSCLC patients with advanced stage IV disease by quantitative fluorescent immunohistochemical analysis of ex vivo tumor samples.

MATERIALS AND METHODS

Case Selection and Clinical Data Collection

This study was approved by the University of Calgary Conjoint Faculties Research Ethics Board, in accordance with the Tri-Council Policy Statement on Research with Human Subjects. Clinical data were collected retrospectively through chart review of NSCLC patients diagnosed at the Tom Baker Cancer Centre (TBCC) from 2003 to 2006 and entered into the Glans-Look Lung Cancer Database. All patients diagnosed during this period as identified by the provincially legislated Alberta Cancer Registry were included. Relevant data were obtained from physician progress notes, pathology reports, diagnostic imaging reports, laboratory results, and other hospital records. Demographic details included age at diagnosis, gender, birthplace, and smoking status; clinical variables included stage of disease, tumor histology, treatment modalities, and outcome data.

Staging was performed according to the American Joint Committee on Cancer tumor, node, metastasis system and reflected the recent 2009 revisions for NSCLC staging. In the new system, patients designated M1a had metastases contained to the thorax (including contralateral lung and malignant pleural effusions), while those patients designated M1b had distant metastases outside of the thorax (bone, brain, viscera, and skin/subcutaneous). Data on actual patient ethnicity were unavailable, so patient origin was used as a surrogate for ethnicity and was determined by the birthplace of the patient. North American origin included patients born in both Canada and the United States. Southeast Asian origin included patients born in China, Japan, Cambodia, Philippines, Indonesia, Korea, Malaysia, and Vietnam. Origin classified as "other" included patients born in Africa, Europe, South America, Australia, South and West Asia, and unknown birthplaces. Smoking status was determined by the attending physician: nonsmoking status was defined as having smoked less than 100 cigarettes total, while a current smoking status was assigned if the patient smoked at the time of diagnosis. Rural or urban status was determined based on the patient's residential postal code at the time of diagnosis. Tumor histology was determined by a pathologist when adequate tissue was available. Those patients whose tumors were designated "histology not otherwise specified (NOS)" included those patients where a specific histological diagnosis could not be made from the available tissue and those without a pathological tissue diagnosis.

Tissue Microarray Generation

All archived formalin-fixed paraffin-embedded tumor samples from stage IV NSCLC patients included in the clinical database were retrieved from Calgary Laboratory Services. Hematoxylin and eosin-stained slides were reviewed by a pathologist to confirm diagnosis, and those deemed to be of sufficient quality were selected and marked for sampling and inclusion into the tissue microarray (TMA). Representative cores (0.6 mm) from each specimen were assembled in triplicate (when adequate material was available) into each TMA (25–45 specimens per TMA) using a Beecher Manual Tissue Microarrayer

(Beecher Instruments Inc., Sun Prairie, WI). Normal lung tissue specimens, normal tonsil tissue, and Hela cells were also included as controls.

Fluorescent Immunohistochemical Staining

After TMA construction, 5-μm-thick sections were cut from the TMA block and deparaffinized in xylene, rinsed in ethanol, and rehydrated. Heat-induced epitope retrieval was performed by heating slides to 121°C in a citrate-based buffer (pH 6.0) Target Retrieval Solution (Dako, Mississauga, ON, Canada) for 3 minutes in a decloaking chamber (Biocare Medical, Concord, CA). Endogenous peroxidase activity was quenched with a 10-minute incubation of peroxidase block (Dako) followed by a 15-minute protein block (Signal Stain, Cell Signaling, Danvers, MA) to eliminate nonspecific antibody binding. Slides were stained overnight in a humidified chamber at room temperature with Signal Stain protein block (Cell Signaling) with a 1:500 dilution of anti-pan-cytokeratin mouse monoclonal antibody (Dako) to identify tumor cells, combined with a 1:25 dilution of anti-CXCR4 rabbit mAb (clone UMB2, Biotrend, Köln, Germany).[34] The following day, slides were washed with tris-buffered saline and tween 20 (TBST) wash buffer (Dako), and corresponding secondary antibodies were applied for 60 minutes at room temperature: goat anti-mouse antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone from the DAKO EnVision+ system (Dako) and a 1:200 dilution of Alexa-555-conjugated goat anti-mouse antibody (Invitrogen, Burlington, ON, Canada). The slides were washed with TBST wash buffer (Dako) and incubated for 5 minutes with the TSA-Plus Cy5 tyramide signal amplification reagent (PerkinElmer, Woodbridge, ON, Canada). After three washes in TBST wash buffer, the TMA slides were mounted with ProLong Gold antifade mounting medium containing DAPI (Invitrogen) and stored at 4°C until use.

Automated Image Acquisition and Analysis

Automated image acquisition was performed using the HistoRx PM-2000, which has previously been described in detail.[35] Briefly, high-resolution monochromatic 8-bit digital images (resulting in 256 discrete intensity values per pixel of an acquired image) were obtained for every histospot on the TMAs using filters specific for DAPI to define the nuclear compartment, Cy3 to define cytokeratin-positive NSCLC cells and the tumor cytosolic compartment, and Cy5 to define the target biomarker CXCR4. Pixels were then written to image files as a function of power (Power [P] = [Pixel Intensity/256]/exposure time) to help compensate for experimental variations in staining intensity.

Images were taken for each channel for future use with the AQUAsition program, version 2.2.1.7, as previously described.[35] Briefly, a tumor specific mask was generated to distinguish the NSCLC cells from normal tissue by thresholding the pan-cytokeratin images. Thresholding created a binary mask that identified the presence or absence of tumor cells by the presence of a pixel that was "on" or "off," respectively. Thresholding levels were verified and adjusted if necessary, by spot-checking a small sample of images to determine an optimal threshold value. All images were then processed using this optimal threshold value, and all subsequent image manipulations involved only image information from the masked area. The target CXCR4 signal in the masked area was tabulated and used to generate tumor-specific AQUA scores, which reflect the average signal intensity per tumor area. Images were validated according to the following: (1) more than 10% of the tissue area is pan-cytokeratin positive, (2) more than 50% of the image was usable (i.e., not compromised due to overlapping or out of focus tissue). Unusable areas within each image were manually cropped so that they were excluded from the final analysis.

