UNIVERSITY OF CALGARY

In Vitro and Clinical Studies on the Role of the CXCR4/SDF-1 Chemokine Axis in Non-Small Cell Lung Cancer

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

MAY, 2011

UNIVERSITY OF
CALGARY

Abstract

The CXCR4/SDF-1 chemokine axis has been shown to play a significant role in the metastatic process *in vitro* and is associated with a poor clinical outcome in several cancers, including non-small cell lung cancer (NSCLC). This thesis explored the role of CXCR4 in NSCLC using two different approaches: 1) the role of CXCR4 in cellular migration was investigated by assessing the migrational capacity of NSCLC cell lines after CXCR4/SDF-1 axis inhibition, and 2) clinical outcome in stage IV and resected early stage NSCLC patients was investigated in relation to the expression of CXCR4 in *ex vivo* tumor specimens. These studies demonstrated that SDF-1 induces *in vitro* migration of NSCLC cells and that inhibition of either CXCR4 or SDF-1 results in decreased cellular migration. Intrestingly, it was additionally found that CXCR4 was an independent prognostic biomarker in females with stage IV NSCLC. Together, the results of these investigations suggest that CXCR4 plays an important, yet complex role in the metastasis of NSCLC.

Acknowledgements

First and foremost I have to thank my supervisor and mentor, Dr. Gwyn Bebb for his guidance, support, advice and encouragment throughout this whole experience. And for his unwavering confidence in me, without which I don't think I would be where I am today. I would like to thank my family and friends for their support, understanding and patience in putting up with an often exhausted and stressed out graduate student like myself. In particular, I would like to thank Anna and Mike for their willingness to listen to the daily trials and tribulations of grad school, and for convincing me to stick with it in my many moments of uncertainty. I also have to thank the Magliocco Lab, in particular Alexander Klimowicz, for their indispensable collaboration and endless expertise. I also have to thank the other members of the Bebb and Morris labs, especially Huong, for their training in the lab and willingness to pass on their vast scientific knowledge. And lastly, I want to thank everyone else who helped me from the Translational Laboratories and the Tom Baker Cancer Centre, for making my time here that much easier and more enjoyable.

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AJCC	American Joint Committee on Cancer
ATCC	American Type Culture Collection
BAC	Bronchioloalveolar Carcinoma
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CT	Computed Tomography
DFS	Disease Free Survival
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
FACS	Fluorescent Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Adminstration
FFPE	Formalin Fixed Paraffin Embedded
FITC	Fluorescein isothiocyanate
GPCR	G Protein Coupled Receptor
G-CSF	Granulocyte Colony Stimulating Factor
HIF-1	Hypoxia Inducible Factor 1
HIV	Human Immunodeficiency Virus
HRT	Hormone Replacement Therapy
IASLC	International Association for the Study of Lung Cancer

IHC	Immunohistochemistry
IgG	Immunoglobulin G
MAb	Monoclonal Antibody
MAPK	Mitogen Activation Protein Kinase
MMP	Matrix Metalloproteinase
MTA	Material Transfer Agreement
NF-κB	Nuclear Factor κB
NSCLC	Non Small Cell Lung Cancer
OS	Overall Survival
PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PET	Positron Emission Technology
PI3K	Phosphatidylinositol 3 Kinase
RNA	Ribonucleic Acid
SCLC	Small Cell Lung Cancer
SDF-1	Stromal Cell Derived Factor-1
SEER	Survival, Epidemiology and End Results
siRNA	Short Interfering Ribonucleic Acid
TMA	Tissue Micro Array
TKI	Tyrosine Kinase Inhibitor
TNM	Tumor Node Metastasis
VEGF	Vascular Endothelial Growth Factor

WHIM	Warts, Hypogammaglobulinemia, Infections and Myelokathexia
WHO	World Health Organization

Epigraph

Patience and tenacity are worth more than twice their weight of cleverness.
-Thomas Huxley

Science is simply common sense at its best, that is, rigidly accurate in observation, and merciless to fallacy in logic.
-Thomas Huxley

Chapter One: INTRODUCTION

Excerpts from this chapter were published as a review in December 2008 in the Journal of Thoracic Oncology. S. Otsuka, and G. Bebb (2008). "The CXCR4/SDF-1 chemokine receptor axis: a new target therapeutic for non-small cell lung cancer." J Thorac Oncol **3**(12): 1379-83.

Lung cancer continues to be the most common cause of cancer death in North America, killing more people every year than breast, colon and prostate cancer combined (CCS 2011; NCI 2011). According to the Canadian Cancer Society, an estimated 24,100 people were diagnosed with lung cancer in Canada in 2010 with (CCS 2011), of which approximately 85% will eventually succumb to their disease. There are two main types of lung cancer – small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for approximately 85% of all lung cancers, while SCLC accounts for the remaining 15% of cases (Sher et al. 2008).

Since the majority of NSCLC patients present with advanced or metastatic disease, overall five-year survival is less than 15% (Jemal et al. 2006). The adoption of platinum based chemotherapy has pushed median overall survival in the metastatic setting from 4-6 months to 8-10 months (Scagliotti et al. 2002; Schiller et al. 2002), yet despite the advent of targeted therapy (Kris et al. 2003; Sandler 2003) metastatic NSCLC remains incurable. Just as discouraging is the high rate of distant recurrence for resected stage I and II disease, despite adjuvant treatment (Olaussen et al. 2006; Winton et al. 2005). Similarly, the use of combined modality concurrent chemo-radiation for unresectable stage III cases provides long term remission in only 15-20% of patients with the majority succumbing to distal metastatic disease (Rigas et al. 2007). Without doubt, the primary source of morbidity and mortality in NSCLC is metastatic spread. The need for new strategies that reduce metastasis and limit the early stage dissemination of NSCLC cannot be overstated.

The following thesis addresses this issue of metastasis by attempting to investigate one of the most crucial aspects of the metastatic process, cellular migration, and in particular, what role the CXCR4/SDF-1 chemokine/receptor axis has in the migration of NSCLC cell lines *in vitro.* Demonstration of successful inhibition of the CXCR4/SDF-1 axis could open the door to possible therapeutic avenues in the prevention and treatment of NSCLC metastasis. This thesis also attempts to shed light on the clinical relevance of CXCR4 in NSCLC patients by assessing the expression of CXCR4 in patient tissue specimens and examines its association with clinical outcome.

1.1 Non Small Cell Lung Cancer

1.1.1 NSCLC Epidemiology, Etiology, and Carcinogenesis

Once a rare cancer (0.5% of all malignancies at the turn of the 20th century)(Spiro et al. 2005), lung cancer is now the most commonly diagnosed malignancy in the world and the number one cancer killer for both men and women (Coate et al. 2009; Jemal et al. 2009). Since the beginning of the 20th century, the incidence of lung cancer has risen dramatically, primarily due to increases in smoking. Recently, smoking rates have declined in many developed countries, but despite this, smoking (including second hand smoke) still contributes to approximately 85% of lung cancer cases and is still the predominant cause of lung cancer worldwide (Alberg et al. 2005; Parkin et al. 1994; Spira et al. 2004). And unfortunately, smoking rates are still increasing in many developing countries such as China (Zhang et al. 2003), which means that the overall global incidence of lung cancer is likely going to continue to rise (Molina et al. 2008).

Tobacco smoke is known to contain multiple carcinogens that contribute to the carcinogenesis of lung cancer including NSCLC. Approximately 60-70 definitive as well as potential carcinogens have been identified in cigarette smoke, however polycyclic aromatic hydrocarbons (PAH's) and nitrosamine compounds appear to have the most direct and damaging carcinogenic effects (Hecht 2002). PAH's and nitrosamines are metabolized to reactive metabolites in lung tissue and can directly form DNA adducts in cells (Hecht et al. 1993; Phillips 2002). The presence of DNA adducts increases the rate of DNA repair which can lead to the formation of mutations, and can also prevent the binding of various polymerases which can lead to DNA strand breaks causing genetic instability that ultimately leads to carcinogenesis of the lung (Phillips 2005; Shrivastav et al. 2010). Cigarette smoke also contains many compounds which can indirectly promote carcinogenesis. For example, radioactive elements such as polonium are deposited in the lungs where they emit alpha particles (Khater 2004); free radical species can induce oxidative damage (Rahman 2003); and certain metals (such as cadmium and chromium) may enhance the carcinogenicity of other DNA damaging agents (Costa 1997; Waalkes 2003). In addition, cigarette smoke is known to trigger prolonged inflammatory reactions in the lungs which can also increase the risk of developing lung cancer (Coussens et al. 2002; Rahman 2003).

However, only about 20% of smokers will ever develop lung cancer suggesting that genetic factors may predispose certain individuals to develop this disease. Individual differences or mutations in genes involved in DNA repair pathways (Bailey-Wilson et al. 2004; Wei et al. 1996; Zheng et al. 2007; Zienolddiny et al. 2006), carcinogen metabolism and detoxification (Liu et al. 2005), and cell cycle control (Burke et al. 2005;

Coate et al. 2009) may influence individual susceptibility to, and the development of lung cancer. These gene mutations may be acquired somatic mutations, or inherited germ line mutations. Familial aggregation of lung cancer has been demonstrated which suggests there may be a genetic basis to this disease (Bailey-Wilson et al. 2004; Hung et al. 2008; Hwang et al. 2003; Li et al. 2004). Studies have shown associations between loci on chromosome 6 (Bailey-Wilson et al. 2004) and chromosome 15 (Hung et al. 2008) with increased susceptibility to lung cancer. In addition, germ line mutations in the P53 (Hwang et al. 2003), ERCC1 (Yu et al. 2008), and EGFR genes (Bell et al. 2005; Li & Hemminki 2004), among others (Bailey-Wilson et al. 2004), have also been reported to increase risk.

On the other hand, up to 20% of the patients diagnosed with lung cancer have never smoked suggesting that there are other contributing factors in the carcinogenesis of lung cancer. Environmental cigarette smoke (Husgafvel-Pursiainen et al. 2000), other environmental factors such as air pollution (Boffetta 2006; Vineis et al. 2006), certain occupational exposures (Boffetta et al. 1997; Siemiatycki et al. 2004), and exposure to radon (Darby et al. 2005) may play a role. In fact, recent work has suggested that lung cancer in patients who have never smoked may actually be considered a different disease than lung cancer caused by the carcinogens in cigarette smoke (Subramanian et al. 2007; Sun et al. 2007).One difference is that lung cancer in never smokers tends to have a different genetic and molecular profile. For example, there are differences in the spectrum of p53 and EGFR gene mutations in smokers versus never smoker (Rudin et al. 2009; Samet et al. 2009). There are also other variations in the age of onset, histology and

response to targeted therapy between in lung cancer patients who have smoked compared to those who have not (Samet et al. 2009).

1.1.2 NSCLC Staging, Histology, Treatment and Prognosis

As stated before, the majority of NSCLC patients present with advanced disease (Herbst et al. 2008; Molina et al. 2008). This is primarily due to rapid disease course and the absence of symptoms in the early stages of the disease. Most patients are symptomatic at the time of diagnosis, and common symptoms can include prolonged cough, dypsnea or hemoptysis. Only in about 10% of cases is the cancer found in a patient without symptoms, for example, on routine chest x-ray performed for unrelated reasons. Pathological diagnoses of NSCLC are generally made by either histological (resection or tissue biopsy) or cytological (fine needle aspiration, bronchial washing, pleural effusion or sputum sample) means, the method used being dependent primarily on tumor size and location, as well as age and patient comorbidities (mainly other smoking related issues such as chronic obstructive pulmonary disease, emphysema and coronary heart disease). It also is not uncommon for either patient and/or clinician to decide that pursuing a pathological diagnosis of NSCLC is unnecessary. This is normally based on advanced age, patient comorbidities, or the absence of viable treatment options regardless of diagnosis.

Specific pathological diagnoses are made when adequate tissue is available. This includes the tissue histopathology (usually based on cellular/tissue morphology and sometimes immunohistochemical stains), as well as grade or extent of cellular differentiation. According to the World Health Organization (WHO) and International

Association for the Study of Lung Cancer (IASLC), the major subtypes of NSCLC are squamous cell carcinoma, adenocarcinoma (which includes bronchioloalveolar carcinoma (BAC)), large cell carcinoma (which includes large cell neuroendocrine carcinoma), adenosquamous carcinoma, carcinomas with sarcomatous elements, and carcinoid tumors (both typical and atypical). Up until recently, it was only necessary to differentiate the tumor into either the small cell lung cancer (SCLC) or NSCLC classifications due to differences in treatment plans for the two sub-types of lung cancer. However, with the development of newer NSCLC targeted agents (such as EGFR TKIs and Bevacizumab) which behave differently in tumors of different NSCLC histologies (usually squamous versus non-squamous differentiation), it is becoming more important for pathologists to attempt to provide a specific NSCLC histological diagnosis (Gazdar 2010).

Squamous cell carcinoma and adenocarcinoma are by far the most commonly encountered tumor histologies (Herbst et al. 2008). Squamous cell carcinoma was once the commonest NSCLC histology diagnosed in most countries, however, adenocarcinoma is now the most common histology in both North America and Asia (Travis et al. 1995). Squamous cell carcinoma is still the dominant tumor histology seen in some European countries, mainly in Eastern European, however the incidence of adenocarcinoma has been steadily increasing (Janssen-Heijnen et al. 2003; Radzikowska et al. 2002). This trend towards a decreasing incidence of squamous cell carcinoma may be due to declining rates of smoking and/or the adoption of low tar filter cigarettes in many countries (Gazdar 2010; Janssen-Heijnen & Coebergh 2003; Stellman et al. 1997; Thun et al. 1997).

Once a pathological diagnosis (if possible) of NSCLC is made, the patient's disease must be staged in order to determine the full extent of the disease, which in turn will determine the appropriate treatment options (Molina et al. 2008). NSCLC is staged according to the TNM (Tumor, Nodes, Metastasis) system using diagnostic imaging means (ie. computed tomography (CT), positron emission technology (PET imaging), nuclear bone scan) as well as more invasive staging techniques (bronchoscopy and mediastinoscopy) (Marom et al. 1999; Molina et al. 2008; Valk et al. 1995). Recently, there has been a revision (7^{th} Edition) (AJCC 2010) to the TNM system for NSCLC staging by the American Joint Committee on Cancer (AJCC) (Table 1.1). A summary of the AJCC's previous 6^{th} Edition of NSCLC staging can be found in Appendix A. According to the new staging system, patients are designated stage I if their disease is limited to a solitary lung mass less than 7 cm in size. The primary and most successful treatment for patients with stage I disease is resection (lobectomy, wedge resection or pneumonectomy) as long as the patient has no comorbidities which preclude surgery (Molina et al. 2008). Stage II patients differ from stage I patients in that they have lobar or hilar lymph node involvement in addition to a lung nodule, or a solitary lung nodule exceeding 7 cm in diameter. These patients are also treated with surgery and adjuvant chemotherapy if it is indicated (Arriagada et al. 2004; Molina et al. 2008; Socinski 2004; Winton et al. 2005).

Stage III disease comprises a heterogeneous group of patients, which includes stage IIIA, defined as ipsilateral mediastinal lymph node involvement, but may also include ipsilateral lung involvement (ie. different lobe than the primary tumor in the same lung), or large tumor size (> 7cm) plus at least hilar node involvement. These patients are

treated with surgery if indicated, but more commonly with combined chemo-radiotherapy with curative intent. Patients designated Stage IIIB include those with contralateral mediastinal or hilar nodes, those with any neck lymph node involvement (cervical or supraclavicular) or those with both ipsilateral lung as well as mediastinal involvement. The primary treatment for stage IIIB patients may also include combined chemo-radiotherapy (Sause et al. 1995), or palliative chemotherapy and/or radiotherapy (Pfister et al. 2004).

Lastly, stage IV patients include those whose disease has metastasized locally (to the contralateral lung or pleural effusion; designated M1a) or distantly (bone, brain, liver, adrenals etc.; designated M1b). Stage IV NSCLC is considered incurable. Treatment is palliative and may include platinum based systemic chemotherapy (D'Addario et al. 2005; Pfister et al. 2004) and/or palliative radiation for local symptom control. Targeted therapies, such as those targeting the epidermal growth factor receptor (EGFR) (Erlotinib) or vascular endothelial growth factor (VEGF), may be indicated for second or third line systemic therapy, for those who fail first line therapy (Kris et al. 2003), or in conjunction with standard chemotherapy (Sandler et al.). In specific cases, first line EGFR tyrosine kinase inhibitor (TKI) targeted therapy using the drug Gefitinib, is indicated for those patients whose tumors harbour a mutation in the EGFR (Lynch et al. 2004; Mok et al. 2009; Paez et al. 2004).

In terms of prognosis, diagnostic stage is the most relied upon indicator of patient prognosis and survival estimates are usually quoted based purely on disease stage. According to the National Cancer Institute’s Survival, Epidemiology, and End Results

(SEER) Database (SEER 2011), the 5 year relative survival rates for patients with local (stage I and II), regional (stage III) and distant (stage IV) stage disease are 49%, 16% and 2% respectively, and the overall five year survival rate for all patients with NSCLC is only 15%. Although diagnostic stage is the primary indicator of survival, there are other factors which may be prognostic indicators in NSCLC. Histologic tumor grade has been shown to be an independent prognostic factor in patients with unresectable disease. Patients with poorly differentiated tumors have an increased risk of death compared to patients with well differentiated tumors (Sun et al. 2006). Also, it has been shown that gender is an independent prognostic marker, with females exhibiting increased survival compared to males (Cerfolio et al. 2006; Visbal et al. 2004) regardless of disease stage. Other potential prognostic factors include weight loss and performance status (in advance stage patients) (Albain et al. 1991), and tumor size and vascular invasion of the tumor (in patients with early stage resected disease).

TABLE 1.1 American Joint Committee on Cancer non small cell lung cancer TNM staging 7th Edition and relative 5 year survival rates

Relative 5 year Survival	NSCLC Stage	T	N	M
Local 49%	**Stage IA**	T1a, 1b	N0	M0
	Stage IB	T2a	N0	M0
	Stage IIA	T1a, 1b	N1	M0
		T2a	N1	M0
		T2b	N0	M0
	Stage IIB	T2b	N1	M0
		T3	N0	M0
Regional 16%	**Stage IIIA**	T1, T2	N2	M0
		T3	N1, N2	M0
		T4	N0, N1	M0
	Stage IIIB	T4	N2	M0
		Any T	N3	M0
Distant 2%	**Stage IV**	Any T	Any N	M1a, 1b

TNM Descriptors *

T – Primary Tumor
- T1 Tumor 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without evidence of invasion more proximal than the main bronchus
  - T1a Tumor ≤ 2 cm in greatest dimension
  - T1b Tumor > 2 cm but ≤ 3 cm in greatest dimension
- T2 Tumor more than 3 cm but not more than 7 cm; or tumor with any of the following features – involves main bronchus (2 cm or more distal to the carina), invades visceral pleura
  - T2a Tumor > 3 cm but ≤ 5 cm in greatest dimension
  - T2b Tumor > 5 cm but ≤ 7 cm in greatest dimension
- T3 Tumor > 7 cm or one that directly invades any of the following: chest wall, diaphragm, phrenic nerve, medisastinal pleura, parietal pericardium; or tumor in main bronchus less than 2 cm distal to the carina but without involvement of the carina
- T4 Tumor of any size that invades any of the following: mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina; or separate tumor nodule(s) in a different ipsilateral lobe to that of the primary.