Statistical Analysis

Descriptive statistics compared the frequencies of measured patient and pathological features between the full, TMA, and non-TMA cohorts, as well as the male and female expression groups within the TMA cohort. A cut-point to create two groups from the maximum CXCR4 expression levels was found using a method based on the log-rank test statistic.[36] The relationships between CXCR4 scores and clinicopathological variables of interest were evaluated using Fisher's exact test with mid-p adjustment for categorical data and two-sample Student t test for the age variable. Equivalence between the TMA and non-TMA groups was evaluated using a multinomial goodness-of-fit approach for the categorical variables and a two-sample t test for the continuous variable. Survival analyses assessed the equivalence of the survival experiences between the TMA and non-TMA groups,[37] tested the observed differences in the survival experiences of low and high expressors, and evaluated clinicopathological features in Cox proportional hazards (PH) regression models. PH assumptions were assessed using scaled Schoenfeld residual plots and trend test statistics.[38] Validation of the final Cox PH regression model was based on the c (concordance) index derived from Somers' D_{xy} rank correlation, using 200 bootstrap samples.[39,40] All analyses were conducted with SAS/STAT software (Version 9.2) SAS System for Unix[41] and R software (version 2.11).[42]

RESULTS

Patient Characteristics

Description of Full Clinical Cohort

Between January 2003 and December 2006, 832 patients were diagnosed with stage IV NSCLC at the Tom Baker Cancer Centre. Patient demographics and clinical characteristics for all patients (full cohort) included in the clinical analysis are summarized in column 2 of Table 1. Median age was 69 years, 51.4% were male, 85.8% were ex- or current smokers, 7.8% were of Southeast Asian origin, and 65.9% had M1b disease. In terms of tumor histology, 43.8% were adenocarcinomas, 18.9% squamous cell carcinomas, 32.3% NOS, and 5.1% were other histology (large cell carcinoma, bronchioloalveolar carcinoma, and adenosqamous carcinoma). Treatment varied widely and was largely heterogeneous in the cohort: 21.0% of patients received no treatment, 7.0% chemotherapy alone, 55.7% palliative radiotherapy

TABLE 1. Demographic Details of Patients with Stage IV NSCLC

	Full Cohort (n = 832) n (%)	TA Cohort (n = 170) n (%)	Non-TA Cohort (n = 662) n (%)
Gender			
Female	404 (48.6)	84 (49.4)	320 (48.3)
Male	428 (51.4)	86 (50.6)	342 (51.7)
Histology			
Adenocarcinoma	364 (43.8)	91 (53.5)	273 (41.2)
Squamous cell	157 (18.9)	49 (28.8)	108 (16.3)
Large cell	24 (2.9)	3 (1.8)	21 (3.2)
BAC	12 (1.4)	5 (2.9)	7 (1.1)
Adenosquamous	6 (0.7)	1 (0.6)	5 (0.8)
NOS	269 (32.3)	21 (12.4)	248 (37.5)
Radiotherapy			
No	233 (28.0)	31 (18.2)	202 (30.5)
Yes	599 (72.0)	139 (81.8)	460 (69.5)
Systemic therapy			
No	638 (76.7)	128 (75.3)	510 (77.0)
Yes	194 (23.3)	42 (24.7)	152 (23.0)
No. of lines if yes			
1	116 (59.8)	26 (61.9)	90 (59.2)
2	42 (21.7)	5 (11.9)	37 (24.3)
3	24 (12.4)	8 (19.1)	16 (10.5)
4	10 (5.2)	3 (7.1)	7 (4.6)
6	2 (1.0)	0 (0.0)	2 (1.3)
EGFR TKI therapy			
No	763 (91.7)	154 (90.6)	609 (92.0)
Yes	69 (8.3)	16 (9.4)	53 (8.0)
Distant	548 (65.9)	121 (71.2)	427 (64.5)
Local	284 (34.1)	49 (28.8)	235 (35.5)
Smoking status			
Current	268 (32.2)	53 (31.2)	215 (32.5)
Ex	446 (53.6)	87 (51.2)	359 (54.5)
Never	86 (10.3)	23 (13.5)	63 (9.5)
Unknown	32 (3.9)	7 (4.1)	25 (3.8)
Origin			
North American	609 (73.2)	124 (72.9)	485 (73.3)
Southeast Asian	65 (7.8)	16 (9.4)	49 (7.4)
Other	158 (19.0)	30 (17.6)	128 (19.3)
Region			
Rural	105 (12.6)	26 (15.3)	79 (11.9)
Urban	727 (87.4)	144 (84.7)	583 (88.1)
Age			
Mean (SD)	68.1 (11.2)	66.4 (10.7)	68.5 (11.2)
Median	69	67	69
Range	32–96	32–88	39–96

Values are given as N (%).

NSCLC, non-small cell lung cancer; TA, tissue array; BAC, bronchioloalveolar carcinoma; NOS, not otherwise specified.

alone, and 16.4% received both chemotherapy and palliative radiotherapy at some point during the course of their disease (data not shown). In addition, 8.3% of patients received treatment with epidermal growth factor receptor kinase inhibitors (includes both alone and with palliative radiotherapy).

Description of Cohort in TMA

Of the 832 stage IV patients included in the clinical analysis, 290 patients had diagnostic or resected tissue specimens available (not including 21 patients who had tissue biopsies in locations other than Calgary). The remaining 521 patients did not have a tissue diagnosis (n = 107), had tissue biopsies unavailable for retrieval (n = 12), or were diagnosed based on cytological tissue specimens (fine needle aspirate, bronchial washing, bronchoalveolar lavage wash, thoracentesis, or sputum sample) (n = 402), which were not suitable for inclusion into the TMAs. Ultimately, only 170 patients had tissue samples deemed of sufficient quality for TMA incorporation and analysis. Seven of these patients had two separate biopsy samples included into TMAs and AQUA scores, which were averaged before analysis. The 177 tumor specimens consisted of tissue obtained from primary tumor (n = 101) or metastatic deposits (n = 76) (including distant metastases and lymph nodes).

The demographics and clinical characteristics of the 170 patients included in the molecular analysis are summarized in column 3 of Table 1. Median age was 67 years, 50.6% were male, 82.4% were current or ex-smokers, 9.4% were of Southeast Asian ethnicity, 71.2% presented with M1b disease, 5.3% received only chemotherapy, 62.4% only radiotherapy, 19.4% both therapies, and 12.9% no therapy (data not shown). In addition, 53.5% of the patients had adenocarcinoma tumor histology, 28.8% squamous cell carcinoma, and only 12.4% of patients had tumor histology not otherwise specified (NOS), which was significantly less than in the clinical cohort as a whole.

Patients with tissue suitable for inclusion into TMAs had similar characteristics to the stage IV cohort of patients without available tissue. Table 1 (columns 4–7) demonstrates that most predictor variables were equivalent using a strict tolerance value (10%), when the TMA sample was compared with the non-TMA reference group with two exceptions: the TMA group included higher proportions of patients who received radiotherapy, more patients diagnosed with adenocarcinoma or squamous cell carcinoma, and a corresponding lower proportion of patients whose diagnoses were NOS. The non-TMA cohort (n = 662) had a median overall survival (MOS) of 3.75 months (95% confidence interval [CI] = 3.29–4.37) versus 5.22 months (95% CI = 3.71–6.05) for those patients in the TMA cohort (n = 170). Comparison of the Kaplan-Meier survival curves of the two cohorts using a log-rank test of equivalence showed that the curves never separated more than 10% over the study duration, which was within our strict equivalence interval (Figure 1). Thus, the overall unadjusted survival of the 170 patients whose tumors were suitable for TMA inclusion was very similar to those whose tumors were not suitable for TMA inclusion, suggesting that they are reasonably representative of stage IV patients as a whole.

CXCR4 Expression by Quantitative Fluorescent IHC in Stage IV NSCLC Patient Specimens

Quantitative fluorescent IHC was successfully completed for all patients included in the TMAs. Automated quantitative analysis (AQUA) was performed on the images

created after tissue CXCR4 staining, and an AQUA score representing the tumor-specific, non-nuclear CXCR4 receptor expression for each patient tissue specimen was obtained. The value of AQUA measurements using "keratin masking" enables accurate determination of CXCR4 expression within

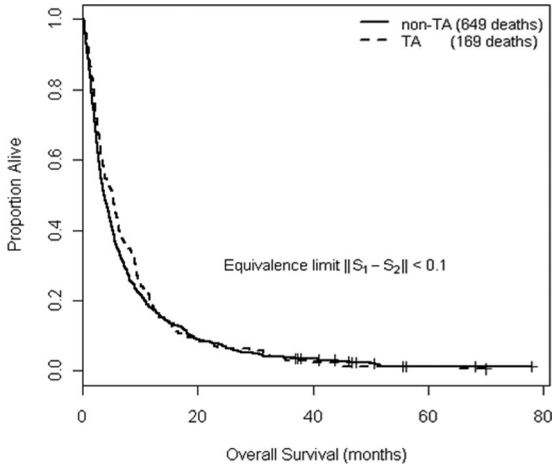


FIGURE 1. Kaplan-Meier survival curve comparing the overall survival of the tissue array (TA) and non-TA cohorts. The curves never deviate more than 10% over the study duration.

only the epithelial cells of the cancer. To verify the specificity of the UMB2 rabbit monoclonal antibody used to detect CXCR4, Hela cells were used as positive controls while normal human tonsil tissue was also used as both a positive and negative control. Hela cells showed strong CXCR4 staining. In the tonsil, germinal center cells expressed high levels of CXCR4, mantle zone cells expressed moderate levels of CXCR4, and cells of the surrounding lymphoid tissue expressed low levels of CXCR4. In the absence of the CXCR4 antibody, no specific staining in the tonsil was observed (Figure 2*A*).

Normal lung displayed CXCR4 staining only in endothelial cells of the alveolar capillaries. There was no staining in the lung epithelial cells. The lung tumors displayed a range of CXCR4 expression with some cases devoid of significant expression and others displaying marked cytoplasmic expression (Figure 2*B*). In all cases, there seemed to be strong CXCR4 expression in the associated endothelial cells of the capillaries (Figure 2*B*, middle panel). The cytoplasmic expression pattern, the lack of nuclear expression, and the CXCR4 expression in the endothelial cells of the capillaries are all consistent with the original characterization of the anti-CXCR4 rabbit monoclonal antibody used in this study.[34] The mean AQUA score for the 170 patients was 2512.44 (SD 1371.74). When plotted on a frequency histogram, the distri-