N – Regional Lymph Nodes
- N0 No regional lymph node metasatsis
- N1 Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes
- N2 Metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes
- N3 Metastasis in contralateral mediastinal or hilar nodes, or involvement of the supraclavicular or cervical lymph nodes

M – Distance Metastasis
- M0 No distant metastasis
- M1 Distant metastasis
  - M1a Separate tumor nodule(s) in a contralateral lobe; tumor with pleural nodules or a malignant pleural or pericardial effusion
  - M1b Distant Metastasis outside the thorax

* Adopted from the Surveillance, Epidemiology and End Results (SEER) database (1975-2007), National Cancer Institute (SEER 2011)

† Adopted from the American Joint Committee on Cancer (AJCC) 7th Edition. (AJCC 2010)

1.1.3 Gender Discrepancies in Lung Cancer

Once a disease affecting mainly men, lung cancer incidence has increased dramatically in females over the latter half of the 20th century. In fact, some have termed the increased incidence of lung cancer in women a "contemporary epidemic" (Patel 2009; Patel et al. 2004). This has been primarily due to the change in smoking rates for females earlier on in the century. In contrast, the incidence of lung cancer in males has reached a plateau and is reversing the ratio of females to males with lung cancer (Paggi et al. 2010). There also may be gender differences in the susceptibility to the carcinogens present in cigarette smoke. Studies have suggested that females who smoke may have a higher risk of developing lung cancer than their male counterparts (Henschke et al. 2004; Zang et al. 1996). In particular, it seems that females may be more susceptible to the DNA damage (DNA adducts, p53 gene mutations) caused by PAH's in tobacco smoke (Kure et al. 1996; Mollerup et al. 1999; Toyooka et al. 2003), in addition to a decreased ability to repair the damage done to DNA (Wei et al. 2000). These factors may lead to the reported increased risk of lung cancer in women compared to men, however there are other conflicting reports which failed to find a gender difference in the risk of lung cancer (Bain et al. 2004; Freedman et al. 2008).

In addition to differences in incidence rates, there are gender discrepancies in many other aspects of this disease. In terms of age of diagnosis, females tend to be diagnosed with lung cancer at a younger age than males (Paggi et al. 2010). It was demonstrated in multiple studies that the median age of diagnosis for women was significantly younger than that of males (Hsu et al. 2009; Radzikowska et al. 2002; Wakelee et al. 2006). On the other hand, it appears that even among non smokers, women

are at increased risk of lung cancer. Many have reported that there is a larger proportion of non smoking women than non smoking men with lung cancer (de Perrot et al. 2000; Wu et al. 1996a). Interestingly, it seems that non smoking women of Asian ethnicity are particularly at risk of developing lung cancer (Hsu et al. 2009; Wakelee et al. 2007). And due to the link between smoking and tumor histology, there also tends to be a larger proportion of adenocarcinomas and bronchioloalveolar carcinomas in women when compared to men, who tend to be diagnosed with a larger proportion of squamous cell carcinomas (de Perrot et al. 2000; Radzikowska et al. 2002; Thun et al. 1997), although there also may be other causes for the gender difference seen in tumor histology.

In addition, it is also well known that there are gender-based outcome differences in NSCLC, both in treatment responses and in survival (Patel et al. 2004). Evidence exists in both early stage resected disease (Cerfolio et al. 2006; Ferguson et al. 2000; Minami et al. 2000), as well as in more advanced disease (Albain et al. 1991; Hsu et al. 2009; Radzikowska et al. 2002; Visbal et al. 2004; Wakelee et al. 2006) that females tend to have a better clinical outcome and increased survival when compared to males, despite the potential increased susceptibility of developing lung cancer in females. This also includes differences in treatment responses between the sexes (Shafer et al. 2009). It seems that regardless of histology, stage or specific therapy (surgery, radiotherapy, chemotherapy), women tend to have better responses to various treatment modalities than men, particularly platinum based chemotherapy (Albain et al. 1991; Ferguson et al. 2000; Werner-Wasik et al. 2000).

Furthermore, it is now evident that there are gender dependent differences in the responses to some novel targeted agents. For example, many clinical studies have shown that women tend to have better responses to the epidermal growth factor receptor tyrosine kinase inhibitor's (EGFR TKI's), in particular, women who are of South East Asian ethnicity, non smokers and have adenocarcinoma tumor histology (Kris et al. 2003; Lynch et al. 2004; Shepherd et al. 2005). Molecular analyses have subsequently shown that better response rates are seen in those individuals harbouring an activating mutation in the epidermal growth factor receptor (EGFR), rendering it constitutively active, and that these mutations are also more common in females (Paez et al. 2004; Rosell et al. 2009; Toyooka et al. 2007), although the etiology of this gender difference is unexplained.

Lastly, is the potential hormonal influence on development and outcome in NSCLC. It has long been known that the sex hormone estrogen plays a role in the pathogenesis and growth of breast, endometrial and ovarian cancers, and this has been exploited by the use of estrogen receptor (ER) and aromatase inhibitors now used extensively in the treatment of breast cancer (Tamoxifen) (Thomas et al. 2005). There are two types of ERs, ERα which is localized in the cytoplasm, and ERβ which is localized in the cell nucleus (Kawai et al. 2005) .There is evidence that a significant proportion of NSCLC tumors express ERα and/or ERβ (Beattie et al. 1985; Cagle et al. 1990; Omoto et al. 2001; Stabile et al. 2002), and that there may be a gender dependent difference in ER expression (Fasco et al. 2002; Kaiser et al. 1996). Despite this, there does not seem to be a consensus on whether the expression of ERα or ERβ has any bearing on clinical outcome in NSCLC (Kawai et al. 2005; Schwartz et al. 2005).

In addition, it was reported that different concentrations of sex hormones may play a role in the gender discrepancies seen in cancer susceptibility (Patel 2005; Taioli et al. 1994). It has been suggested that sex hormones are capable of modulating the activity of enzymes involved in DNA repair (Wei et al. 2000) and carcinogen metabolism (Kirsch-Volders et al. 2010) and that estrogen may be capable of producing DNA adducts directly (Yager et al. 1996). This is further supported by the evidence that there is an increased risk of lung cancer in post menopausal women taking hormone replacement therapy (HRT) (Adami et al. 1989; Ganti et al. 2006; Moore et al. 2003; Taioli & Wynder 1994).

1.2 Chemokines

Chemokines are a class of small chemoattractant cytokines with sizes ranging from 8-14 kDa (Zlotnik et al. 2000). Chemokines make up the largest family of cytokines and are the major regulators of cell trafficking and adhesion in the body (Schier 2003). Chemokines bind and activate a subset of seven transmembrane spanning G protein coupled receptors present on the surface of target cells (Murphy et al. 2000). To date approximately 50 chemokines and 20 chemokine receptors have been identified (Zlotnik & Yoshie 2000). Chemokines have roughly 30-70% homology in amino acid sequence and are divided into 4 major families (C, CC, CXC, CX_3C), although only 2 have been extensively characterized - the alpha and beta chemokines (Zlotnik & Yoshie 2000). The alpha chemokines are termed CXC chemokines and are named as such because their N-termini cysteine residues are separated by one amino acid. The beta chemokines on the other hand have adjacent cysteine residues in the N terminus region and are thus termed

CC chemokines. For the most part, there is considerable redundancy in the chemokine system allowing a chemokine to bind more than one receptor and vice versa, suggesting a certain amount of flexibility in signalling and regulation (Mantovani 1999; Murphy et al. 2000; Zlotnik et al. 2006).

The CXC class of chemokines can be further classified based on whether or not the chemokine has an ELR (Glu Leu Arg) motif in its N terminus region (Belperio et al. 2000). ELR+ CXC chemokines are angiogenic factors and are capable of regulating the directional migration of endothelial cells and have been shown to stimulate angiogenesis *in vivo*. On the other hand, ELR- CXC chemokines are actually capable of inhibiting angiogenesis (Strieter et al. 1995). Members of the ELR+ CXC chemokines include, but are not limited to CXCL1, CXCL2, CXCL6 and CXCL8 (Strieter et al. 1995).

In addition to the various chemokine classifications based primarily on structure, there are functional classifications for chemokines. They can be classified as either ‘inflammatory’ or ‘homeostatic’ based on their primary functional roles. The majority of chemokines function to regulate cell recruitment to sites of tissue infection and inflammation (Moser et al. 2004). These chemokines are inducible and play a crucial role in the host immune defence system by recruiting specific leukocyte populations (including lymphocytes, neutrophils, monocytes, and eosinophils) to sites of tissue damage and infection and are highly upregulated by inflammatory stimuli such as antigens, polyclonal stimulants, cell irritants and cytokines (Baggiolini et al. 1994; Taub et al. 1994).

On the other hand, some chemokines have a homeostatic function and are constitutively expressed in certain tissues or organs and play a role in immune surveillance by regulating the homeostatic trafficking of leukocytes (Moser & Willimann 2004). In addition, it is now known that chemokines are also expressed by many other cell types such as endothelial and epithelial cells, stromal cells, neurons as well as smooth muscle cells (Rollins 1997) and have roles in dendritic cell development, Th1 and Th2 responses, and angiogenesis (Zlotnik & Yoshie 2000). Lastly, chemokines are now believed to play a crucial role in many aspects of the malignant process, from primary tumor growth and development to metastasis (Tanaka et al. 2005; Zlotnik 2006).

1.3 The CXCR4/SDF-1 Chemokine Axis

The CXCR4 chemokine receptor and its ligand, stromal cell derived factor (SDF-1) constitute a receptor/chemokine axis that has attracted great interest in the past decade partly because CXCR4 is a target for HIV binding and entry into cells (Deng et al. 1996; Feng et al. 1996), but more recently due to its emerging role in malignancy. SDF-1 is a member of the alpha family of chemokines and is alternatively named CXCL12 (Zlotnik & Yoshie 2000). There are two main splice variants of SDF-1, SDF-1α and SDF-1β which both bind to CXCR4 with comparable affinity, however SDF-1α is the most predominant variant (Hesselgesser et al. 1998). SDF-1 is rather unique in that it is structurally a ELR- chemokine, however it is the one non ELR+ CXC chemokine that is still capable of promoting angiogenesis (Salcedo et al. 2003). In addition, it is the only CXC chemokine in which its gene is located on chromosome 10, where as all other CXC chemokine genes are located on chromosome 4. The SDF-1 gene also displays

remarkable homology between species (93% identical between murine and human) (Shirozu et al. 1995).

CXCR4 is a well described chemokine receptor that has a role in many facets of cellular movement. It consists of 352 amino acids and is a member of the G-protein coupled receptor (GPCR) superfamily (Loetscher et al. 1994). It was initially cloned from leukocytes (Loetscher et al. 1994) but has since been shown to be expressed by other cell types (Rossi et al. 2000). Similarly, SDF-1 was initially isolated from a bone marrow stromal cell line (Nagasawa et al. 1994) and was found to be a potent lymphocyte chemoattractant (Bleul et al. 1996b). Since then it has been demonstrated that SDF-1 is constitutively secreted by fibroblasts in several different organs/tissues including bone marrow, lymph nodes, lung, liver and muscle (Ratajczak et al. 2003; Zou et al. 1998).

Most chemokines bind more than one receptor and the majority of chemokine receptors bind more than one chemokine. Up until very recently there was however one exception in that the chemokine SDF-1 bound exclusively to CXCR4, and CXCR4 had SDF-1 as its only ligand (Horuk 2001). This exclusive relationship suggested that this chemokine axis played a uniquely important biological role. There is now evidence that SDF-1 binds to another chemokine receptor (see p 30) called CXCR7/RDC1 (Balabanian et al. 2005), which was cloned decades ago but only recently was linked to SDF-1 (Burns et al. 2006). However, the role of CXCR7 in normal physiology as well as in malignancy has yet to be fully investigated.

1.3.1 CXCR4/SDF-1 Normal Physiological Function

The CXCR4/SDF-1 axis plays an important biological role both during development and later on in adult life. During embryogenesis the CXCR4/SDF-1 axis plays a role in the directional migration of CXCR4+ endoderm and mesoderm layer cells to a SDF-1 gradient established by the ectoderms cells of the gastrula (Raz et al. 2009). It is also involved in organogenesis and large vessel development in the embryo as suggested by the complementary expression patterns of SDF-1 and CXCR4 in hematopoietic, cardiac, vascular and neuronal embryological systems (Zou et al. 1998), as well as the high levels of SDF-1 seen in areas of neovascularization (McGrath et al. 1999). In fact, Mcgrath *et al* demonstrated that CXCR4 was the most abundant chemokine expressed during early embryogenesis in a murine model of development, and that CXCR4 expression actually distinguished migrating from non-migrating tissues (McGrath et al. 1999).

In addition, the CXCR4/SDF-1 axis has a function in embryological hematopoiesis. It was demonstrated in knock-out studies that mice lacking the SDF-1 gene had significant defects in the colonization of embryonic bone marrow by hematopoietic stem cells derived from the foetal liver (Nagasawa et al. 1996). This resulted in a significant decrease in the number of B-cell and myeloid progenitor cells in the bone marrow causing a severe disruption in hematopoiesis. In subsequent studies with SDF-1 knockout mice, it was demonstrated that absence of the SDF-1 gene caused serious defects in the development of the heart (particularly aortopulmonary septum defects), brain and large vessels (Bagri et al. 2002; Lazarini et al. 2003; Nagasawa et al. 1996; Zou et al. 1998) supporting the role of this chemokine axis in organogenesis and

large vessel development. There is additional evidence from studies with CXCR4 gene knockouts, where it was demonstrated that the absence of the CXCR4 gene caused severe defects in hematopoiesis and organogenesis (Fuchs et al. 2004; Zou et al. 1998) reinforcing the importance of this chemokine axis during embryogenesis. Both CXCR4 and SDF-1 gene knockouts are lethal to developing embryos and as a result they die in utero.

Later on in adult life, the primary role of the CXCR4/SDF-1 axis is in the homing/retention of hematopoietic/lymphopoietic stem cells, pre T and B lymphocytes in the bone marrow (Aiuti et al. 1997; Ma et al. 1999; Petit et al. 2002) as well as the trafficking of these cells to sites of tissue inflammation (Bleul et al. 1996b). It is also involved in the development of these cells and plays a role in the survival of fully developed lymphocytes and the generation of memory T cells (Klein et al. 2004). Most important, is the role of CXCR4/SDF-1 axis in the homing of hematopoetic stem cells to the bone marrow, where stem cell niches are created to aid in the growth and differentiation of the cells (Fuchs et al. 2004). SDF-1 is constitutively released by bone marrow stromal cells which acts as a chemotactic agent to sequester the CXCR4+ stem cells in this environment (Aiuti et al. 1997; Dorshkind 1990; Sugiyama et al. 2006). In fact, it has been suggested that SDF-1 is the only chemokine which is capable of eliciting a chemotactic response from hematopoietic stem cells (Kucia et al. 2005; Wright et al. 2002), which further demonstrates the important role of the CXCR4/SDF-1 axis in stem cell homing to, and retention in the bone marrow (Aiuti et al. 1997; Burger et al. 2009; Lapidot et al. 2005; Peled et al. 1999).

Interestingly, this particular function of the CXCR4/SDF-1 has recently been exploited in the context of stem cell transplantations (Burger et al. 2006; Devine et al. 2004; Flomenberg et al. 2005a). It has been shown that an antagonist to CXCR4, AMD 3100, either alone or in combination with granulocyte colony-stimulating factor (G-CSF), is capable of inducing the mobilization of hematopoietic stems cells into the peripheral blood for harvesting for autologous stem cell transplant (Broxmeyer et al. 2005; Cashen et al. 2007; Flomenberg et al. 2005b; Liles et al. 2005). Blocking CXCR4 using the CXCR4 antagonist causes a decrease in SDF-1 induced CXCR4 activation and the accompanying chemotactic signals provided by the SDF-1 secreting bone marrow stromal cells, allowing the release of the hematopoietic stem cells from the bone marrow. These findings resulted in the recent approval of AMD 3100 by the United States Food and Drug Administration (FDA), under the trade name Mozobil (Genzyme, Cambridge, MA, USA), for stem cell mobilization in patients with non-hodgkins lymphoma and multiple myeloma undergoing autologous stem cell transplantations (Kessans et al. 2010; Pusic et al. 2010). In addition, studies have also indicated that this chemokine axis plays a role after stem cell transplantation by directing the newly engrafted hematopoietic stem cells into the bone marrow environment (Lapidot et al. 2005; Peled et al. 1999).

There are also other important homeostatic functions which the CXCR4/SDF-1 chemokine axis is involved in. For example, in the adult brain, it has been suggested that CXCR4 and SDF-1 play a role in the modulation of neurotransmission based on the finding that CXCR4 is constitutively expressed in mature neurons and an increase in intracellular calcium (Ca^{2+}) was seen upon stimulation with SDF-1 (Limatola et al. 2000). Also, since being identified on the surface of hematopoietic stem cells it has been

demonstrated that CXCR4 is also expressed on the surface of various tissue-committed stem cells (Ratajczak et al. 2004) and is involved in the trafficking of these stem cells to sites of tissue damage. Although SDF-1 is constitutively secreted by stromal and endothelial cells in various organs such as the lungs, liver, heart and brain (Pituch-Noworolska et al. 2003; Ratajczak et al. 2003), it has been found to be upregulated in these tissues during times of tissue damage such as myocardial infarction and stroke (Abbott et al. 2004; Hu et al. 2007; Wojakowski et al. 2004), toxic liver damage (Kollet et al. 2003), hemorrhage (Ratajczak et al. 2004), and total body irradiation (Ponomaryov et al. 2000). It has been proposed that the increase in SDF-1 in these tissues during periods of tissue damage is required to chemoattract CXCR4+ stem cells necessary for tissue repair and regeneration (Ceradini et al. 2004).

Supporting this idea is the evidence that CXCR4 and SDF-1 are upregulated in response to hypoxic stimuli. Both CXCR4 and SDF-1 have been shown to be upregulated in endothelial and various types of tumor cells in response to HIF-1α (Ceradini et al. 2004; Schioppa et al. 2003; Staller et al. 2003; Zagzag et al. 2005) as well as NF-κB (Helbig et al. 2003) which are produced in response to hypoxia. Lastly, it has also been suggested that the CXCR4/SDF-1 axis may be involved in cell proliferation and survival (Broxmeyer et al. 2003), however the evidence has been controversial (Libura et al. 2002).