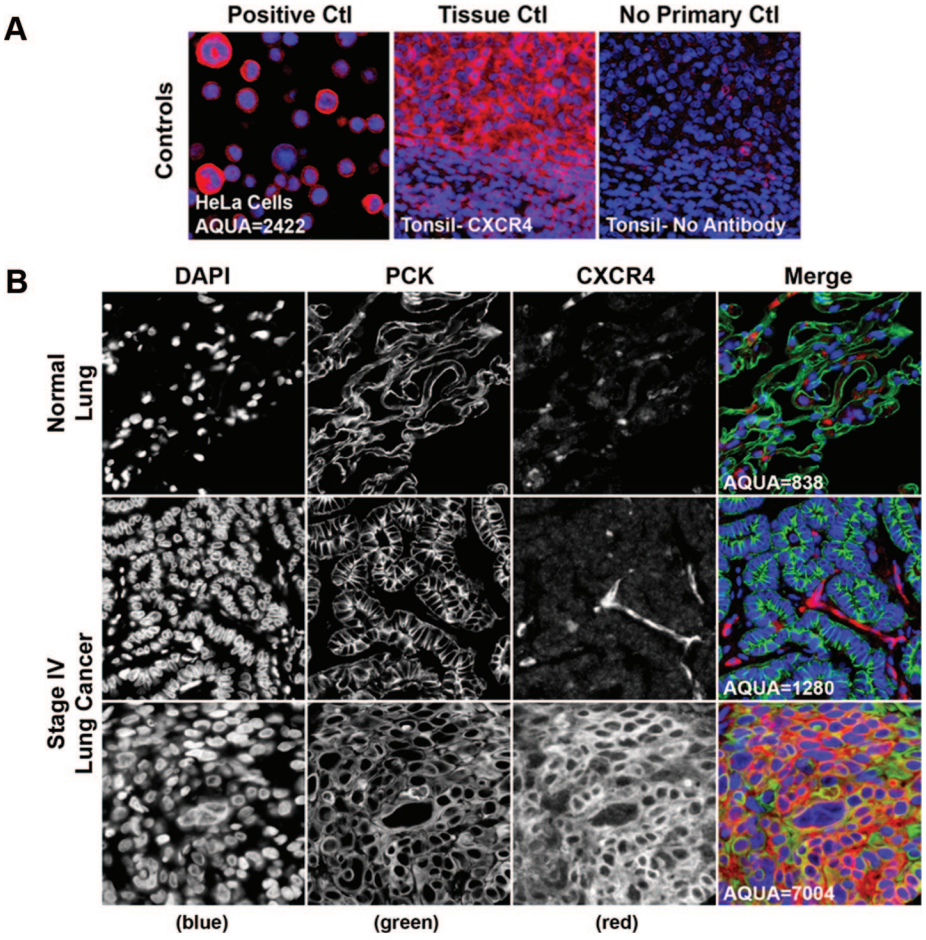


FIGURE 2. CXCR4 immunohistochemistry (IHC) staining. *A*, CXCR4 staining in positive control Hela cells and tonsil tissue with and without the primary antibody. *B*, Interrogation of non-small cell lung cancer (NSCLC) samples on tissue microarray (TMA) for CXCR4 expression by IHC using the HistoRx/AQUA platform. CXCR4 is expressed in normal lung tissue only in the endothelial cells and is frequently expressed in NSCLC with a cytomembranous distribution.

TABLE 2. Demographic Details of Patients with High and Low CXCR4 Expression, Stratified by Gender

	Females (n = 84)			Males (n = 86)		
	Low (n = 74)	High (n = 10)	p^a	Low (n = 67)	High (n = 19)	p^a
Histology						
Adenocarcinoma	45 (60.8)	4 (40)	0.37	38 (56.7)	4 (21.1)	0.0074
Squamous cell	16 (21.6)	5 (50)		15 (22.4)	13 (68.4)	
Large cell	1 (1.4)	0 (0)		2 (2.9)	0 (0)	
BAC	3 (4.1)	0 (0)		2 (2.9)	0 (0)	
Adenosquamous	0 (0)	0 (0)		1 (1.5)	0 (0)	
NOS	9 (12.2)	1 (10)		9 (13.4)	2 (10.5)	
Radiotherapy						
No	10 (13.5)	3 (30)	0.11	15 (22.4)	3 (15.8)	0.64
Yes	64 (86.5)	7 (70)		52 (77.6)	16 (84.2)	
Systemic therapy						
No	54 (72.9)	9 (90)	0.35	50 (74.6)	15 (78.9)	0.77
Yes	20 (27)	1 (10)		17 (25.4)	4 (21)	
Metastases						
Distant	52 (70.3)	7 (70)	0.86	49 (73.1)	13 (68.4)	0.67
Local	22 (29.7)	3 (30)		18 (26.9)	6 (31.6)	
Smoker						
Current	19 (2.7)	4 (40)	0.23	21 (31.3)	9 (47.4)	0.60
Ex	36 (48.7)	5 (50)		37 (55.2)	9 (47.4)	
Never	16 (21.6)	0 (0)		6 (8.9)	1 (5.3)	
Unknown	3 (4.1)	1 (10)		3 (4.5)	0 (0)	
Origin						
North American	51 (68.9)	10 (100)	0.44	45 (67.2)	18 (94.7)	0.22
Southeast Asian	8 (10.8)	0 (0)		7 (10.4)	1 (5.3)	
Other	15 (20.3)	0 (0)		15 (22.4)	0 (0)	
Age						
Mean (SD)	66.6 (11.9)	71.6 (10.3)	0.18	65.7 (9.9)	64.9 (8.4)	0.73
Median	68.5	75.5		65	67	
Range	32–88	48–83		43–85	46–76	

Values are given as N (%).
[a] Based on Fisher exact test with mid-p adjustment for categorical data and two-sample student's t test with unequal variances for age variable.
BAC, bronchioloalveolar carcinoma; NOS, not otherwise specified.

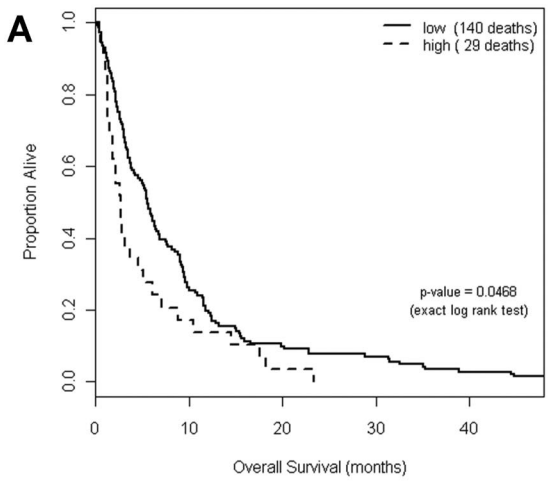
bution of CXCR4 AQUA scores was right skewed and ranged from a minimum of 536.00 to a maximum of 8317.73 (median 2227.31).

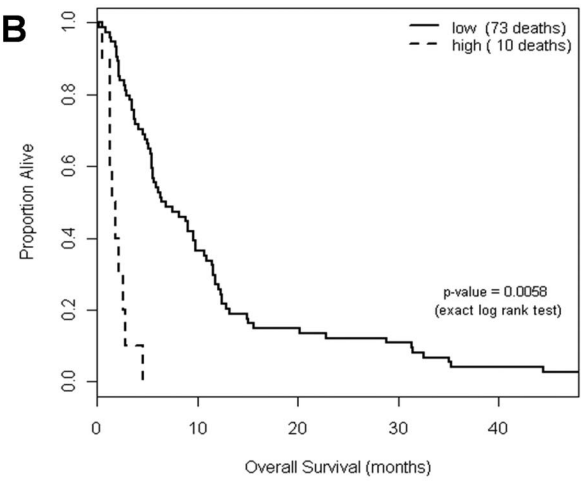
To divide the patients into high and low CXCR4 expressing groups, an AQUA score cut-point of 3371.00 was determined using a log-rank test statistic method and confirmed graphically with plots of martingale residuals from a null model against the AQUA score.[38] Twenty-nine (17.1%) patients had an AQUA score more than 3371.00 and thus were considered high expressors; the remaining 141 patients were considered to have low CXCR4 expression. Patient demographics of the two CXCR4 expression groups by gender are summarized in Table 2.