1.3.2 Molecular Pathways Associated with CXCR4/SDF-1

The CXCR4/SDF-1 axis is a major regulator of cell trafficking in the body and as such, its biological effects are associated with the ability of SDF-1 to induce chemotaxis,

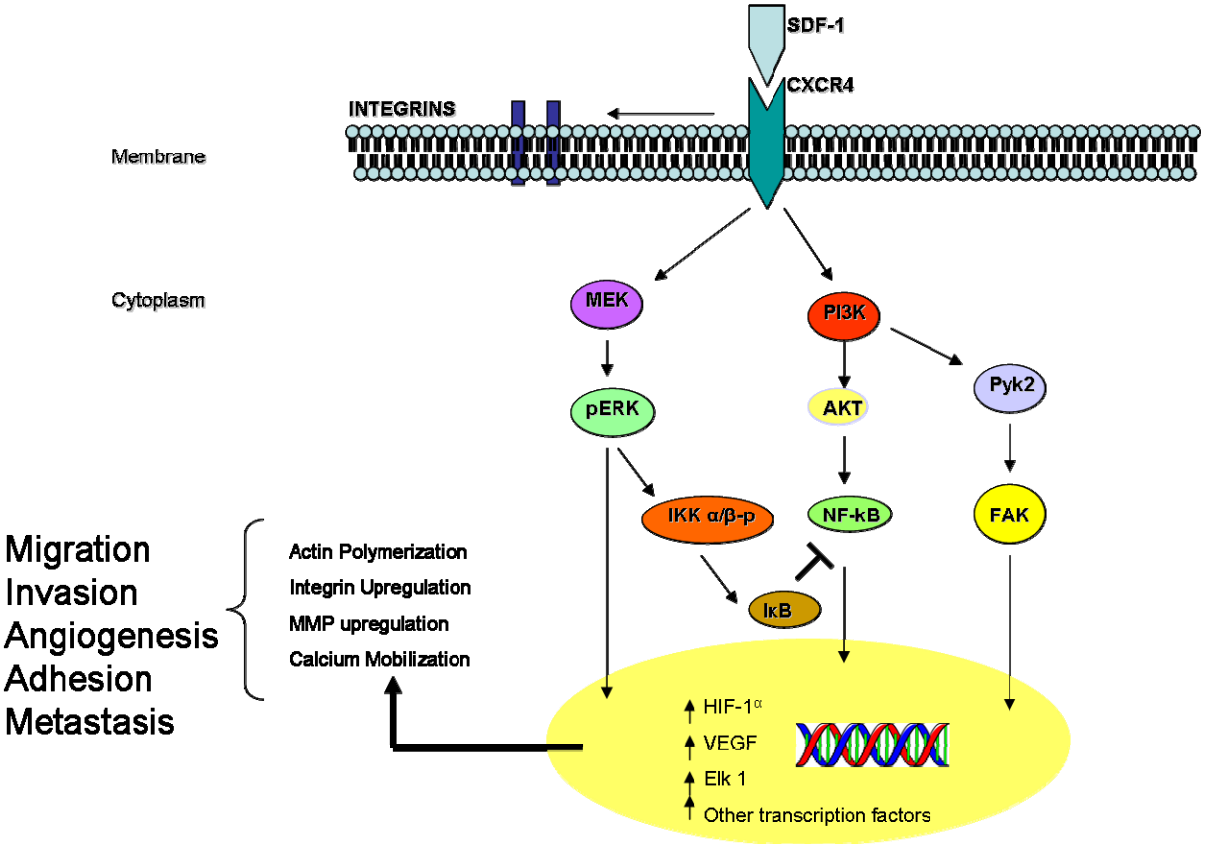
migration, adhesion, invasion and the secretion of angiopoietic factors when it binds CXCR4 (Kucia et al. 2005; Teicher et al. 2010). After binding SDF-1, CXCR4 undergoes dimerization which is thought to be required for further signalling (Vila-Coro et al. 1999). It has also been suggested that the SDF-1/CXCR4 complex is then incorporated into lipid rafts where is associates with members of the Src family of kinases (Zaman et al. 2008). CXCR4 is a member of the GPCR family of receptors, though it may also signal through G protein independent means (Busillo et al. 2007). For example, it has been proposed that CXCR4 activates the JAK/STAT pathway in a G protein independent manner (Vila-Coro et al. 1999). However, a greater proportion of CXCR4 signalling is G protein dependent, as SDF-1 bound CXCR4 activates, via a Gαi protein, a number of downstream signal transduction pathways. Both the PI-3K/AKT axis and the mitogen activated protein kinase (MAPK) pathways are activated and play a role in chemotaxis and cell migration (Ganju et al. 1998; Peng et al. 2005; Vicente-Manzanares et al. 1999). Studies revealed that activation of the CXCR4/SDF-1 axis resulted in rearrangements of cytoskeletal proteins such as F-actin filaments, which is a crucial process in cell motility (Libura et al. 2002). Furthermore, it has been shown that activation of these pathways also results in the secretion of various matrix metalloproteinases (MMP's), including MMP-2 and MMP-9 which are involved in the invasion of cells through the basement membrane (Fernandis et al. 2004; Janowska-Wieczorek et al. 2000; Tang et al. 2007).

In addition, the activation of CXCR4 increases intracellular calcium concentration and induces phosphorylation of focal adhesion components such as FAK and Pyk2 (Oonakahara et al. 2004; Phillips et al. 2005), as well as other molecules downstream of MAPK and PI-3K such as IKK α/β and NF-κB (Huang et al. 2009a). It is also involved in

the production and secretion of the angiopoietic factor VEGF and evidence suggests that the CXCR4/SDF-1 axis upregulates the expression of VEGF via this pathway (Liang et al. 2007). Furthermore, the CXCR4/SDF-1 axis induces, also through the PI-3K/AKT and MAPK pathways, the expression of several cell surface integrins such as VLA-4 and VLA-5 (Huang et al. 2007; Kijowski et al. 2001; Peled et al. 2000). This upregulation of integrins enhanced the integrin-mediated adhesion to fibronectin in hematopoietic cells (Kijowski et al. 2001). It was demonstrated by Peled *et al* that activation of CXCR4 by SDF-1 led to firm adhesion and transendothelial migration of hematopoietic stem cells *in vitro* (Peled et al. 2000).

The importance of the PI-3K/AKT and MAPK pathways in CXCR4/SDF-1 axis signalling is supported by studies which demonstrated that blocking either pathway inhibited CXCR4 activated cell migration in a pre B cell line (Ganju et al. 1998). Ultimately, through these pathways, SDF-1 bound CXCR4 can induce cytoskeletal rearrangement, adhesion to endothelial cells, polarized migration of cells to specific organs and the secretion of angiopoietic factors, all important components of the metastatic process (Hillyer et al. 2003; Kucia et al. 2005).

FIGURE 1.1. Molecular pathways activated by CXCR4. Stromal cell derived factor (SDF-1) bound-CXCR4, possibly after incorporation into lipid rafts, acts via Gαi, to activate the phosphatidylinositol-3-kinase (PI3K) and mitogen activated protein kinase (MAPK) signaling pathways. Activated CXCR4 increases intracellular calcium mobilization and induces phosphorylation of focal adhesion components such as FAK and Pyk2. The activated signal transduction pathways contribute to chemotaxis, cell migration, and secretion of various matrix metalloproteinases (MMP's) including MMP-2 and MMP-9. Figure adapted from (Otsuka et al. 2008).


SDF-1
CXCR4
INTEGRINS
Membrane
Cytoplasm
MEK
PI3K
Pyk2
pERK
AKT
IKK α/β-p
NF-kB
FAK
IκB
HIF-1α
VEGF
Elk 1
Other transcription factors
Migration
Invasion
Angiogenesis
Adhesion
Metastasis
Actin Polymerization
Integrin Upregulation
MMP upregulation
Calcium Mobilization

1.3.3 CXCR4 Pathophysiology

Data from *in vivo* knockout studies demonstrate that the complete absence of CXCR4 is lethal and as such, embryos die *in utero*. However, there are a number of pathophysiologies related to CXCR4 and/or SDF-1 and their functions. Due to the central role of the CXCR4/SDF-1 axis in lymphocyte trafficking, this chemokine axis is involved in a host of immunological and inflammatory conditions such as allergic airway disease (Gonzalo et al. 2000), and rheumatoid arthritis (Nanki et al. 2000) as well as an immunodeficiency disease called WHIM syndrome (Warts, Hypogammaglobulinemia, Infections and Myelokathexia which comprise the major symptoms of this disorder).

This condition is characterized by peripheral neutropenia but normal or above normal neutrophil levels in the bone marrow (Wetzler et al. 1990; Zuelzer 1964). This condition is often inherited as an autosomal dominant trait and is caused by a heterozygous truncating mutation in the C-terminus tail of the CXCR4 receptor (Hernandez et al. 2003). Patients with WHIM syndrome often have a markedly reduced number of circulating B lymphocytes but the T cell subsets are relatively unaffected. It has been proposed that the mutation in the CXCR4 receptor is an activating mutation and thus increases the receptors chemotactic responsiveness to SDF-1 (Hernandez et al. 2003) leading to the retention of the affected cells in the bone marrow. This suggests that the C-terminus portion of the receptor is essential for receptor desensitization and protects its hyperactivity (Haribabu et al. 1997).

In addition, it appears that the CXCR4/SDF-1 axis may play a role in some other pathophysiologies possibly linked to abnormal angiogenic processes. For example,

multiple studies have suggested a role for CXCR4/SDF-1 in the pathogenesis of idiopathic pulmonary fibrosis (Antoniou et al. 2010; Phillips et al. 2004; Strieter et al. 2007). It was demonstrated in one study that there were decreased expression levels of both SDF-1 as well as VEGF in patients with the disease versus normal controls (Antoniou et al. 2010), suggesting that a decrease in angiogenesis may play a role in the development of this disease. However, another study showed increased SDF-1 levels in the lungs of patients with fibrosis (Phillips et al. 2004), clearly indicating that further investigation into the role of the CXCR4/SDF-1 axis in pulmonary fibrosis is needed.

Lastly, CXCR4 has long been known to be associated with HIV, which is particularly important in the context of malignancy as inhibitors to the CXCR4 receptor developed for use as anti-HIV drugs, could also prove effective as anti-cancer therapies. It has been shown that CXCR4 functions as the primary co-receptor for the entry of T-cell tropic (T-tropic) HIV strains (HIV-1 virus) as it was demonstrated that transfection of CD4+ cells with CXCR4 complementary DNA allowed these cells to become permissive to infection by T-tropic strains of HIV (Deng et al. 1996; Feng et al. 1996). When CD4+ cells come in contact with the virus, envelope gp120 first binds to the CD4 receptor which results in the exposure of co-receptor binding sites in gp120. Co-receptor binding then triggers the fusion and entry of the viral genome into the host cell (Wu et al. 1996b). Similarly, the chemokine receptor CCR5 is a co-receptor for the entry of macrophage tropic (M-tropic) strains of HIV (Choe et al. 1996).

Before its function as a chemokine receptor was identified CXCR4 was formerly termed the LESTR/fusin receptor, and was originally isolated from a human blood

monocyte cDNA library and cloned (Loetscher et al. 1994). Subsequently, it was shown that SDF-1 was the natural ligand for LESTR/fusin and the receptor name was changed to CXCR4 in accordance with the current chemokine receptor nomenclature (Bleul et al. 1996a; Oberlin et al. 1996). It was hypothesized that because SDF-1 was the natural ligand for CXCR4, binding of CXCR4 by SDF-1 might inhibit the binding and entry of HIV into CXCR4+ cells, which was subsequently proven (Bleul et al. 1996a; Oberlin et al. 1996). It has also been demonstrated that HIV binding and entry via CXCR4 can be inhibited by small molecular inhibitors of the CXCR4 receptor (Donzella et al. 1998) and a great deal of research has been focused on using CXCR4 inhibition strategies as a method to stop the spread of HIV infection (De Clercq et al. 2001; Seibert et al. 2004; Tamamura et al. 2007).

1.4 Discovery of CXCR7

About 20 years ago, the CXCR7 gene was cloned from the cDNA library of a dog thyroid and thus termed Receptor Dog cDNA 1 (RDC1) (Libert et al. 1989; Libert et al. 1990; Maksym et al. 2009). RDC1 was considered an orphan receptor until it was recently discovered that it bound to the chemokines SDF-1 and CXCL11 and its name was changed to CXCR7 (Burns et al. 2006; Joost et al. 2002). CXCR7, like CXCR4, is a 7 transmembrane spanning receptor, is highly conserved across species (Heesen et al. 1998), and is also a co-receptor for HIV (strains which are neither T – nor M tropic) (Balabanian et al. 2005; Shimizu et al. 2000). It is also capable of binding the chemokine CXCL11 (alternatively called I-TAC, which also binds CXCR3) which actually may compete with SDF-1 for binding to CXCR7 (Burns et al. 2006).

It was shown in a study by Burns and colleagues, that CXCR7 protein was highly expressed in a range of transformed cell lines, but was noticeably absent in the majority of non transformed cell lines, and appears to have roles in cell growth/proliferation, survival, and cell adhesion (Burns et al. 2006). Furthermore, CXCR7 has been shown to be expressed on several tumor cell lines from breast, prostate and lung carcinomas, and played a role in tumor development and growth in mouse models of cancer (Iwakiri et al. 2009; Miao et al. 2007; Wang et al. 2008). Similarly, it was demonstrated that CXCR7 was expressed in patient tumor samples from a variety of human cancers and may be associated with a worse clinical outcome (Goldmann et al. 2008; Iwakiri et al. 2009; Miao et al. 2007; Schutyser et al. 2007).

In terms of the mechanisms through which CXCR7 exerts its biological effects, there seems to be some controversy. On one hand, there is preliminary evidence that activated CXCR7 signals through downstream MAPK and PI3K signalling pathways (A. P. Jillella 2008; Hartmann et al. 2008; Thelen et al. 2008; Wang et al. 2008). On the other hand, many reports have suggested that CXCR7 is incapable of coupling to G proteins and instead functions as a non signalling receptor in many cell types (Rajagopal et al. 2010). In these cells, CXCR7 may instead just form heterodimers with CXCR4 to enhance SDF-1 activated CXCR4 signalling (Levoye et al. 2009; Sierro et al. 2007). It has also been proposed more recently that CXCR7 may act as ligand scavenger or ‘molecular sink’ for SDF-1, acting to sequester the ligand in certain regions or tissues in order to regulate the levels of SDF-1 in the microenvironment and produce or strengthen SDF-1 chemotactic gradients (Boldajipour et al. 2008; Haraldsen et al. 2006). Studies have shown that after SDF-1 binding to CXCR7, it is quickly internalized where it

undergoes degredation and in this way is able to influence the local SDF-1 concentration (Luker et al. 2010).

This particular finding suggests a potentially crucial and indirect role of CXCR7 in the directional migration of cells resulting from SDF-1 activated CXCR4, and in fact, many studies have demonstrated the crucial role of CXCR7 in the regulation of SDF-1/CXCR4 induced chemotaxis of various cell types, including tumor cells (Naumann et al. 2010; Torisawa et al. 2010; Zabel et al. 2009). As for the ability of CXCR7 to directly mediate SDF-1 induced chemotaxis, the findings are also conflicting. For example, an early study by Balabanian *et al* suggested that a monoclonal antibody to the CXCR7 receptor was capable of inhibiting the migration of T lymphocytes in response to SDF-1 (Balabanian et al. 2005). However, in many subsequent studies since then, it has been found that SDF-1 activated CXCR7 was incapable of directly inducing chemotaxis in T cells or any other cell line tested (Boldajipour et al. 2008; Burns et al. 2006; Hartmann et al. 2008). Clearly, the role of the CXCR7/SDF-1 pathway in cell trafficking and migration have yet to be fully clarified.

Although much about the role of CXCR7 in development and malignancy has yet to be worked out, it will be important to consider both the independent function of CXCR7/SDF-1 as well as any potential interaction with the CXCR4/SDF-1 axis, when interpreting any past research reported under the premise that CXCR4 and SDF-1 were exclusive, as well as all future findings in relation to the role of CXCR4 in cancer.

1.5 Role of the CXCR4/SDF-1 Axis in Malignancy

Since the identification of CXCR4 as a chemokine receptor there has been much focus on the CXCR4/SDF-1 chemokine axis and its various roles, including in malignancy. The concept of "seed and soil" (Paget 1889) has always implied that metastatic dissemination of circulating cancer cells is not a random process, but instead is dependent on specific characteristics of the cell and distal environment. A stromal chemokine such as SDF-1 and a cell based receptor such as CXCR4 may help account for this phenomenon (Hart et al. 2005; Libura et al. 2002). The metastatic process can be viewed as being comprised of two main stages; the malignant cell must first acquire the capability to migrate and break away from the primary tumor to become blood or lymphatic borne before then homing in on its metastatic destination. The potential role for the CXCR4/SDF-1 axis in metastasis can be envisaged in both these critical areas:

1. *The process of cell migration and escape from the site of the primary tumor:* After binding its ligand, CXCR4 induces a series of specific cellular changes. It causes cytoskeletal rearrangement, modulates adhesion to endothelial cells and facilitates polarized migration of different types of cells to specific organs (Cho et al. 2006; Hillyer et al. 2003). The process of migration is a critical step in allowing the malignant cells to move away from its source of origin and gain access to circulating lymph fluid or blood (Kang et al. 2005; Sutton et al. 2007).

2. *The homing of the separated cell once in the circulation to distant sites of metastasis:* SDF-1, the only described ligand for CXCR4, is known to be constitutively released by stromal cells from bone marrow, lung, and liver, all of

which are common sites of metastasis (Pituch-Noworolska et al. 2003). It has been well established that the CXCR4/SDF-1 axis plays a critical role in leukocyte trafficking and homing of stem cells (Lapidot et al. 2005; Wang et al. 2006). Similarly, it is believed that organ selective metastasis shares similarities with the homing of stem cells (Geminder et al. 2001). Evidence to suggest that many cancers, including non small cell lung cancer cells exploit this system to generate metastases has been steadily accumulating.

The first evidence of the CXCR4/SDF-1 axis' direct role in tumor metastasis came from a pivotal study by Muller and colleagues, where it was demonstrated that the most abundant of the chemokine receptors in breast cancer was CXCR4 and that CXCR4 expression was significantly up-regulated in breast cancer cells compared with normal mammary epithelial cells in which CXCR4 was undetectable (Muller et al. 2001). They also looked at the expression of SDF-1 within the body and found markedly increased levels of SDF-1 in lymph nodes, bone marrow, lungs and liver which are all preferential sites of breast cancer metastasis. Furthermore, it was shown *in vivo* that the administration of an anti-CXCR4 monoclonal antibody resulted in markedly reduced lung metastasis in a mouse model, providing the first evidence that CXCR4 inhibition may be a useful strategy in the prevention of metastasis (Muller et al. 2001).

Since this study there has been a growing body of evidence that the SDF-1/CXCR4 axis plays an essential role in the metastasis of many types of tumor cells. In fact, there is evidence to show that CXCR4 plays a role in 23 types of cancer, more than any other chemokine receptor studied to date (Kakinuma et al. 2006; O'Hayre et al.

2008). Retrospective studies in breast (Chu et al. 2010; Kang et al. 2005; N. T. Holm 2008), and other cancers such as ovarian (Jiang et al. 2006), head and neck (Hu et al. 2005) and pancreatic carcinoma (Koshiba et al. 2000) have provided evidence that high expression of the CXCR4 receptor on primary tumor specimens correlates with a poorer clinical outcome. *In vitro* studies in prostate cancer (Hart et al. 2005), nasopharyngeal carcinoma (Hu et al. 2005), pancreatic cancer (Mori et al. 2004) cell lines and haematological malignancies (Burger et al. 2005; Juarez et al. 2003), have shown that inhibition of the CXCR4/SDF-1 chemokine axis resulted in decreases in migration and invasion of these cell lines. Lastly, *in vivo* studies in renal cell carcinoma (Pan et al. 2006), breast cancer (Muller et al. 2001; Smith et al. 2004) and head and neck cancer (Yoon et al. 2007) have demonstrated that *in vivo* inhibition of the CXCR4/SDF-1 axis significantly decreased the amount of metastases seen in mouse models of cancer.

1.5.1 CXCR4 and Non Small Cell Lung Cancer

Similar, yet less extensive studies have also been carried out with regard to the role of the CXCR4/SDF-1 chemokine axis in non small cell lung cancer (NSCLC). In one study, Su *et al* looked at the relationship between CXCR4 expression and the presence of metastatic disease in patients NSCLC, but also sought to investigate whether modulation of CXCR4 expression would alter the metastatic potential of NSCLC cells *in vitro* (Su et al. 2005). They demonstrated that the expression of CXCR4, both at the mRNA level (as assessed by reverse transcription-PCR) and the protein level (as assessed by ELISA), was up-regulated in lung cancer tissue specimens compared to normal lung tissue. To confirm

their results they also assessed protein expression by immunohistochemistry (IHC) and found that there was positive CXCR4 staining in almost all of the NSCLC tumor tissue but no expression in the normal lung tissue. Staining of the CXCR4 protein was seen mainly in the cytoplasm and/or the cell membrane, and when the level of CXCR4 expression (high versus low) was correlated with clinicopathological features it was seen that a high level of CXCR4 was associated with the presence of metastatic disease.