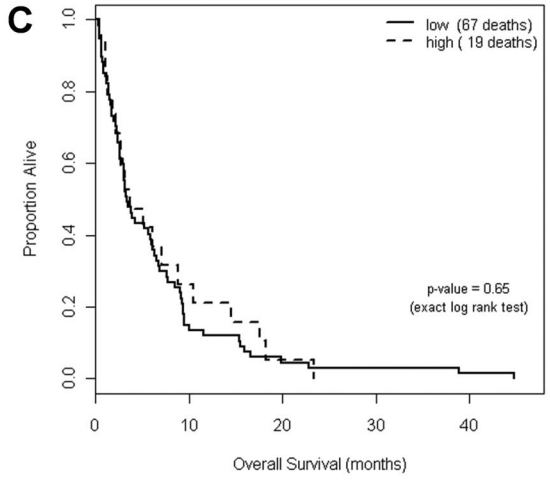
Association of CXCR4 Expression and Overall Survival

Overall survival was the main outcome of interest, with only one patient censored at the study end date, June 8, 2010. Potential confounding factors were forced into all Cox PH regression models regardless of statistical significance; these included all treatment variables (systemic therapy, radiotherapy, and epidermal growth factor receptor tyrosine kinase inhibitor [EGFR TKI] treatment), age (years), gender, smoking status, histology, TMA batch, and location of metastases (thoracic or distant). Initial multivariable models assessed the importance of CXCR4 AQUA score status (high versus low) jointly with region of residence, tissue biopsy site, race, and race-smoking status interactions. Reduced models (dropped race, race-smoking status interactions, and tissue biopsy site), based on likelihood ratio statistics ($p \leq 0.05$), next assessed CXCR4 AQUA score status interactions with radiotherapy, histology, systemic therapy, metastases location, gender, and EGFR TKI treatment.

Two factors associated with overall survival in the final Cox PH regression model included (1) EGFR TKI treatment and (2) CXCR4 AQUA score status-gender interaction. Receiving EGFR TKI treatment reduced the risk of dying by 0.48 (95% CI = 0.22–1.01), which was marginally above the statistical significance value of 0.05. Of greater interest is the influence of gender on outcome in the CXCR4 high expressors. Women with high CXCR4 expression had more than







D

	Low Expression	High Expression
Females	HR 1.00 MOS 6.4 mo (5.4, 9.8)	HR 5.36 (2.44, 11.79) MOS 1.6 mo (0.5, 2.6)
Males	HR 2.07 (1.42, 3.02) MOS 3.4 mo (2.6, 6.0)	HR 1.77 (0.98, 3.22) MOS 3.7 mo (1.9, 8.8)

FIGURE 3. Kaplan-Meier survival curves comparing the overall survival between the high and low CXCR4 expression groups: (*A*) whole tissue microarray (TMA) cohort, (*B*) females, (*C*) males. (*D*) Summary table of the median overall survival and HRs for high and low expressing groups stratified on gender. High expressing females have a 5.4 times increased risk of death compared with the low expressors and a median overall survival (MOS) of only 1.6 months.

fivefold increased risk of death relative to women with low expression levels (hazard ratio [HR] = 5.36; 95% CI = 2.44–11.79), whereas men were about twice as likely to die regardless of expression level (high expression levels: HR = 1.77, 95% CI = 0.98–3.22; low expression levels: HR = 2.07, 95% CI = 1.42–3.02).

Kaplan-Meier survival curves were also generated comparing the overall survival between the high and low CXCR4 expressing groups (Figure 3*A*). The high expression group (AQUA score ≥3371.00) had a significantly poorer survival experience compared with the low expression group ($p = 0.047$ from exact log-rank test statistic), with a MOS of 2.7 months (95% CI = 1.9–5.2) compared with a MOS of 5.6 months (95% CI = 4.2–6.9). The gender-CXCR4 interaction found in the Cox PH model suggests that this difference in outcome is driven primarily by the female patients with high CXCR4 expression. Kaplan-Meier survival curves comparing the overall survival of the high versus low CXCR4 expressors in females and males can be seen in Figures 3*B*, *C*, respectively. In females, the patients with high CXCR4 expression have a significantly decreased overall survival when compared with those with low CXCR4 expression ($p = 0.006$) with a MOS of 1.6 months (95% CI = 0.5–2.6) for high expressors versus 6.4 months (95% CI = 5.4–9.8) for the low expressors. In males, no significant difference can be seen in survival between the two expression groups ($p = 0.65$) with a MOS of 3.4 months (95% CI = 2.6–6.0) for the low expressors and 3.7 months (95% CI = 1.9–8.8) for the high expressors. Median overall survival and HRs for the high and low expression groups separated by gender are summarized in Figure 3*D*.

Because systemic therapy and radiotherapy failed to meet the PH assumption, they were incorporated as stratifying factors into the model. Diagnostic plots assessed the functional form of age and the presence of influential or outlying observations. Internal model validation was carried out using 200 bootstrap samples. It revealed slight overfitting (over-estimation of the regression coefficients) by up to 38%

but still acceptable discrimination ability (c-index or area under the receiver operating curve was 0.71).[40]

Association of CXCR4 Expression and Histology

The relationship between CXCR4 AQUA score status and histology type was investigated to determine whether this could be impacting the significant gender-CXCR4 AQUA score status interaction. It should be noted that histology (overall or any subtype) was not significant in the final Cox PH regression model, which did adjust for gender, nor was the three-way interaction between histology, gender, and CXCR4 (CXCR4 score modeled as continuous; results not shown). There was no difference in the distribution of histology subtypes between males and females ($p = 0.63$, result not shown), but there was between the high and the low CXCR4 expression groups ($p = 0.00036$, result not shown). In the high CXCR4 expression group, there was a smaller proportion of individuals with adenocarcinomas and a higher proportion with squamous cell carcinomas. When these distributions are examined within gender strata, it becomes apparent that these differences are much more pronounced among the males ($p = 0.0074$) than the females ($p = 0.37$). Clearly, the small number of men and women in the high CXCR4 groups limits further assessment; however, the earlier analyses show that histology differences are not solely driving the observed gender-CXCR4 group interaction.