In addition, they also demonstrated that down-regulation of CXCR4 in NSCLC cell lines by transfection of a CXCR4 anti-sense nucleotide fragment resulted in a significant decrease in the migration, invasion and adhesion of these NSCLC cells *in vitro*. Similarly, blocking of the CXCR4 receptor by a neutralizing antibody also inhibited the migration, invasion and adhesion of these cell lines (Su et al. 2005).

In vivo studies have also suggested that CXCR4 may play a role in the metastasis of NSCLC. In a study by Belperio *et al* it was shown *in vivo* that preferential sites of lung cancer metastasis had significantly higher levels of SDF-1 protein expression than the primary tumor or plasma levels suggesting that a chemotactic gradient could be established between the site of the primary tumor and commons sites of NSCLC metastasis (Belperio et al. 2004). Also, in vivo neutralization of SDF-1 by an anti-SDF-1 monoclonal antibody resulted in a significant decrease of NSCLC metastases to several organs including the adrenal glands, liver, lung, brain and bone marrow. Similarly, Xu *et al* demonstrated that there was a decrease NSCLC metastasis to the lung, in a murine model of NSCLC (tail vein injection of NSCLC cells) when CXCR4 was down regulated using siRNA (Xu et al. 2009).

Several studies have examined the association between the expression of CXCR4 or SDF-1 and clinical outcome. The results of these studies however, have been somewhat inconclusive. Spano *et al* assessed the expression of CXCR4, by semi-quantitative immunohistochemistry (IHC), in NSCLC tumors resected from patients with stage I disease and correlated expression with prognosis (Spano et al. 2004). They found that CXCR4 was present in the cytoplasm of the tumor cells of all tissue specimens tested, but was absent in the normal lung tissue. There was also strong nuclear staining in a significant number of tumor specimens. There was no significant correlation between CXCR4 expression and age, gender, smoking history or tumor size but there was a positive correlation between CXCR4 nuclear expression and better prognosis. Patients with high nuclear expressing tumors had a better 5 year overall survival (OS) than those patients with tumors with negative nuclear CXCR4 expression. They found no association between cytoplasmic CXCR4 expression and outcome.

Similarly, Minamiya *et al* reported that high CXCR4 expression, as assessed by semi-quantitative real time reverse transcription PCR, was associated with a better clinical outcome and longer 5 year disease free survival (DFS) in early stage resected patients with adenocarcinoma tumor histology (Minamiya et al.). Likewise, Wagner and Colleagues demonstrated that high cyto-membranous CXCR4 expression (also as assessed by semi-quantitative IHC) was an independent prognostic marker of worse survival, and that nuclear CXCR4 conferred a survival benefit in patients with adenocarcinoma (Wagner et al. 2009). On the other hand, other studies have demonstrated that high nuclear staining of CXCR4 was associated with lymph node

metastasis (Na et al. 2008) and high cytoplasmic or nuclear staining was an indicator of a worse prognosis (Wald et al. 2006).

The role of SDF-1 has also been studied in NSCLC but again, the results appear inconclusive. Some studies report that there is significant expression of SDF-1 in tumors from early stage resected cases of NSCLC as well as in normal lung tissue, and that tumor expression of SDF-1 is associated with clinicopathological features such as nodal metastasis and increased disease recurrence rates (Wagner et al. 2009; Wald et al. 2006). On the other hand, another study demonstrated that SDF-1 is not highly expressed in NSCLC tumors or in NSCLC tumor cell lines but is expressed in adjacent lung tissue (Phillips et al. 2003). And yet another study found that SDF-1 was expressed in both small cell and non small cell lung cancer cell lines, but was absent in normal bronchial epithelial cell lines, and that neutralizing SDF-1 with and anti-SDF-1 antibody resulting in a decrease in the growth and migration of lung tumor cell lines *in vitro* (Imai et al. 2010).

On this basis it can be suggested that the CXCR4/SDF-1 axis may play an important yet incompletely defined role in the development and metastasis of non small cell lung cancer. The experiments outlined in this thesis are designed to investigate this role in more detail using two approaches: 1) the role of CXCR4 in cellular migration was investigated by assessing the influence of CXCR4/SDF-1 axis modulation on NSCLC cell line migration *in vitro*, and 2) the association between clinical outcome in stage IV and resected early stage NSCLC patients and CXCR4 expression was investigated using

a newly created lung cancer clinical pathological database and novel quantitative immunohistochemistry (IHC) technology.

1.6 Hypotheses

1) Successful inhibition of the CXCR4/SDF-1 chemokine axis will reduce the migrational capacity of NSCLC lines *in vitro*. Anti-SDF-1 strategies will be more effective than CXCR4 inhibition strategies in reducing NSCLC cell line migration, as this approach will also inhibit any potential effects of SDF-1/CXCR7 interaction.

2) High CXCR4 receptor expression on NSCLC tissue samples will correlate with a poorer clinical outcome in both resected early stage as well as in advanced disease.

Chapter Two: INHIBITION OF NSCLC CELL LINE MIGRATION BY MODULATION OF THE CXCR4/SDF-1 AXIS *IN VITRO*

2.1 Introduction

Cell migration plays a fundamental role in a number of important biological processes, including (but not limited to) development and embryogenesis, hematopoiesis, inflammation and immune function, and tissue repair. Not surprisingly, cell migration or aberrant cellular migration is also involved in many disease states, including cancer. It is envisaged that cell migration is one of the most important processes involved in tumor progression and metastasis. It is integral to the process of metastasis right from the time the tumor cell breaks away from the primary tumor and intravasates into the blood stream, to the moment the tumor cell embeds into a distant organ to create a metastasis.

The chemokine family of cytokines has long been known to be involved in many facets of cellular movement (Schier 2003). Given the prominent and well studied role of chemokines in the directional migration of leukocytes (Taub & Oppenheim 1994; Zlotnik & Yoshie 2000), it was a logical step to the investigation of the role of chemokines in tumor cell migration (Zlotnik & Yoshie 2000). In particular, the mechanisms utilized in the regulation of lymphocyte and tissue committed stem cell trafficking to sites of tissue inflammation and damage, as well as in the regulation of hematopoietic stem cell homing to the bone marrow, seem to share many similarities with the organ selective metastasis of malignant tumor cells. The CXCR4/SDF-1 chemokine axis plays a central role in the above mentioned processes and it is possible that the tumor cells exploit the function of this axis in order to facilitate metastasis (Geminder et al. 2001; Lapidot et al. 2005; Wang et al. 2006).

About 10 years ago, Muller and colleagues provided the first evidence that the CXCR4 chemokine receptor was one of the most important chemokine receptors in breast

cancer. They reported two major findings: 1) that of all the chemokine receptors, CXCR4 had the highest expression in malignant breast tissue; and 2) that there was significantly increased secretion of SDF-1 in the lymph nodes, bone marrow, lungs and liver which are all preferential sites of breast cancer metastasis (Muller et al. 2001). These findings supported the hypothesized function of the CXCR4/SDF-1 axis in organ selective metastasis. Since then, many researchers have reported evidence of the role CXCR4 in many cancers, including specific evidence on the role of CXCR4 in *in vitro* tumor cell migration, invasion, adhesion and angiogenesis (Burger et al. 2005; Hart et al. 2005; Hong et al. 2006; Ohira et al. 2006; Scotton et al. 2002; Su et al. 2005). Taken together, these studies support a crucial role for the CXCR4/SDF-1 chemokine axis in many central processes which together lead to metastatic dissemination, and have suggested that this axis has the potential to be a therapeutic target.

Various inhibitors to the CXCR4 receptor have been developed which have aided investigation into the role of the CXCR4/SDF-1 axis in metastasis and the utility of targeting CXCR4 as an anti-cancer approach (Burger & Peled 2009). The first of the CXCR4 inhibitors were developed during HIV research in an attempt to find efficacious anti-HIV therapies. Many were actually discovered before it was known that CXCR4 was one of the co-receptors for HIV entry into T cells, and were isolated based purely on their anti-HIV properties, without knowing the mechanism of action (Burger & Peled 2009; De Clercq et al. 1992; Nakashima et al. 1992). However, many of them have crossed over into cancer research and more recently, new ones have been developed specifically for assessment as anti-cancer therapies.

However, the various inhibitors to CXCR4 and SDF-1 has never been compared in their effectiveness at inhibiting the migration of NSCLC cell lines. Studies have traditionally only examined one or at the most two methods of CXCR4/SDF-1 axis inhibition in only one or two NSCLC cell lines (Huang et al. 2007; Su et al. 2005) so comparisons between multiple CXCR4/SDF-1 inhibitors could not be made. Furthermore, there are no reports to date of the effect of simultaneously neutralizing both CXCR4 and SDF-1 on the migration of NSCLC tumor cells lines. So, we set out to test a range of available CXCR4 and SDF-1 inhibitors to assess their efficacy in inhibiting the migrational capacity of several NSCLC cell lines. Specific inhibition of the CXCR4/SDF-1 axis was carried out using a number of strategies:

(1) *Antagonists of the CXCR4 receptor:* because of the role of this axis in HIV infection there are a number of small molecular inhibitors to the CXCR4 receptor on the market. AMD 3100 is a non-competitive antagonist and selective small molecular inhibitor which reversibly binds the CXCR4 receptor and inhibits binding of SDF-1 and the subsequent signal transduction (Donzella et al. 1998; Fricker et al. 2006). It has been shown to block the entry of HIV T-tropic strains into cells (Donzella et al. 1998) as well as to inhibit the migration of a number of tumor cell lines including ovarian (Jiang et al. 2007) and multiple myeloma (Alsayed et al. 2007). AMD 3100 was also recently approved by the FDA under the trade name Mozobil (Genzyme, Cambridge, MA, USA), in combination with G-CSF for stem cell mobilization in patients with non-Hodgkins lymphoma and multiple myeloma (Pusic & DiPersio 2010).

A second CXCR4 antagonist, CTCE 9908, is a peptide analog of SDF-1 which inhibits the CXCR4 receptor and has been shown in studies to inhibit the migration of some cancer cell lines (Kim et al. 2007). It has also been efficacious in reducing metastasis in some preclinical *in vivo* models of cancer (Huang et al. 2009b; Porvasnik et al. 2009; Richert et al. 2009).

(2) *Monoclonal antibody to the CXCR4 receptor*: This approach is analogous to other therapeutic monoclonal antibodies: anti CD20 (lymphoma), anti EGFR (colon cancer) and anti HER2 (breast cancer). Limited data exists on the utility of this approach.

(3) *Anti-SDF-1 antibody*: This strategy aims to 'mop up' or neutralize the ligand to CXCR4, render the medium deficient in SDF-1 and prevent chemoattraction from taking place. This approach is analogous to the anti-VEGF (Bevacizumab) approach which is widely used at treatment in colon cancer (Jenab-Wolcott et al. 2009).

The main experimental approach utilized in this project is a migration assay using the Boyden Chamber to quantify the inhibitory effect of CXCR4/SDF-1 axis disruption strategies on the migration of NSCLC cell lines *in vitro*. Chemoattractants (increasing concentrations of SDF-1) added to the lower wells of the chamber act on a known number of cells in suspension in the upper wells which are separated by a collagen coated synthetic polycarbonate filter. Cell migration is quantified by counting, under microscopy, cells that have moved from the upper wells, through the pores in the filter and have adhered to the underside of the filter. Putative inhibitors of migration can be

incubated with the cells and the migration inhibitory effect measured in a replicable, quantitative manner.

2.2 Materials and Methods

2.2.1 Cell culture and CXCR4/SDF-1 axis inhibitors

Three non small cell lung cancer (NSCLC) cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). H1299: a human large cell lung cancer line derived from the lymph node and has a homozygous partial deletion of p53 and lacks p53 expression, H460: a human large cell lung cancer derived from a pleural effusion, and A549: a non small cell lung cancer cell line. The H1299 and H460 cell lines were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) supplemented with 1% hepes, 1% glucose and 1% sodium pyruvate. The A549 cell line was cultured in DMEM/F12 medium (Invitrogen) and 10% FBS. In addition, *Hela* (also purchased from ATCC) cells were used as a positive control for CXCR4 for the quantitative IHC assessment, as well as the western blot and FACS analysis (Appendix 2). And 786-0, a renal carcinoma cell line (ATCC) was used as a negative CXCR4 control for the western blot and FACS analyses. Both cell lines were cultured in RPMI 1640 medium plus 10% FBS. All cell lines were grown in an incubator at a controlled environment of 5% CO_2 and at 37^{o}C.

Recombinant human SDF-1α and two of the CXCR4/SDF-1 inhibitors (AMD 3100 and anti-CXCR4 MAb) were purchased from Sigma (Saint Louis, MO, USA). AMD 3100 is a non-competitive antagonist and selective small molecular inhibitor which inhibits the CXCR4 receptor. The anti-CXCR4 monoclonal antibody (clone 44716.111)

is a mouse IgG2b isotype, and neutralizes cell surface CXCR4 activity. The third CXCR4 inhibitor, CTCE 9908 was made available to us through a Materal Transfer Agreement (MTA) by British Canadian BioSciences Corporation, a Vancouver based biotechnology company. Lastly, both the monoclonal and polyclonal anti-SDF-1 antibodies were purchased from R&D Systems (Minneapolis, MN, USA).

2.2.2 Cell line array construction

The NSCLC cell lines (H1299, H460 and A549) and the control lines *(Hela* and 786-0*)* were cultured as above until approximately 1 x 10^7 cells per cell line were available for collection. Collected cells were spun down into a cell pellet in an eppendorf tube, and the supernatant removed. Cells in the cell pellet were gently washed with PBS and then spun down again into cell pellets (x2). Cells were then fixed in formalin for 30 minutes on ice. Cells were once again spun down and the supernatant removed. HistoGel Processing Gel (Thermo Fisher Scientific, Waltham, MA, USA) heated to 50 degrees to liquefy, and then cooled slightly was added to cell pellets (in a 50:50 ratio HistoGel to volume of cells) and gently mixed. Cells were allowed to cool again on ice for 30 minutes to allow the histogel to solidify. Cell/histogel pellets were then placed in a tissue cassette, labelled and stored in a formalin jar. Formalin fixed cell pellets were then embedded in paraffin blocks (within 24 hours from time of fixation) by Calgary Laboratory Services (CLS).

Slides were then cut from the formalin fixed paraffin embedded (FFPE) tissue blocks for each cell line and stained with hematoxylin and eosin. Stained slides were reviewed by Dr. Alexander Klimowicz from Dr. Anthony Magliocco's pathology

laboratory, to select and sample representative areas of the cell pellet. These representative cores (0.6 mm) from each specimen were assembled in triplicate in a cell line array using a Beecher Manual Tissue Microarrayer (Beecher Instruments Inc. Sun Prairie, WI, USA).

2.2.3 Quantitative IHC

After cell line array construction, 5μm thick sections were cut from the array 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, USA). Slides were stained overnight in a humidified chamber at room temperature with Signal Stain protein block (Cell Signaling, Danvers, MA, USA) 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 (Biotrend, Köln, Germany). The following day, slides were washed with 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 TM + 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 cell line array slides were mounted with ProLong® Gold anti-fade 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 (Camp et al. 2002). 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 TAs 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)) in order 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 (Camp et al. 2002). 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) >10% of the tissue area is pan-cytokeratin positive, 2) >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.

2.2.4 Migration assay

Migration of the NSCLC cell lines was assessed using the 48 well mini-Boyden chamber (Neuroprobe, Gaithersburg, MD, USA) migration assay. Before each experiment, the chamber was rinsed with sterile distilled water and left to air dry. Meanwhile, the synthetic polycarbonate filter (Neuroprobe, Gaithersburg, MD, USA) (11 microns thick with 12 micron pores) - which is used to separate the upper and lower wells of the chamber – was marked to later determine proper orientation. It was then coated in type 1 rat tail collagen (1 mg/ml) for 1 hour, then washed in Phosphate Buffer Solution (PBS) and air dried. SDF-1 was added to the lower wells at concentrations of 0, 50, 100 and 200 ng/ml for the response to SDF-1 assays, and 0 and 100 ng/ml for the CXCR4/SDF-1 inhibitor assays. Cells were added to the upper wells at a concentration of 10×10^5 cells/ml (or 5×10^4 cells/well). For assays using the CXCR4 inhibitors, the inhibitor was incubated with the cells at the appropriate concentrations for 30 minutes before the cells were placed in the upper wells. For assays using the anti-SDF-1 antibody, it was placed in the bottom wells along with the SDF-1. Medium used for both the upper and lower chambers was serum free and contained only culture supplements (if indicated).

Chambers were then incubated for a period of 6 hours in an incubator at 37◦C and 5% CO_2. After incubation, the chamber was disassembled and the cells in solution discarded. The non migrating cells on the top side of the filter were wiped off with a Wiper Blade (Neuroprobe) and the filter was fixed in methanol for 2 minutes to fix the migrating cells adhered to the underside of the filter. After fixation, the filter was stained with hematoxylin for 4 minutes in order to visualize the migrating cells. After drying, the filter was mounted on microscope slides. Cells were counted in 5 different fields at 20x magnification using a microscope. Baseline levels of migration were optimized to allow statistically significant decreases to be measured with successful inhibition of the migration process.

Results from individual experiments were converted from absolute number of migrating cells into migration indexes to control for varying conditions between experiments. The migration index represents the fold increase in the number of migrating cells in response to the chemoattractant over the spontaneous migration in response to control medium.

2.2.5 Statistical analysis

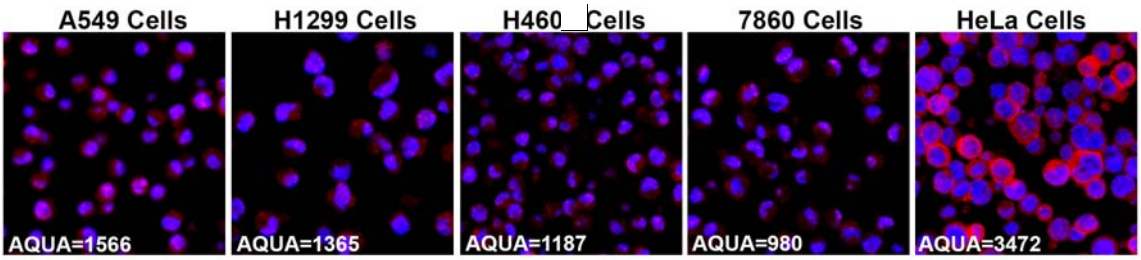
For each migration assay, quadruplicate wells were used for each data point and averaged, and each experiment was run in triplicate. Results for each individual migration assay were converted to migration indexes as described above and averaged between experiments. Statistically significant differences in migration to the various experimental conditions (chemoattractant, and inhibitors) were tested using a one tailed student's *t*-test. Differences in migration were considered statistically significant if $p \leq 0.05$.

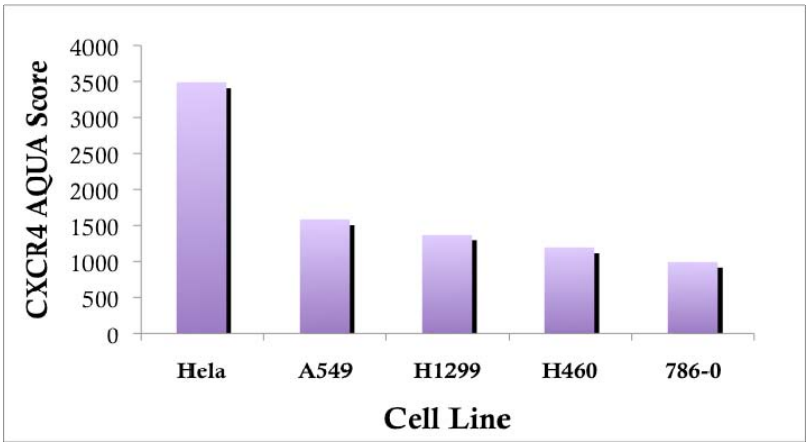
2.3 Results

2.3.1 Confirmation that NSCLC cell lines express the CXCR4 chemokine receptor

Before assessing the migration of the non small cell lung cancer cell lines, tests to confirm that the NSCLC cell lines expressed the chemokine receptor of interest, CXCR4, were carried out. Quantitative immunohistochemistry (IHC) utilizing the HistoRx platform was employed to assess the expression of the CXCR4 chemokine receptor in a previously prepared tissue array constructed from the three NSCLC cell line pellets. Varying levels of CXCR4 expression were seen in all NSCLC cell lines tested (Figure 2.1). CXCR4 tumor AQUA scores for the NSCLC cells lines ranged from a minimum of 1187 for the H460 cell line to a maximum score of 1566 for the A549 cell line. The positive control *Hela* cell line had an CXCR4 AQUA score of 3472 indicating very high expression of the CXCR4 receptor. Staining was seen predominantly in the cell membrane and cytoplasm of the cells, with very little if any staining seen in the nucleus of the cells. In addition to the quantitative IHC, cell fractionation followed by western blot, as well as FACS analysis were also used by the Bebb lab to confirm the expression and localization of CXCR4 in the NSCLC cell lines (data in Appendix B).

FIGURE 2.1. Expression of the chemokine receptor CXCR4 in NSCLC cell lines by quantitative immunohistochemistry using the HistoRx platform. Hela cells were used as a positive control. Pink fluorescent staining in the top panel indicates positive CXCR4 staining. Graphical representation of the CXCR4 AQUA expression scores for the NSCLC cell lines and Hela control cells is shown in the graph.





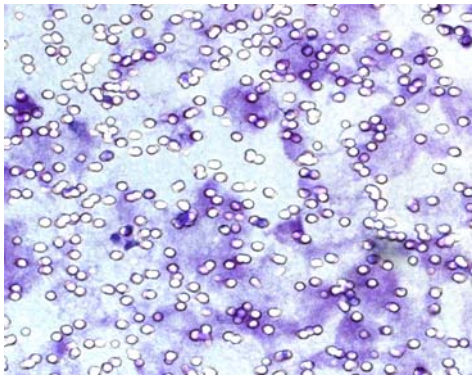
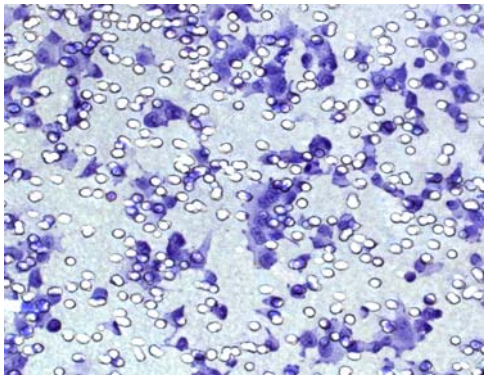
2.3.2 Optimization of migration assay protocol

Previous experiences with similar chemotaxis assays were carried out under varying conditions. Incubation periods, cell seeding densities, medium components and filter pore sizes varied between studies (Hu et al. 2005; Huang et al. 2007; Mori et al. 2004; Muller et al. 2001; Su et al. 2005), so before the formal assessment of NSCLC cell line migration was done, tests were completed to establish optimal conditions for the migration of non small cell lung cancer cell lines H1299, A549 and H460. The final protocol used was adapted based on the manufacturers suggestions as well as a protocol in a previous study by Su *et al* (Su et al. 2005), in which a similar migration assay was used with two different NSCLC cell lines.

A series of experiments were designed to determine the optimal migration conditions for the NSCLC cell lines, with a specific focus on optimizing the protocol in order to carry out the experiments in serum free medium. Previous migration assays run in the Bebb laboratory used the following protocol: medium containing 10% FBS, 48 hour incubation period, and a cell seeding density of 10×10^4 cells/ml. When switching to serum free media, it was found that a significantly shorter incubation period of 6 hours and an increase in seeding density (10×10^5 cells/ml) was required to produce results (which was more in concordance to the previously mentioned references). In serum free media it was found that after the 48 hour incubation period, all that was seen on the filter when examined was cellular debris and very few, if any, live cells remained (Figure 2.2). In order to prevent cellular death during the migration assays, the incubation period was reduced and the cell seeding density was increased. In addition, preliminary data from the Bebb lab established that the size of the pores in the filter influenced migration, with

filters containing 8 μm pores impeding migration compared to filters with 12 μm pores, and thus all further migration assays were carried out using filters containing 12 μm pores.

FIGURE 2.2. Visual comparison of results before and after migration assay protocol optimization. Synthetic filter under 20x magnification from two migration assays with the H1299 cell line in serum free medium. a) results of an experiment using an incubation period of 48 hours and the cellular debris which is all that remained. b), results of an experiment, after protocol optimization, using all the same experimental conditions, but with an incubation period of only 6 hours, and the resulting live migrating cells which remain.

a)  **b)** 

2.3.3 NSCLC cell lines migrate in response to SDF-1 in vitro

Once optimal conditions for migration were established, and expression of the CXCR4 chemokine receptor was confirmed in the NSCLC cell lines, cellular migration was quantified in response to a range of concentrations of the CXCR4 ligand SDF-1. Concentrations of SDF-1 ranging from 0 – 200 ng/ml were chosen based on a literature search. Previous studies in lung cancer cell lines as well as other cancers demonstrated that a SDF-1 concentration of 100 ng/ml would yield maximum migration in similar *in vitro* migration assays (Mori et al. 2004; Su et al. 2005), and one study even suggested that at concentrations greater than 100 ng/ml, suppression of migration was seen (Mori et al. 2004). In addition to confirming that the cell lines migrated in response to SDF-1, optimal levels of migration to SDF-1 were determined to allow statistically significant decreases to be measured with successful inhibition of the migration process in the next series of migration assays to be performed.

Migration assays using the Boyden chamber were carried out for all three NSCLC cell lines (H1299, A549 and H460 cell lines). Concentrations of SDF-1 at 0 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml were added to the lower wells of the chamber as a chemoattractant. Cells in suspension were seeded at a density of 10×10^5 cells/ml (5×10^4 per well) in the upper wells of the chamber. After 6 hours incubation at 37◦C, the migrating cells were stained and counted at 20x magnification (Figure 2.3). Each experimental condition was tested in quadruplicate wells and each experiment was repeated 3 times and averaged. Results from individual experiments were converted from absolute number of migrating cells into migration indexes to control for varying conditions between experiments.

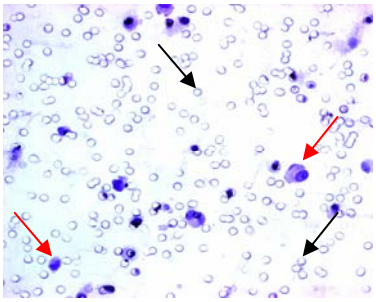
For the NSCLC cell line H1299, migration increased in a dose dependent manner to increasing concentrations of SDF-1, with a significant increase in migration seen to all concentrations of SDF-1 tested (Figure 2.4). There was a 3.35 fold increase in migration in response to 100 ng/ml SDF-1 compared to baseline levels of migration in the absence of added SDF-1 (p = 0.007). For the H460 and A549 cell lines, statistically significant increases in migration were seen to all concentrations, however to a lesser extent than for the H1299 cell line. For A549 cells, maximal migration was observed at 200 ng/ml, with a 2.6 fold increase in migration compared to control (p = 0.005), and a 1.9 fold increase in migration was seen at 100 ng/ml (p = 0.02). For the H460 cell line, maximum migration was actually seen at 50 ng/ml, however due to the large standard error, this was the least statistically significant concentration tested (p = 0.03) for this cell line. On the other hand, there was a only a 1.5 fold increase in migration seen to 100 ng/ml of SDF-1 but this was of greater statistical significance (p = 0.002). It can be concluded then that the NSCLC cell lines were indeed capable of migrating in response to SDF-1 with optimal migration seen at 100 ng/ml.

Interestingly, a similar trend was observed in the decrease or levelling off of migration in response to 200 ng/ml SDF-1 when compared to migration in response to 100 ng/ml for two out of the three cell lines, as reported by Mori *et al* in their studies with pancreatic cancer cell lines (Mori et al. 2004). However, the mechanism for this 'supraoptimal concentration' of SDF-1 and corresponding decrease in migration was not explored in this study.

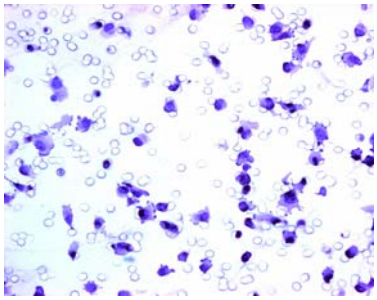
FIGURE 2.3. An example of the migrating cells under microscopy at 20x magnification. Each picture shows the porous membrane with adhered migrating cells in response to 0 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml SDF-1 in H1299, A549, H460 and Hela cell lines. The black arrows in the first image indicate the 12 micron pores in the membrane, red arrows indicate hematoxylin stained migrating cells.

H1299

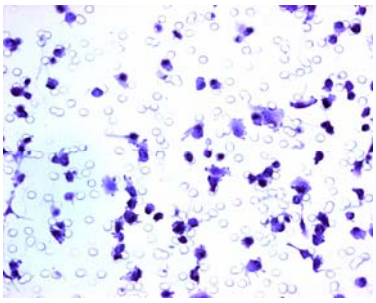
0 ng/ml SDF-1



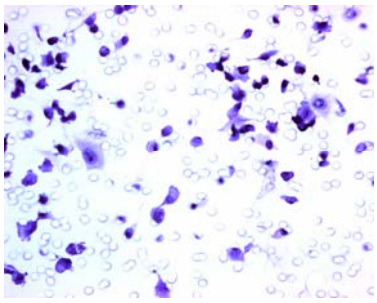
50 ng/ml SDF-1



100 ng/ml SDF-1

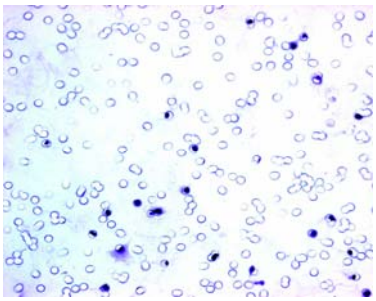


200 ng/ml SDF-1

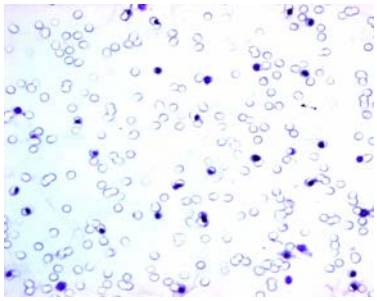


H460

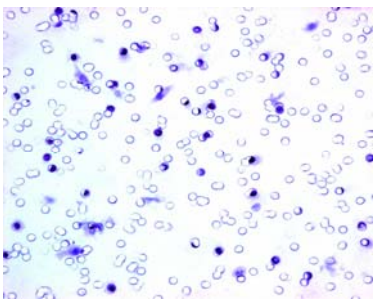
0 ng/ml SDF-1



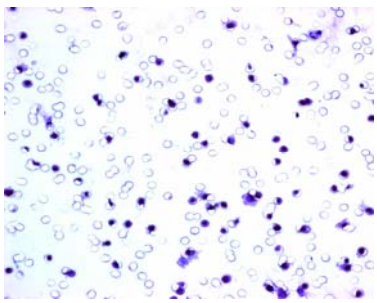
50 ng/ml SDF-1



100 ng/ml SDF-1

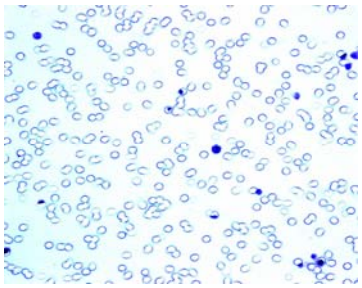


200 ng/ml SDF-1

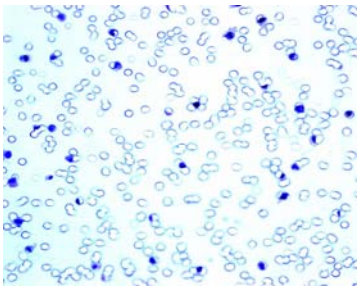


A549

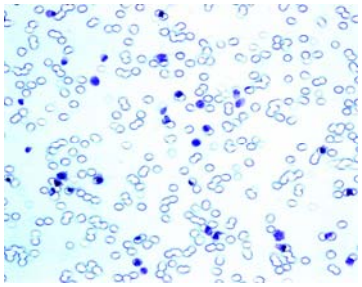
0 ng/ml SDF-1



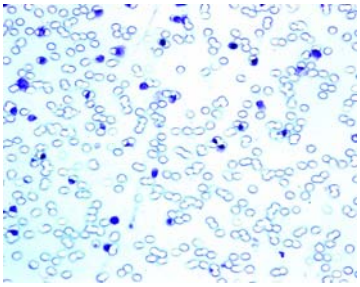
50 ng/ml SDF-1



100 ng/ml SDF-1

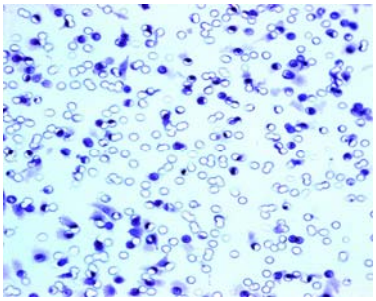


200 ng/ml SDF-1

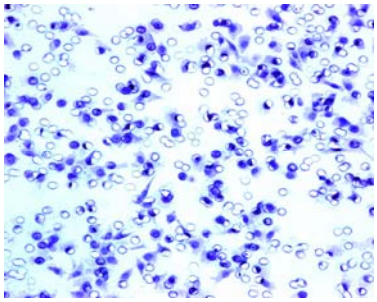


Hela

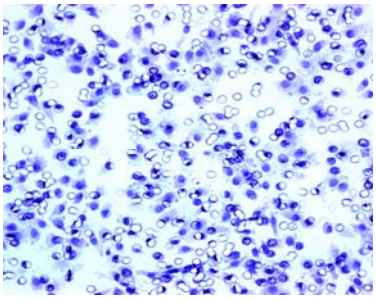
0 ng/ml SDF-1



50 ng/ml SDF-1



100 ng/ml SDF-1



200 ng/ml SDF-1

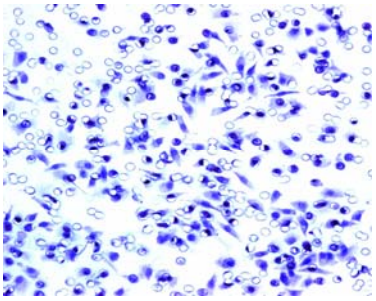
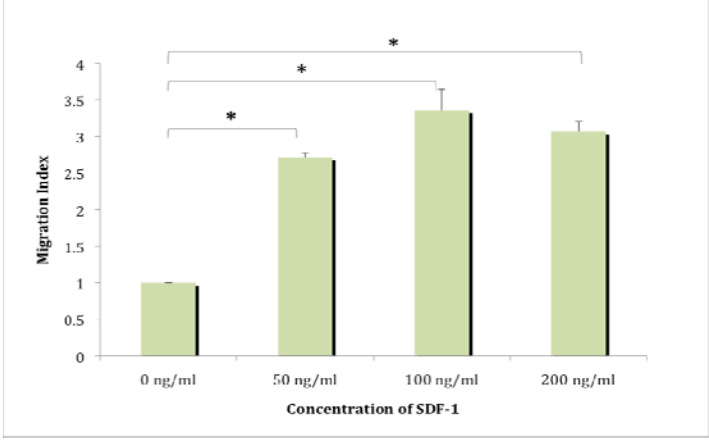
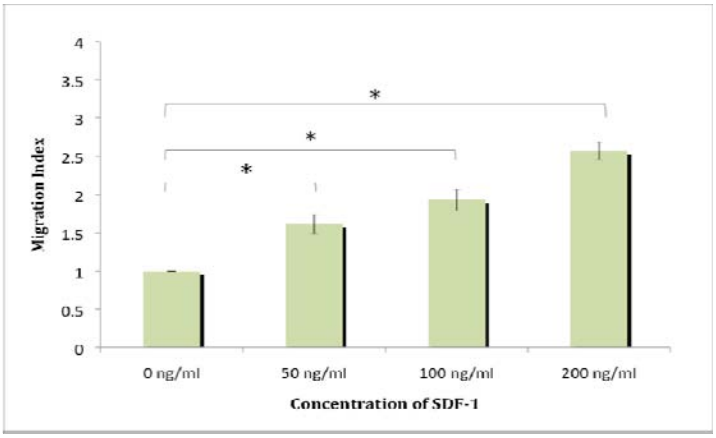


FIGURE 2.4. Cell migration of NSCLC cell lines in response to SDF-1. Migration in reponse to 50, 100 and 200 ug/ml of the CXCR4 ligand SDF-1 in H1299, A549 and H460 non-small cell lung cancer cell lines (NSCLC). * indicates significance at $p < 0.05$ between the indicated conditions using a one tailed students *t*-test.

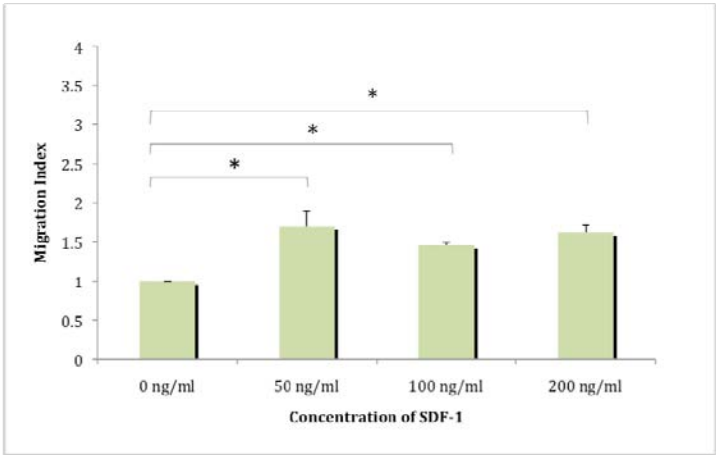
H1299



A549



H460



2.3.4 Inhibition of CXCR4 reduces the migrational capacity of NSCLC cell lines

After confirming that the NSCLC cell lines were capable of migrating in response to SDF-1, a series of CXCR4 inhibitors were tested to assess whether it was possible to decrease SDF-1 induced migration by blocking the receptor. The inhibitors used consisted of a small molecular inhibitor to the CXCR4 receptor (AMD 3100), a small peptide inhibitor to the receptor (CTCE 9908) and an anti-CXCR4 monoclonal antibody (MAb). Many of these CXCR4 inhibitors have been tested *in vitro* in other studies with NSCLC cell lines, however never in the same study where the relative efficacies of each inhibitor could be compared with one another.