DISCUSSION

Evidence increasingly suggests that the CXCR4/SDF-1 chemokine axis is important in the development and progression of several tumor types, particularly breast cancer. In NSCLC, the evidence is more controversial: a number of studies have examined CXCR4 expression and association with outcome in early-stage NSCLC, but there are little data on CXCR4 in advanced disease. Our results confirm that CXCR4 is expressed by the malignant component of a tumor mass in almost all cases of stage IV NSCLC and that its expression can be described as being mainly cytomembranous with little expression in the nucleus. These findings support CXCR4 as a potential therapeutic target for NSCLC. Several anti-CXCR4 compounds have been developed for treating human immunodeficiency virus, thus allowing rapid transition into clinical trials.

In addition, our results also suggest that CXCR4 expression appears to be a prognostic biomarker in stage IV NSCLC. We report that in our cohort of patients, high expression of the CXCR4 receptor as assessed by quantitative IHC conferred a significantly worse prognosis in the stage IV NSCLC patients studied. Moreover, it appears that this survival difference is a gender-dependent effect, because only the females are negatively affected by high CXCR4 receptor expression with a five times greater risk of death compared with those with low expression. In contrast, no significant difference was seen in overall survival between the high and low expressing groups in the male population.

This gender difference in the correlation of CXCR4 receptor expression with clinical outcome is an intriguing finding and has not been previously reported. Our study did not provide a clear explanation for this phenomenon; however, a gender-based molecular-dependent difference in outcome in NSCLC is not improbable. It is generally accepted that there are gender-based outcome differences in NSCLC in early resectable disease[43–45] and in more advanced disease.[46–49] More recently, clinical experience,[3,50,51] and now molecular analyses,[52,53] has shown that responses to the EGFR TKIs and the activating mutations underpinning such responses are more common in females, although the etiology of this difference is unexplained. However, unlike our study, most of these reports associate female gender with improved outcomes and longer survival.

Interestingly, there have been recent reports of a positive regulatory loop between the CXCR4/SDF-1 chemokine axis and estrogen receptor (ER) signaling pathways, which influence both ER- and CXCR4-dependent gene expression and ultimately tumor cell growth in vitro.[54] Some studies have demonstrated that a significant proportion of NSCLC tumors express ERs[55,56] and that there may be a gender-dependent difference in ER expression.[57,58] Despite this, there does not seem to be a consensus on whether ER expression has any bearing on clinical outcome in NSCLC.[59,60] It can be postulated that if ERs were also present in tumors that express high levels of CXCR4, a significant increase in both CXCR4- and ER-dependent gene transcription (including SDF-1) could occur specifically in females due to the positive regulatory loop between the two receptors, accelerating progression and metastasis and resulting in the subsequent decrease in survival. If that is the case, then factors influencing estrogen concentration such as menopausal status or obesity (factors not explored in this retrospective analysis) may influence survival of female patients with NSCLC.

Our study has several attributes that strengthen its validity. A sample size of 170 interrogatable specimens compares favorably with other studies of molecular analysis in stage IV disease.[61,62] A low proportion of analyzable samples is a conspicuous feature of many studies of advanced NSCLC (even those based on clinical trials that include molecular correlative studies in their design) and highlights one of the challenges inherent in any translational work in metastatic lung cancer. We were also able to demonstrate that our interrogated TMA population is representative of all stage IV patients in our database. Furthermore, by making use of AQUA technology and a better quality antibody (UMB-2), we were able to analyze CXCR4 expression quantitatively using IHC in all specimens studied and determine more precisely the specific localization of the receptor.

In previous studies assessing the expression of CXCR4 in lung tumors, localization of the receptor was generally seen in both the nucleus and cytoplasm/membrane of NSCLC tumor cells.[29–32] However, there has been a great deal of inconsistency in these studies in terms of the associations found between the expression and localization of CXCR4 and clinical outcome. Nuclear CXCR4 expression has been associated with a better prognosis,[29] has had no effect on outcome,[31] and has also been associated with lymph node metastasis.[32] On the other hand, total mRNA expression has been associated with a better clinical outcome,[30] whereas

cytomembraneous CXCR4 expression has been shown to confer a worse prognosis.[31]

Much of this inconsistency may be due to the use of undercharacterized mouse monoclonal antibodies in these studies, which have not been thoroughly tested for specificity in formalin-fixed paraffin-embedded tissues,[34] as well as the potential subjectivity in the analysis of semiquantitative IHC staining. Our findings demonstrate that CXCR4 has a predominantly cytomembraneous expression in NSCLC tumor cells and as CXCR4 is known to be a surface receptor, we would expect a greater proportion of the expression seen to be cytomembranous. The UMB-2 rabbit monoclonal antibody used was extensively characterized and shown to accurately detect membrane receptors while showing little staining in the cell nucleus,[34] which is more compatible with the known function and signaling of this receptor.[63]

The possible involvement of the CXCR4/SDF-1 axis in cancer is an attractive pathway to investigate because it helps explain the Paget "seed and soil" phenomenon associated with metastasis. The two key components of metastasis—acquisition of the capability to break away from the primary tumor to become blood or lymph borne and the subsequent ability to home in on its metastatic destination—can both be influenced by the CXCR4/SDF-1 axis. Increased activation of this pathway can confer on the malignant cell a greater ability to migrate and invade,[17,64] whereas the constitutive release by stromal cells from common sites of metastasis such as bone marrow, lung, and liver can guide the circulating cell to home in on its metastatic destination.[65] The role played by the CXCR4/SDF-1 axis in leukocyte trafficking and homing of stem cells[66,67] is likely analogous to organ selective metastasis of cancer stem cells.[68] In this model, metastatic potential of a cell will be determined by surface CXCR4 expression, whereas its destiny will be influenced by local SDF-1 secretion at distal sites. As such, the CXCR4/SDF-1 axis can help explain the "nature" underlying metastatic tendency (CXCR4 expression), as well as the "nurture" of that tendency (SDF-1 secretion at metastatic sites).