The CXCR4 small molecular inhibitor AMD 3100 was capable of significantly inhibiting the migration of all three NSCLC cell lines tested. A concentration of 2 ug/ml was chosen based on preliminary data as well as a literature search of relevant material. For the H1299 cell line, addition of the AMD 3100 inhibitor decreased the migration of cells 23.5% compared to control medium alone (p = 0.03) (Figure 2.5). Similarly, the addition of AMD 3100 decreased the migration of H460 cells by 27.1% (p = 0.005) and 28.8% for the A549 cells (p = 0.009) (Figure 2.5). Although there was a significant decrease in the amount of migration seen in all three NSCLC cell lines with AMD 3100, migration was only reduced to ‘baseline levels’ in the A549 cell line (Figure 2.6). The baseline level of migration represents the spontaneous migration of the cells in the absence of the chemoattractant SDF-1 (blue bars in figure). In the H1299 and H460 cell lines, there is still a significant difference in the migration seen with inhibition of SDF-1 induced migration by AMD 3100 and baseline migration levels. In other words, AMD

3100 was incapable of completely blocking the SDF-1 induced migration of the H1299 and H460 cell lines.

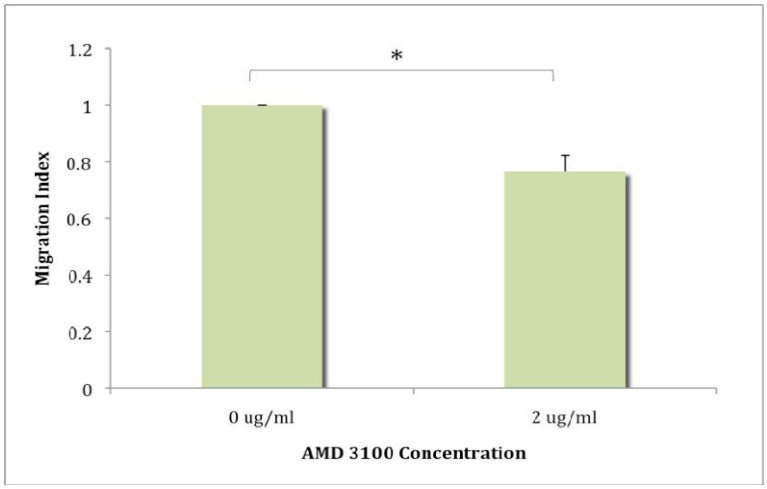
The CXCR4 small peptide inhibitor CTCE 9908 was also capable of significantly decreasing migration in all cell lines tested, and to a greater extent than AMD 3100. A CTCE 9908 concentration of 100 ug/ml was chosen based on the manufacturer's suggestions as well as preliminary data. A 31.5% decrease in migration was seen with neutralization of the CXCR4 receptor by CTCE 9908 for the H1299 cell line (p = 0.0002), a decrease of 31.0% for the A549 cells (p = 0.02) and 47.0% for the H460 cell line (p = 0.02) (Figure 2.7). In addition, unlike the previous CXCR4 inhibitor, AMD 3100, CTCE 9908 was capable of decreasing the migration of all three NSCLC cell lines to baseline levels (Figure 2.8).

The last CXCR4 inhibitor assessed was a monoclonal antibody (MAb) to the CXCR4 receptor which binds to the ligand binding region of the receptor and thereby prevents the binding of SDF-1. A concentration of antibody at 20 ug/ml was chosen also based on preliminary data and the manufacturer's suggestions. The CXCR4 MAb was only capable of significantly decreasing the migration in the H460 and A549 cell lines but not in the H1299 cells. Only a 6.8% decrease in migration was seen in the H1299 cells and was not statistically significant (p = 0.40). For the A549 and AH460 cell lines, the addition of the CXCR4 MAb decreased the migration of the cells by 23% (p = 0.03) and 25.8% (p = 0.02), respectively (Figure 2.9). Furthermore, the CXCR4 MAb reduced migration to baseline levels in only the H460 cell line (Figure 2.10). Taken together, these results suggest that it is possible to significantly decrease the migration of NSCLC cell line cells by effectively blocking or neutralizing the CXCR4 chemokine receptor. It

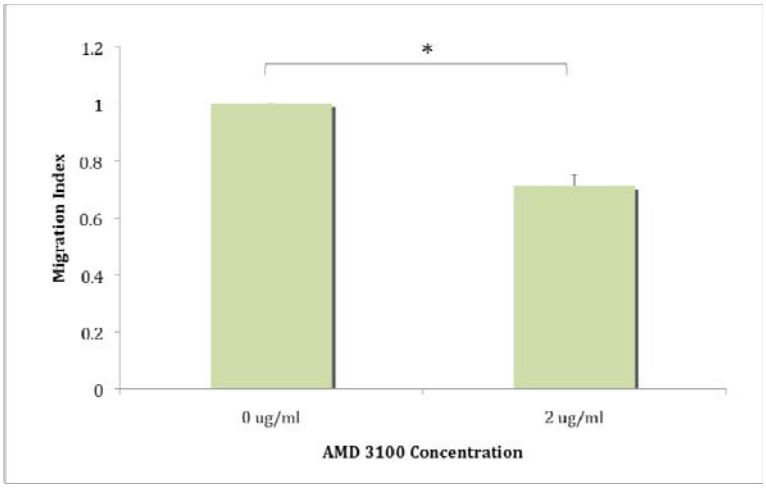
also appears that there are differences in the efficacy of the various CXCR4 inhibitors in their ability to inhibit migration, which may be inhibitor and/or cell line specific. Similarly, the inhibitors were not always capable of entirely inhibiting SDF-1 induced migration of the NSCLC cell lines which could indicate incomplete inhibition of the CXCR4/SDF-1 chemokine axis with the use of these inhibitors.

FIGURE 2.5. Inhibition of NSCLC cell migration by the CXCR4 small molecular inhibitor AMD 3100. 2 ug/ml AMD 3100 significantly decreased the migration of H1299, A549 and H460 non-small cell lung cancer cell lines (NSCLC) in response to 100 ng/ml SDF-1. * indicates significance at $p < 0.05$ between the indicated conditions.

H1299



A549



H460

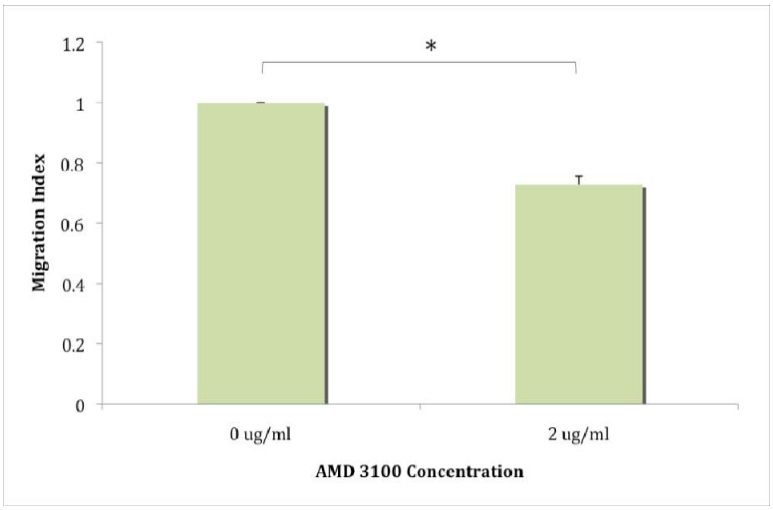


FIGURE 2.6. The CXCR4 inhibitor AMD 3100 was not effective in decreasing the migration of all NSCLC cell lines to baseline migration levels. The blue bars in the graph below indcate baseline levels of migration – the spontaneous migration seen in the absence of the chemoattractant SDF-1. Only in the A549 cell line was AMD 3100 capable of decreasing migration to baseline levels. In the H1299 and H460 cell lines there was still a significant increase in migration in comparison to baseline.

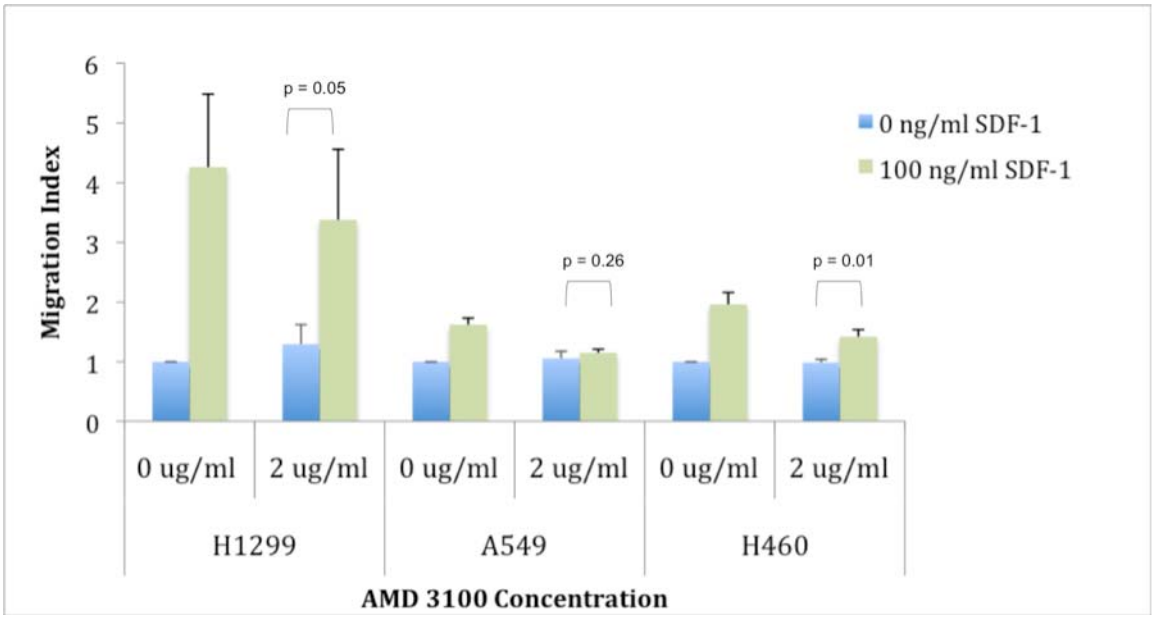
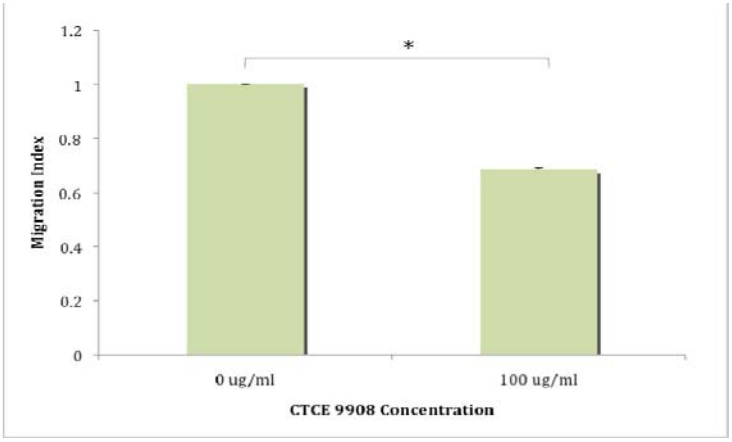
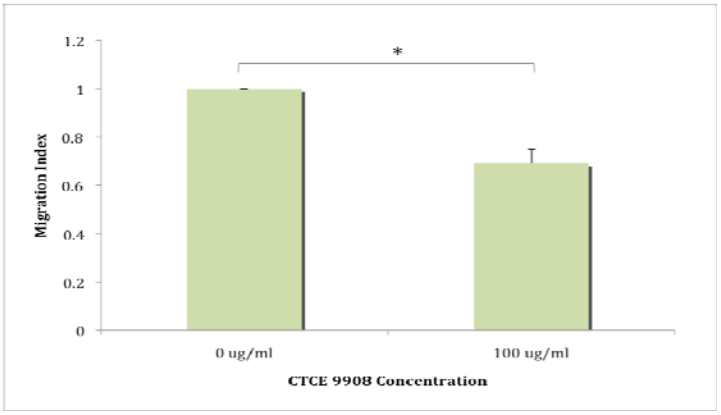


FIGURE 2.7. Inhibition of NSCLC cell migration by the CXCR4 small molecular inhibitor CTCE 9908. 100 ug/ml CTCE 9908 significantly decreased the migration of H1299, A549 and H460 non-small cell lung cancer cell lines (NSCLC) in response to 100 ng/ml SDF-1. * indicates significance at $p < 0.05$ between the indicated conditions.

H1299



A549



H460

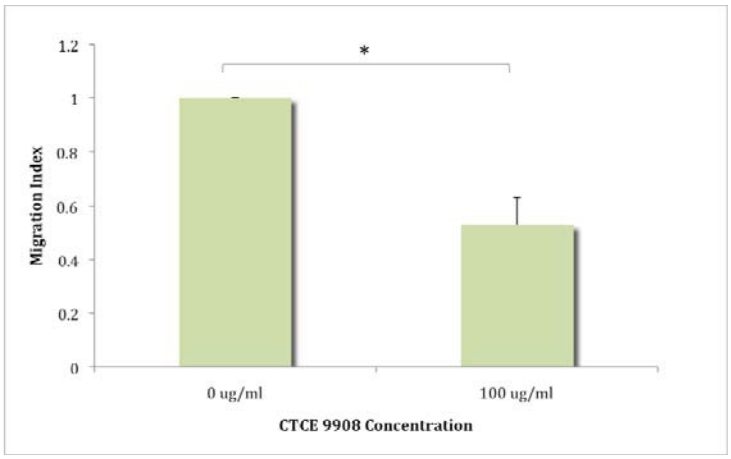


FIGURE 2.8. The CXCR4 inhibitor CTCE 9908 was effective in decreasing the migration of all NSCLC cell lines to baseline migration levels. The blue bars in the graph below indcate baseline levels of migration – the spontaneous migration seen in the absence of the chemoattractant SDF-1. In all cell lines there was no significant difference between the amount of migration after inhibition of CXCR4 by CTCE 9908 and baseline levels (indicated by the non significant *p* values).

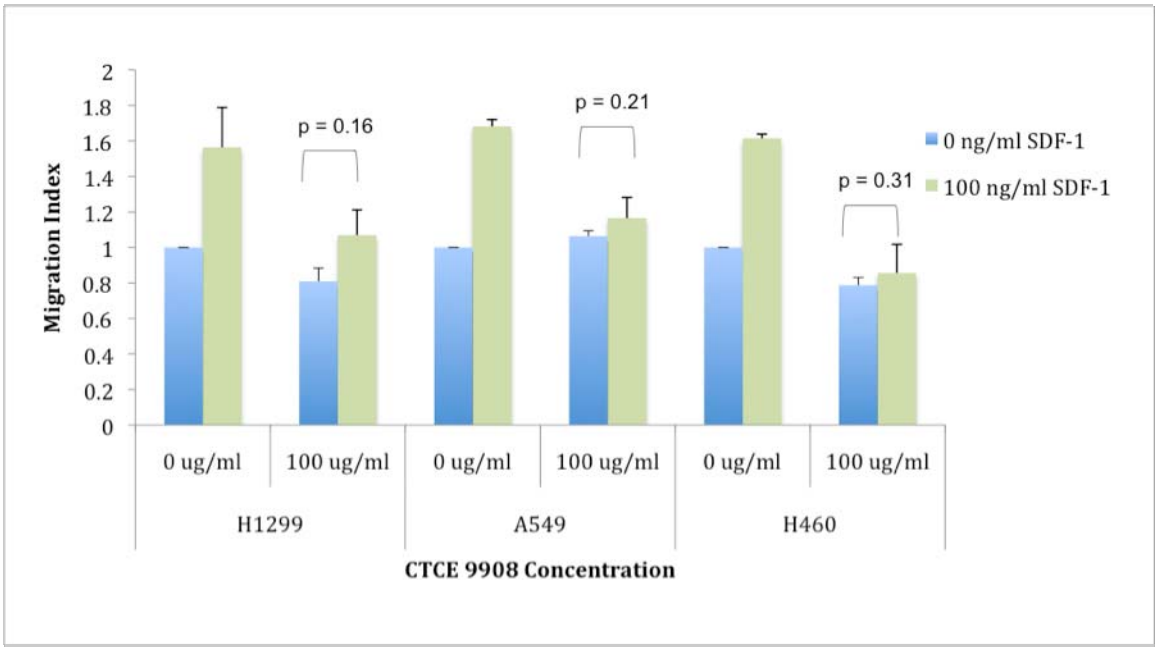
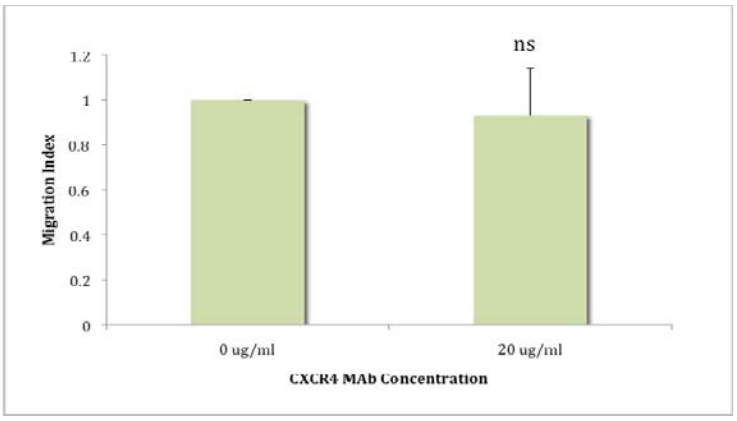
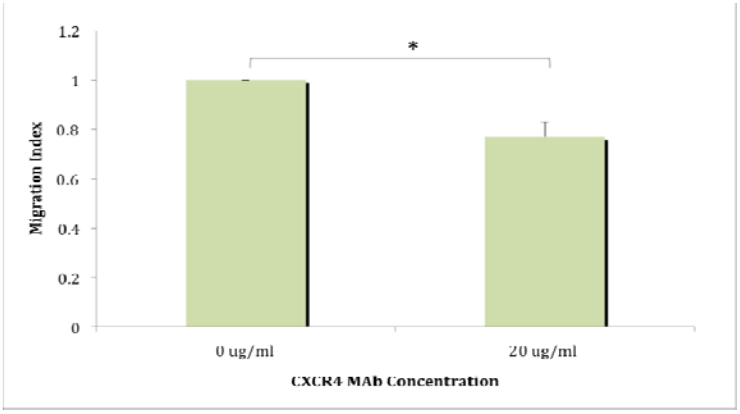


FIGURE 2.9. Inhibition of NSCLC cell migration by an anti-CXCR4 monoclonal antibody (MAb). 20 ug/ml anti-CXCR4 MAb significantly decreased the migration of A549 and H460 non-small cell lung cancer cell lines (NSCLC) in response to 100 ng/ml SDF-1. The anti-CXCR4 MAb was not effective in inhibiting the migration of the H1299 cell line. * indicates significance at $p < 0.05$ between the indicated conditions.

H1299



A549



H460

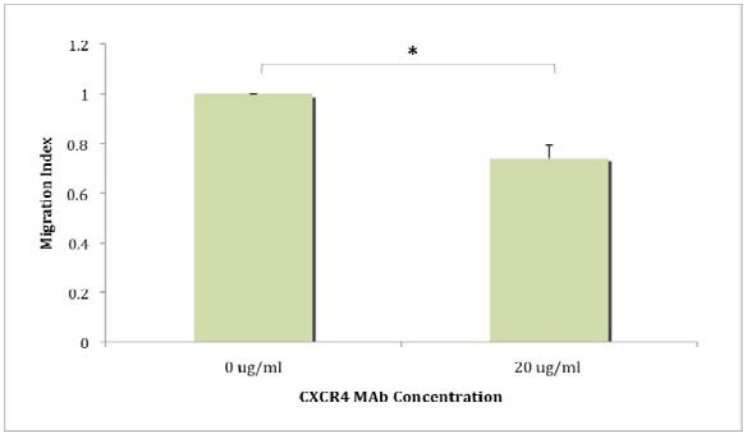
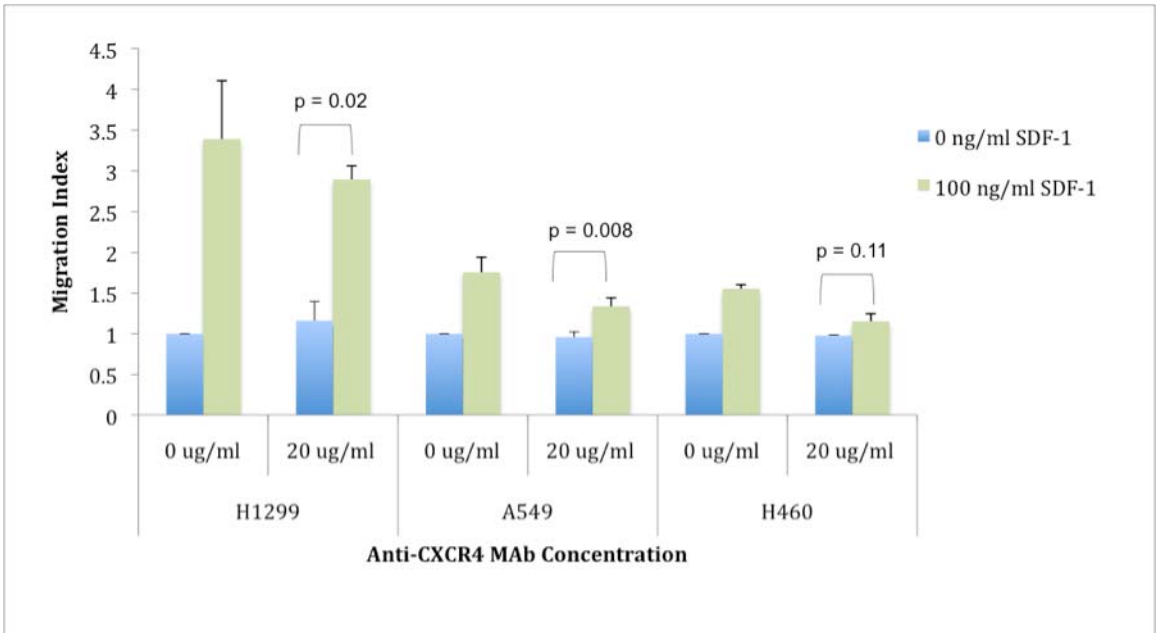


FIGURE 2.10. The anti-CXCR4 monoclonal antibody (MAb) was not effective in decreasing the migration of all NSCLC cell lines to baseline migration levels. The blue bars in the graph below indicate baseline levels of migration – the spontaneous migration seen in the absence of the chemoattractant SDF-1. Only in the H460 cell line was anti-CXCR4 MAb capable of decreasing migration to baseline levels. In the H1299 and A549 cell lines there was still a significant increase in migration in comparison to baseline (indicated by the significant *p* values).



2.3.5 SDF-1 neutralization reduces the migrational capacity of NSCLC cell lines in vitro

Next, the effect of neutralizing, or 'mopping up' the ligand, SDF-1, on migration in the NSCLC cell lines was assessed. As it had been recently discovered that SDF-1 may signal through another receptor, CXCR7, it was possible that complete inhibition of migration was not obtained by blocking the CXCR4 receptor due to the alternate receptor activated by SDF-1. Although the CXCR7 status of the NSCLC cell lines used was unknown, it was postulated that by attempting to neutralize the ligand instead of the receptor, more significant decreases in migration of the cell lines might be seen. As mentioned before, by introducing an anti-SDF-1 antibody directly into the lower chambers along with the SDF-1, would cause the ligand to be 'mopped up' and thereby inhibit the chemoattraction necessary for NSCLC cell migration.

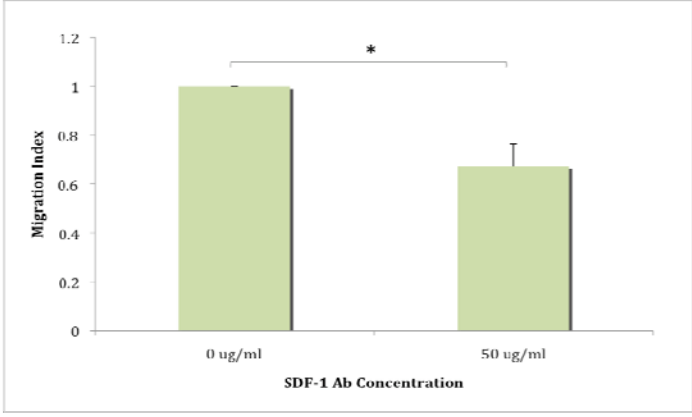
Both a monoclonal as well as a polyclonal anti SDF-1 antibody were used. The MAb was used in the first set of experiments with the H1299 cell line, but was unavailable for purchase for the next set of experiments with the H460 and A549 cell lines. The switch was made to the polyclonal antibody in order to continue with experimentation in a timely manner. For the MAb, a concentration of 50 ug/ml was chosen based on preliminary data as well as the manufacturer's suggestions. For the H1299 cell lines, there was a 32.8% decrease in migration seen with the anti-SDF-1 MAb (p = 0.04) (Figure 2.11). For the polyclonal anti-SDF-1 antibody, a concentration of 10 ug/ml was chosen also based on preliminary data as well as the manufacturer's suggestions. A decrease in migration of 22.0% (p = 0.03) and 20.7% (p = 0.02) was seen

for the A549 and H460 cell lines, respectively (Figure 2.11). And in only the A549 was the SDF-1 Ab capable of decreasing migration to baseline level (Figure 2.12)

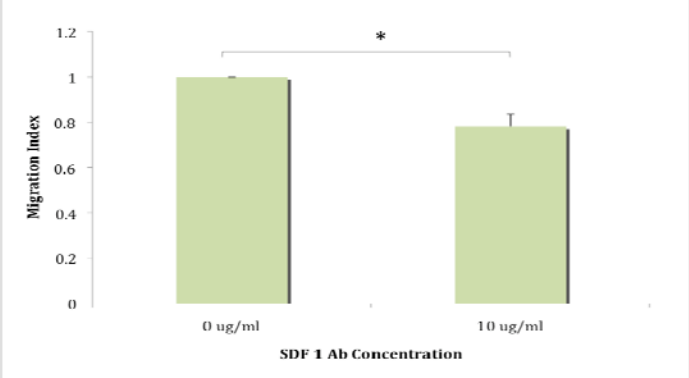
It appears that inhibition of SDF-1 is also capable of decreasing the migrational capacity of the NSCLC cell lines in response to the chemoattractant properties of SDF-1. However, it can be seen that inhibition of the ligand instead of the receptor was not more efficacious in inhibiting the migration of NSCLC cell lines as hypothesized. In fact, it appears that at least for the H460 and A549 cell lines, the anti-SDF-1 antibody tested was slightly less efficacious than the CXCR4 inhibitors tested.

FIGURE 2.11. Inhibition of cell migration by an anti-SDF-1 antibody (Ab). For the H1299 cell line a monoclonal antibody was used at a concentration of 50 ug/ml, and for the A549 and H460 cell lines a polyclonal antibody was used at a concentration of 10 ug/ml. The anti-SDF-1 Ab significantly decreased the migration of H1299, A549 and H460 non-small cell lung cancer cell lines (NSCLC) in response to 100 ng/ml SDF-1. * indicates significance at $p < 0.05$.

H1299



A549



H460

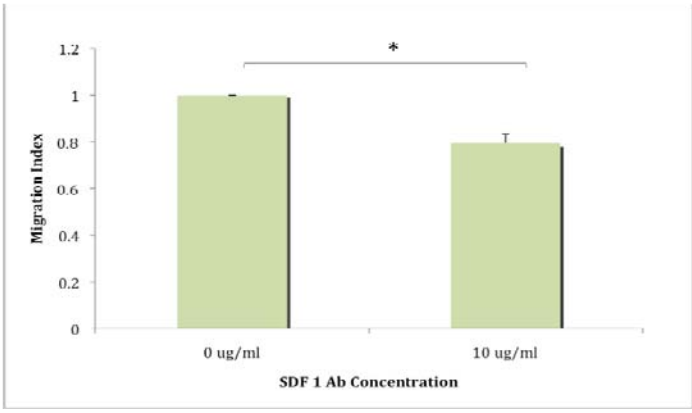
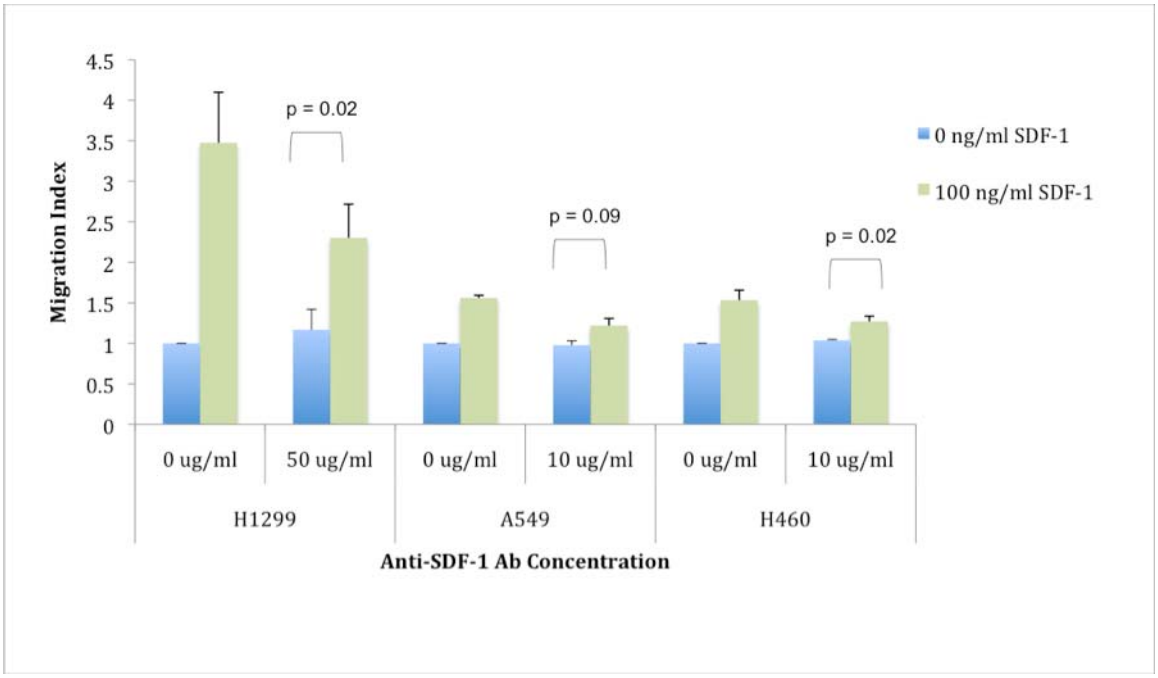


FIGURE 2.12. The anti-SDF-1 antibody (Ab) was not effective in decreasing the migration of any of the NSCLC cell lines to baseline migration levels. The blue bars in the graph below indcate baseline levels of migration – the spontaneous migration seen in the absence of the chemoattractant SDF-1. In all cell lines there was still a significant increase in migration in comparison to baseline (indicated by the significant *p* values).



2.3.6 Effect of blocking both CXCR4 and SDF-1 in combination on the migration of NSCLC cell lines

In order to assess whether blocking both the receptor and ligand in combination resulted in enhanced inhibition of migration over and above what was seen by blocking either the receptor or the ligand individually, the migration of each cell line in response to 100 ng/ml of SDF-1 in the presence of the anti-SDF-1 antibody, the CXCR4 inhibitor CTCE 9908, or both in combination, was assessed. Again, for the H1299 cell line the monoclonal anti-SDF-1 Ab was used at a concentration of 50 µg/ml, and for the A549 and H460 cell lines the polyclonal anti-SDF-1 Ab was used at a concentration of 10 ug/ml. For the H1299 cell line, there was a 25.3% decrease in migration in the presence of the anti-SDF-1 MAb (p = 0.02), a 32.3% decrease with CTCE 9908 (p = 0.01) and a 32.1% decrease in the presence of both CTCE 9908 and the SDF-1 MAb (p = 0.006). There was no significant decrease in migration seen with the addition of the anti SDF-1 antibody over that seen with the CXCR4 inhibitor CTCE 9908.

For the A549 cell line a 15% decrease in migration was seen with the anti-SDF-1 antibody (p = 0.02), 36.4% decrease in migration with the CTCE 9908 (p = 0.001) and a 40.0% decrease in the presence of both the SDF-1 and CXCR4 inhibitors (p = 0.002). Unlike the H1299 cell line however, there was a significant decrease in migration seen with the addition of the anti-SDF-1 Ab to the CTCE 9908 inhibitor (p = 0.02). Lastly, for the H460 cell line both the SDF-1 Ab (17.4%, p =0.005) , the CTCE 9908 (36.9%, p = 0.009) as well as the combination of the two (42.3%, p = 0.005) significantly decreased the migration of this cell line. In addition, the CTCE 9908 was more efficacious in its

ability to decrease the migration of H460 cells when compared to the anti-SDF-1 Ab ($p = 0.01$).

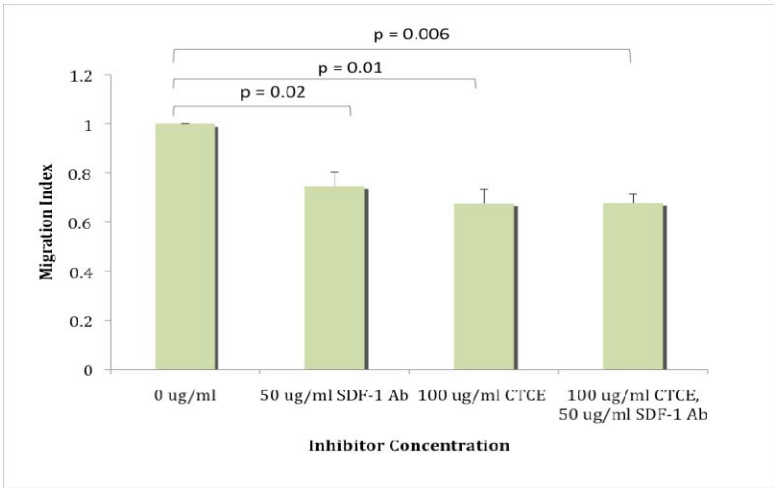
Once the migration assays were completed for all cell lines with the combination of inhibitors, it was possible to compare the relative efficacy of all five methods of CXCR4/SDF-1 axis inhibition (AMD 3100, CTCE 9908, anti-CXCR4 MAb, anti-SDF-1 Ab, and CXCR4 and SDF-1 combination inhibition) to each other for each of the three NSCLC cell lines (Table 2.1). It can be seen that there was some heterogeneity among the methods of CXCR4/SDF-1 axis inhibition in their ability to effectively decrease the migration of the NSCLC cell lines, and these changes appear to be cell line specific (Figure 2.14). When tested for significance, there were no differences in the H1299 cell line between any of the methods of CXCR4/SDF-1 axis inhibition. For the A549 cell line, only CTCE 9908 and the combination of CTCE 9908 and anti-SDF-1 antibody were found to be more effective than the other inhibitors, the CTCE 9908 being superior to the anti-SDF-1 Ab, and the combo being superior to both the anti-SDF-1 Ab and the anti-CXCR4 Ab.

Lastly, for the H460 cells both the CTCE 9908 and the combination of CTCE 9908 and the anti-SDF-1 Ab were the only methods of CXCR4/SDF-1 inhibition which were found to be significantly more effective at decreasing the migration of this cell line. Again, the CTCE 9908 was found to be more effective than the anti-SDF-1 Ab and the combination was more effective than both the anti-SDF-1 Ab as well as AMD 3100. Clearly, there was no one method which was significantly more effective than the other inhibitors for all of the NSCLC lines tested, but the CXCR4 inhibitor CTCE 9908

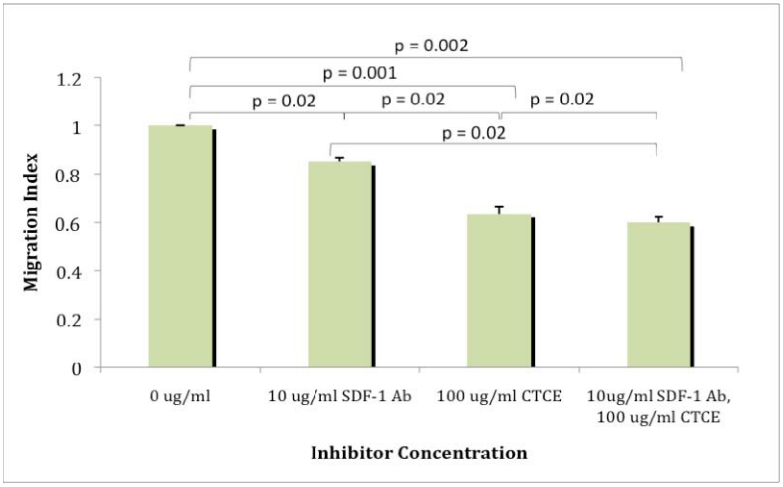
appears to be the most consistent and effective of the CXCR4/SDF-1 axis inhibitors tested. In addition, it appears that there may some utility in combining both SDF-1 neutralization and CXCR4 inhibition, but further studies to test the effectiveness of combinations of inhibitors must be undertaken.

FIGURE 2.13. Inhibition of cell migration by a combination of the CXCR4 inhibitor CTCE 9908 and an anti-SDF-1 antibody (Ab). For the H1299 and H460 cell lines, the combination of anti-SDF-1 MAb and CTCE 9908 was not capable of decreasing migration more than 100 ug/ml CTCE 9908 on its own. On the other hand, for the A549 line, the combination of CTCE 9908 and anti-SDF-1 antibody decreased migration significantly more than either one alone. Significant *p* values are listed.