In summary, we report that CXCR4 is commonly expressed in stage IV NSCLC and is therefore a potential therapeutic target in this disease. In addition, we suggest that CXCR4 may also have a gender-dependent prognostic significance because women whose tumors overexpress this receptor seem to have a significantly worse survival. Further studies are needed to validate these findings in other sample series and to shed light on the possible association between CXCR4 and ER function in NSCLC.

ACKNOWLEDGMENTS

Supported by the ACRI Strategic Initiative for the Treatment of Lung Cancer in Alberta (grant no. 24498), The Look Lung Cancer Initiative at the Tom Baker Cancer Centre and University of Calgary, and the Glans Charity Golf Tournament.

REFERENCES

1. Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 2002;346:92–98.
2. Scagliotti GV, De Marinis F, Rinaldi M, et al. Phase III randomized trial comparing three platinum-based doublets in advanced non-small-cell lung cancer. *J Clin Oncol* 2002;20:4285–4291.
3. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* 2003;290:2149–2158.
4. Sandler AB. Molecular targeted agents in non-small-cell lung cancer. *Clin Lung Cancer* 2003;5(Suppl 1):S22–S28.
5. Winton T, Livingston R, Johnson D, et al. Vinorelbine plus cisplatin vs. observation in resected non-small-cell lung cancer. *N Engl J Med* 2005;352:2589–2597.
6. Olaussen KA, Dunant A, Fouret P, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med* 2006;355:983–991.
7. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000;12:121–127.
8. Schier AF. Chemokine signaling: rules of attraction. *Curr Biol* 2003;13: R192–R194.
9. Baggiolini M, Dewald B, Moser B. Interleukin-8 and related chemotactic cytokines—CXC and CC chemokines. *Adv Immunol* 1994;55:97–179.
10. Ma Q, Jones D, Springer TA. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* 1999;10:463–471.
11. Aiuti A, Webb IJ, Bleul C, et al. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med* 1997;185:111–120.
12. Hillyer P, Mordelet E, Flynn G, et al. Chemokines, chemokine receptors and adhesion molecules on different human endothelia: discriminating the tissue-specific functions that affect leucocyte migration. *Clin Exp Immunol* 2003;134:431–441.
13. Cho HH, Kyoung KM, Seo MJ, et al. Overexpression of CXCR4 increases migration and proliferation of human adipose tissue stromal cells. *Stem Cells Dev* 2006;15:853–864.
14. Kucia M, Reca R, Miekus K, et al. Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. *Stem Cells* 2005;23:879–894.
15. Alsayed Y, Ngo H, Runnels J, et al. Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. *Blood* 2007;109:2708–2717.
16. Fernandis AZ, Prasad A, Band H, et al. Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. *Oncogene* 2004; 23:157–167.
17. Kang H, Watkins G, Parr C, et al. Stromal cell derived factor-1: its influence on invasiveness and migration of breast cancer cells in vitro, and its association with prognosis and survival in human breast cancer. *Breast Cancer Res* 2005;7:R402–R410.
18. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001;410:50–56.
19. Smith MC, Luker KE, Garbow JR, et al. CXCR4 regulates growth of both primary and metastatic breast cancer. *Cancer Res* 2004;64:8604–8612.
20. Jiang YP, Wu XH, Shi B, et al. Expression of chemokine CXCL12 and its receptor CXCR4 in human epithelial ovarian cancer: an independent prognostic factor for tumor progression. *Gynecol Oncol* 2006;103:226–233.
21. Zeelenberg IS, Ruuls-Van Stalle L, Roos E. The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. *Cancer Res* 2003;63:3833–3839.
22. Hu J, Deng X, Bian X, et al. The expression of functional chemokine receptor CXCR4 is associated with the metastatic potential of human nasopharyngeal carcinoma. *Clin Cancer Res* 2005;11:4658–4665.
23. Yoon Y, Liang Z, Zhang X, et al. CXC chemokine receptor-4 antagonist blocks both growth of primary tumor and metastasis of head and neck cancer in xenograft mouse models. *Cancer Res* 2007;67:7518–7524.
24. Koshiba T, Hosotani R, Miyamoto Y, et al. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression. *Clin Cancer Res* 2000;6: 3530–3535.
25. Mori T, Doi R, Koizumi M, et al. CXCR4 antagonist inhibits stromal cell-derived factor 1-induced migration and invasion of human pancreatic cancer. *Mol Cancer Ther* 2004;3:29–37.

26. Su L, Zhang J, Xu H, et al. Differential expression of CXCR4 is associated with the metastatic potential of human non-small cell lung cancer cells. *Clin Cancer Res* 2005;11:8273–8280.
27. Phillips RJ, Burdick MD, Lutz M, et al. The stromal derived factor-1/CXCL12-CXC chemokine receptor 4 biological axis in non-small cell lung cancer metastases. *Am J Respir Crit Care Med* 2003;167:1676–1686.
28. Phillips RJ, Mestas J, Gharaee-Kermani M, et al. Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. *J Biol Chem* 2005;280:22473–22481.
29. Spano JP, Andre F, Morat L, et al. Chemokine receptor CXCR4 and early-stage non-small cell lung cancer: pattern of expression and correlation with outcome. *Ann Oncol* 2004;15:613–617.
30. Minamiya Y, Saito H, Takahashi N, et al. Expression of the chemokine receptor CXCR4 correlates with a favorable prognosis in patients with adenocarcinoma of the lung. *Lung Cancer* 2009;68:466–471.
31. Wagner PL, Hyjek E, Vazquez MF, et al. CXCL12 and CXCR4 in adenocarcinoma of the lung: association with metastasis and survival. *J Thorac Cardiovasc Surg* 2009;137:615–621.
32. Na IK, Scheibenbogen C, Adam C, et al. Nuclear expression of CXCR4 in tumor cells of non-small cell lung cancer is correlated with lymph node metastasis. *Hum Pathol* 2008;39:1751–1755.
33. Wald O, Izhar U, Amir G, et al. CD4+CXCR4highCD69+ T cells accumulate in lung adenocarcinoma. *J Immunol* 2006;177:6983–6990.
34. Fischer T, Nagel F, Jacobs S, et al. Reassessment of CXCR4 chemokine receptor expression in human normal and neoplastic tissues using the novel rabbit monoclonal antibody UMB-2. *PLoS One* 2008;3:e4069.
35. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med* 2002;8:1323–1327.
36. Contal C, O'Quigley J. An application of changepoint methods in studying the effect of age on survival in breast cancer. *Comput Stat Data Analysis* 1999;30:253–270.
37. Wellek S. Testing Statistical Hypotheses of Equivalence. New York, NY: Chapman and Hall/CRC, 2003.
38. Therneau TGP. Modelling Survival Data: Extending the Cox Model. New York, NY: Springer-Verlag, 2000.
39. Somers RH. A new asymmetric measure of association for ordinal variables. *Am Soc Rev* 1962;27:799–811.
40. Harrell FE. Regression Modeling Strategies with Applications to Linear Models, Logistic Regression, and Survival Analysis. New York, NY: Springer-Verlag, 2001.
41. SAS/STAT Software, version 9.2 [program]. Cary, NC: SAS Institute Inc., 2008.
42. A Language and Environment for Statistical Computing [program]. 2.1 version. Vienna: R Foundation for Statistical Computing, 2009.
43. Minami H, Yoshimura M, Miyamoto Y, et al. Lung cancer in women: sex-associated differences in survival of patients undergoing resection for lung cancer. *Chest* 2000;118:1603–1609.
44. Ferguson MK, Wang J, Hoffman PC, et al. Sex-associated differences in survival of patients undergoing resection for lung cancer. *Ann Thorac Surg* 2000;69:245–249; discussion 249–250.
45. Cerfolio RJ, Bryant AS, Scott E, et al. Women with pathologic stage I, II, and III non-small cell lung cancer have better survival than men. *Chest* 2006;130:1796–1802.
46. Radzikowska E, Glaz P, Roszkowski K. Lung cancer in women: age, smoking, histology, performance status, stage, initial treatment and survival. Population-based study of 20 561 cases. *Ann Oncol* 2002;13:1087–1093.
47. Visbal AL, Williams BA, Nichols FC III, et al. Gender differences in non-small-cell lung cancer survival: an analysis of 4,618 patients diagnosed between 1997 and 2002. *Ann Thorac Surg* 2004;78:209–215; discussion 215.
48. Wakelee HA, Wang W, Schiller JH, et al. Survival differences by sex for patients with advanced non-small cell lung cancer on Eastern Cooperative Oncology Group trial 1594. *J Thorac Oncol* 2006;1:441–446.
49. Hsu LH, Chu NM, Liu CC, et al. Sex-associated differences in non-small cell lung cancer in the new era: is gender an independent prognostic factor? *Lung Cancer* 2009;66:262–267.
50. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 2005;353:123–132.
51. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–2139.
52. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–1500.
53. Rosell R, Moran T, Queralt C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009;361:958–967.
54. Sauve K, Lepage J, Sanchez M, et al. Positive feedback activation of estrogen receptors by the CXCL12-CXCR4 pathway. *Cancer Res* 2009;69:5793–5800.
55. Beattie CW, Hansen NW, Thomas PA. Steroid receptors in human lung cancer. *Cancer Res* 1985;45:4206–4214.
56. Cagle PT, Mody DR, Schwartz MR. Estrogen and progesterone receptors in bronchogenic carcinoma. *Cancer Res* 1990;50:6632–6635.
57. Kaiser U, Hofmann J, Schilli M, et al. Steroid-hormone receptors in cell lines and tumor biopsies of human lung cancer. *Int J Cancer* 1996;67:357–364.
58. Fasco MJ, Hurteau GJ, Spivack SD. Gender-dependent expression of alpha and beta estrogen receptors in human nontumor and tumor lung tissue. *Mol Cell Endocrinol* 2002;188:125–140.
59. Kawai H, Ishii A, Washiya K, et al. Estrogen receptor alpha and beta are prognostic factors in non-small cell lung cancer. *Clin Cancer Res* 2005;11:5084–5089.
60. Schwartz AG, Prysak GM, Murphy V, et al. Nuclear estrogen receptor beta in lung cancer: expression and survival differences by sex. *Clin Cancer Res* 2005;11:7280–7287.
61. Lee JJ, Maeng CH, Baek SK, et al. The immunohistochemical overexpression of ribonucleotide reductase regulatory subunit M1 (RRM1) protein is a predictor of shorter survival to gemcitabine-based chemotherapy in advanced non-small cell lung cancer (NSCLC). *Lung Cancer* 2010;70:205–210.
62. Ugocsai K, Mandoky L, Tiszlavicz L, et al. Investigation of HER2 overexpression in non-small cell lung cancer. *Anticancer Res* 2005;25:3061–3066.
63. Ganju RK, Brubaker SA, Meyer J, et al. The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. *J Biol Chem* 1998;273:23169–23175.
64. Sutton A, Friand V, Brule-Donneger S, et al. Stromal cell-derived factor-1/chemokine (C-X-C motif) ligand 12 stimulates human hepatoma cell growth, migration, and invasion. *Mol Cancer Res* 2007;5:21–33.
65. Pituch-Noworolska A, Majka M, Janowska-Wieczorek A, et al. Circulating CXCR4-positive stem/progenitor cells compete for SDF-1-positive niches in bone marrow, muscle and neural tissues: an alternative hypothesis to stem cell plasticity. *Folia Histochem Cytobiol* 2003;41:13–21.
66. Lapidot T, Dar A, Kollet O. How do stem cells find their way home? *Blood* 2005;106:1901–1910.
67. Wang J, Loberg R, Taichman RS. The pivotal role of CXCL12 (SDF-1)/CXCR4 axis in bone metastasis. *Cancer Metastasis Rev* 2006;25:573–587.
68. Geminder H, Sagi-Assif O, Goldberg L, et al. A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the development of bone marrow metastases in neuroblastoma. *J Immunol* 2001;167:4747–4757.