H1299



A549



H460

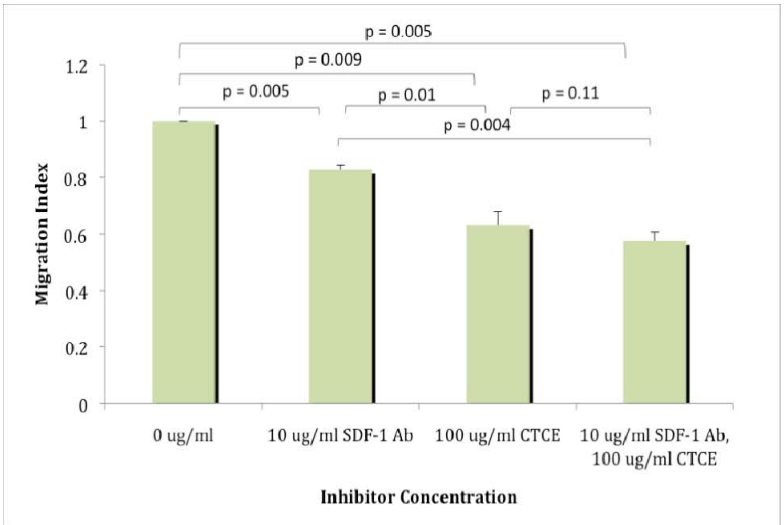
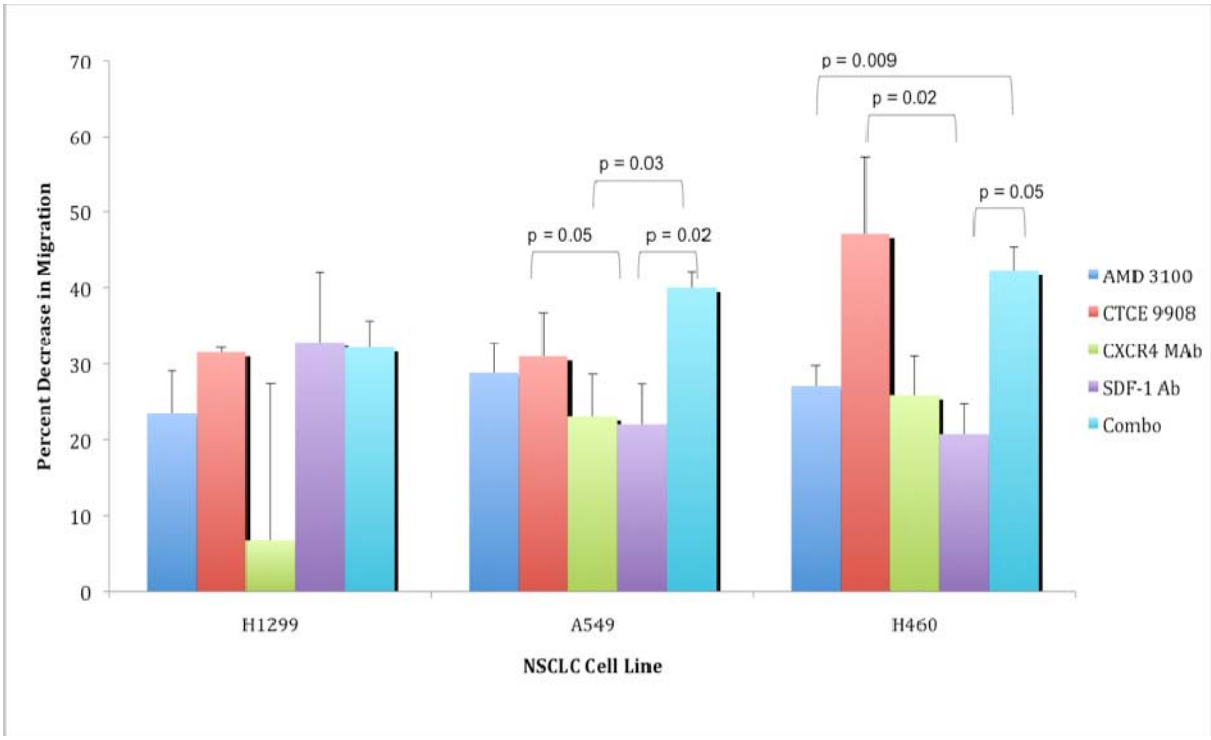


TABLE 2.1. Summary table displaying the average percent decrease in migration seen with each of the five methods of CXCR4/SDF-1 axis inhibition in the three NSCLC cell lines tested.

Cell Line	AMD 3100	CTCE 9908	Anti CXCR4 MAb	Anti SDF-1 Ab	Combo
H1299	23.4%	31.5%	6.8%	32.8%	32.1%
A549	28.8%	31.0%	23.0%	22.0%	40.0%
H460	27.1%	47.0%	25.8%	20.7%	42.3%

FIGURE 2.14. Graphical representation of the average percent decrease in migration seen with each of the five methods of CXCR4/SDF-1 axis inhibition, in the three NSCLC cell lines. No significant difference was seen in the H1299 between the methods of CXCR4/SDF-1 axis inhibition in their ability to decrease cell line migration. For the A549 and H460 cell lines, only the CTCE 9908 and combination of CTCE 9908 and anti-SDF-1 antibody were capable of significantly decreasing migration compared to some of the other inhibitors tested.



2.4 Discussion

The primary cause of mortality in non small cell lung cancer is metastasis, in which migration plays a key role. It is therefore crucial that the mechanisms which tumor cells utilize in order to migrate are investigated in order to have a better understanding of the metastatic process. These experiments demonstrate that NSCLC cell lines express the CXCR4 chemokine receptor, that the CXCR4/SDF-1 chemokine/receptor axis plays a role in the migration of NSCLC cell lines *in vitro,* and that successful inhibition of either CXCR4 or SDF-1 is capable of significantly decreasing the migrational capacity of the cell lines in response to optimal concentrations of SDF-1. In addition, this series of experiments allowed for a comparison of the relative efficacies of the various CXCR4/SDF-1 inhibitors tested, as this has not been reported previously in the literature.

All CXCR4/SDF-1 axis inhibitors tested were effective in significantly decreasing the migration of each of the three NSCLC cell lines assayed (with the exception of the anti-CXCR4 MAb with the H1299 cell line), the CXCR4 small molecular inhibitor CTCE 9908 appearing to be the most consistent and effective in its ability to inhibit migration. It is important to note however, that none of the inhibitors tested were able to completely block the migration of the NSCLC cell lines. This finding was not overly surprising however, as complete inhibition of migration in NSCLC cell lines by modulation of the CXCR4/SDF-1 chemokine axis in previous studies has never been reported (Su et al. 2005). Since the CXCR4/SDF-1 chemokine axis is not the only mechanism that leads to tumor cell migration, it is unlikely that blocking the CXCR4/SDF-1 axis alone would result in the complete absence of cell migration.

It is also important to note that only CTCE 9908 was capable of decreasing migration of all three cell lines down to baseline levels (ie. completely block SDF-1 induced migration) while the other inhibitors of CXCR4 and SDF-1 were able to decrease migration to baseline levels in only one out of the three NSCLC cell lines tested. These results could indicate that either the other CXCR4/SDF-1 inhibitors tested were incapable of completely blocking the activity of CXCR4 or SDF-1, or that there may be an alternate mechanism through which SDF-1 was exerting its migration effects in these cell lines.

This finding was also not unexpected, as it was known that SDF-1 is reported to bind to an alternate chemokine receptor, CXCR7 (Burns et al. 2006; Joost & Methner 2002). Since only one of the SDF-1 receptors was blocked by this experimental approach, it was not inconceivable that SDF-1 was still able to bind to and signal through its alternate receptor to influence the migrational capacity of the NSCLC cell lines. As mentioned in Chapter 1, CXCR7 has been shown to be expressed in a number of tumor cell lines including, breast, prostate and lung tumor cell lines (Miao et al. 2007; Wang et al. 2008), and one study in particular reported that the NSCLC cell line A549 exhibited membrane expression of CXCR7 (Burns et al. 2006). Similarly, it was noted that CXCR7 was expressed in NSCLC patient tumor specimens and may have an association with disease free survival (Iwakiri et al. 2009; Miao et al. 2007).

So, it is very likely that the three NSCLC cell lines tested expressed the chemokine receptor CXCR7, which may have influenced the SDF-1 induced migration of these cell lines. Although most studies have reported that CXCR7 does not play a direct role in the migration of cells and that activated CXCR7 is not capable of eliciting intracellular

calcium mobilization (which is required for cellular migration) (Boldajipour et al. 2008; Burns et al. 2006; Hartmann et al. 2008), many have reported that CXCR7 may play an indirect role in cell motility and chemotaxis (Valentin et al. 2007; Zabel et al. 2009). For example, CXCR7 has been shown to play a role in integrin activation and increased cellular adhesion, both of which could play an indirect role in cell migration (Zabel et al. 2009). Furthermore, it has been reported that CXCR4 and CXCR7 co-expression on cells actually results in enhanced SDF-1 activated calcium mobilization over cells expressing CXCR4 alone (Levoye et al. 2009), so an insignificant amount of CXCR4 activation occurring in the assays, even in the face of CXCR4 inhibition, may have been amplified by the mere presence of CXCR7. The assessment of CXCR7 expression in the NSCLC cell lines was felt to be outside of the scope of this project, however, CXCR7 is the topic of ongoing research in several laboratories.

Whether the inability of the tested CXCR4/SDF-1 axis inhibitors (with the exception of CTCE 9908) to fully inhibit SDF-1 induced cellular migration was simply due to their ineffectiveness in completely blocking the activity of the CXCR4/SDF-1 axis, or due to more complex mechanisms or cross-talk between the CXCR4/SDF-1 axis and SDF-1's other receptor CXCR7, remains to be seen. Assessments to determine the ability of the inhibitors to block SDF-1 induced activation of CXCR4 is currently underway in the Bebb lab. But there is no doubt that other important and complex mechanisms are involved, probably involving CXCR7, and more studies are required in order to fully understand the role of CXCR4 in the migration of NSCLC cells.

These results also suggest that the CXCR4/SDF-1 axis may be a useful therapeutic target for anti-metastatic treatment in NSCLC. Two of the CXCR4 inhibitors tested have already been through early phase clinical trials, although not specifically in lung cancer. CTCE 9908 was well tolerated in a phase I/II clinical trial and preliminary evidence showed that this agent was efficacious in late stage solid tumors (25 patients were included in the trial, with 3 lung cancer patients) (Hotte 2007). Of the 20 patients who received the targeted efficacious dose, 6 had stable disease, and one patient experienced a reduction in the size of their tumor. Also, a phase I trial with AMD 3100 was done in healthy volunteers and was shown to be safe in humans (Hendrix et al. 2000). However, during this trial it was observed that the administration of AMD 3100 resulted in a rapid increase in hematopoietic stem cells to the peripheral blood, which lead to further research into the use of AMD 3100 as a stem cell mobilizer (Broxmeyer et al. 2005; Devine et al. 2004; Liles et al. 2003) as well as the recent approval by the FDA of AMD 3100 for use in combination with G-CSF for stem cell mobilization in patients with non-Hodgkins lymphoma and multiple myeloma (Pusic and DiPersio 2010).

This particular finding raises an interesting point. Inhibition of the CXCR4/SDF-1 chemokine axis may have paradoxical effect in that it may inhibit homing of circulating tumor cells to sites of metastasis as hypothesized, but if there is a sufficient concentration of SDF-1 at the primary tumor, CXCR4/SDF-1 inhibition may also induce the release of tumor cells from the primary site of origin in an analogous fashion to the mobilization of stem cells from the bone marrow. These somewhat conflicting functions of the CXCR4/SDF-1 axis suggest that in order for it to be a useful therapeutic target in the treatment of NSCLC or any other type of cancer, the balance and method of

CXCR4/SDF-1 inhibition will have to be worked out in such a way as to minimize any release of tumor cells from the primary tumor and other unwanted results. Furthermore, the complex interactions of CXCR4/CXCR7/SDF-1 must be taken into account when considering the utility of CXCR4/SDF-1 axis inhibition in the treatment of cancer.

In summary, CXCR4/SDF-1 axis inhibition by a CXCR4 small molecule inhibitor, anti-CXCR4 MAb or anti-SDF-1 Ab is capable of significantly decreasing the migrational capacity of NSCLC cell lines *in vitro.* Of all the inhibitors assessed, the CXCR4 peptide inhibitor CTCE 9908 appeared to be the most effective in inhibiting the migrational capacity of the cell lines, and contrary to the original experimental hypothesis, the anti-SDF-1 approach, nor a combination of CXCR4 and SDF-1 inhibition, was found to be more effective than anti-CXCR4 strategies alone. Furthermore, metastasis involves the cooperation of several processes of which migration is only one. Therefore, approaches that inhibit migration are only targeting only one aspect of an incredibly complex process. However, these findings, as well as other preclinical data stongly suggests that the CXCR4/SDF-1 axis plays a role in not only migration, but many other aspects of the metastatic process such as adhesion, invasion, and angiogenesis, so modulation of the CXCR4/SDF-1 in NSCLC may also inhibit these processes which just inceases the attractiveness of the CXCR4/SDF-1 axis as a therapeutic target.

Chapter Three: Influence of the CXCR4 Chemokine Receptor on Clinical Outcome in NSCLC

This chapter was accepted for publication in the Journal of Thoracic Oncology, in March 2011. Article entitled “CXCR4 Over Expression is Associated with Poor Outcome in Females Diagnosed with Stage IV Non Small Cell Lung Cancer”

3.1 Introduction

Lung cancer is the leading cause of death from cancer worldwide yet 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). 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) pro-inflammatory chemotactic cytokines (Zlotnik & Yoshie 2000), play a predominant role in regulating the homing and trafficking of various leukocyte subpopulations, particularly during inflammation, tissue damage, and infection (Baggiolini et al. 1994; Schier 2003). 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 (Aiuti et al. 1997; Ma et al. 1999), 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 (Alsayed et al. 2007; Cho et al. 2006; Hillyer et al. 2003; Kucia et al. 2005), 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 (Fernandis et al. 2004; Kang et al. 2005; Muller et al. 2001; Smith et al. 2004) ovarian (Jiang et al. 2006), colorectal (Zeelenberg et al. 2003), head and neck (Hu et al. 2005; Yoon et al. 2007), and pancreatic carcinomas (Koshiba et al. 2000; Mori et al. 2004), 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* (Phillips et al. 2003; Su et al. 2005). 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* (Phillips et al. 2005).

Importantly, a role for CXCR4 in NSCLC is supported by retrospective studies in stage I and II NSCLC suggesting that resected patients with high CXCR4 expressing tumors as detected by rtPCR are more prone to metastasis compared to patients with low expressing tumors (Su et al. 2005). In addition, it has been suggested that high CXCR4 expression, as assessed by semi-quantitative IHC, is associated with a significantly decreased overall survival in early stage NSCLC patients. However, when high CXCR4 expression is localized to the cell nucleus only, patients had a better prognosis and longer overall survival (Spano et al. 2004; Wagner et al. 2009). 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. Here, investigations are described aiming to determine if CXCR4 is an independent prognostic biomarker of overall survival in NSCLC patients with advanced stage IV disease by quantitative immunohistochemical analysis of *ex vivo* tumor samples.

3.2 Materials and Methods

3.2.1 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 was 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 was 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 TNM 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 was 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: non-smoking status was defined as having smoked less than 100 cigarettes total, while a current smoking status was assigned if the patient smoked at time of diagnosis. Rural or urban status was determined based on the patient's residential postal code at 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 as well as those without a pathological tissue diagnosis.

3.2.2 Tissue Array Generation

All archived formalin fixed paraffin embedded (FFPE) tumor samples from stage IV NSCLC patients included in the clinical database were retrieved from Calgary Lab Services (CLS). 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, USA). Normal lung tissue specimens were also included in each TMA as controls.

3.2.3 Immunohistochemical Staining and Image Analysis

After tissue microarray construction, 5μm 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, USA). 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, USA) to eliminate non-specific 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)(Fischer et al. 2008). The following day, slides were washed with 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 TM + 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 anti-fade 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 (Camp et al. 2002). 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)) in order 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 (Camp et al. 2002). 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) >10%

of the tissue area is pan-cytokeratin positive, 2) >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.

3.2.4 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 (Contal et al. 1999). 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's *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 (Wellek 2003), tested the observed differences in the survival experiences of low and high expressors, as well as evaluated clinicopathological features in Cox proportional hazards (PH) regression models. Proportional hazards assumptions were assessed using scaled Schoenfeld residual plots and trend test statistics (Therneau T 2000). 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 (Harrell 2001; Somers 1962). All analyses were conducted

with SAS/STAT® software (Version 9.2) SAS System for Unix (SAS/STAT Software, version 9.2 2008) and R software (version 2.11) (R 2009).

3.3 Results

3.3.1 Stage IV Patient Characteristics

3.3.1.1 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 columns 2 and 3 of Table 3.1. 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 cells, 32.3% NOS and 5.1% were other histology (large cell carcinoma, bronchioloalveolar carcinoma, adenosquamous 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 alone while 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).

3.3.1.2 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 either 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, BAL 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 columns 4-5 of Table 3.1. 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 3.1 (columns 4-7), demonstrates that most predictor variables were equivalent using a strict tolerance value (10%), when the TMA sample was compared to the non-TMA reference group with two exceptions: the TMA group included higher proportions of patients who received

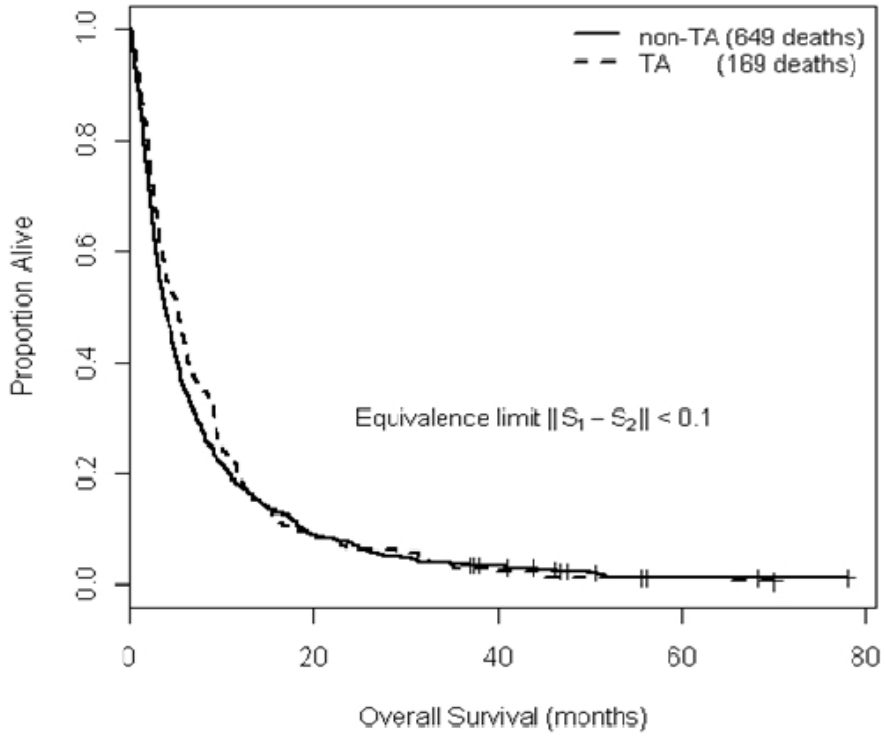
radiotherapy, more patients diagnosed with adeno- or squamous cell carcinomas, and a corresponding lower proportion of patients whose diagnoses were NOS. The non-TMA cohort (n=662) had a median overall survival (MOS) of 3.75 months (95% Confidence Interval (CI) = (3.29, 4.37)) versus 5.22 months (95% 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 the curves never separated more than 10% over the study duration, which was within our strict equivalence interval (Figure 3.1). 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.

TABLE 3.1. Demographic details of stage IV NSCLC patients

	Full Cohort (N = 832)		TA Cohort (N = 170)		non-TA Cohort (N = 662)	
	No.	%	No.	%	No.	%
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
# 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
Metastases at Diagnosis						
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	54.2
Never	86	10.3	23	13.5	63	9.5
Unknown	32	3.9	7	4.1	25	3.8
Ethnicity						
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	

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

FIGURE 3.1. 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.



3.3.2 CXCR4 expression by quantitative IHC in stage IV NSCLC patient specimens

Quantitative fluorescent immunohistochemistry (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 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. Germinal centre 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 3.2A).

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 3.2B). In all cases, there appeared to be strong CXCR4 expression in the associated endothelial cells of the capillaries (Figure 3.2B, 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 (Fischer et al. 2008). The mean AQUA score for the 170 patients was 2512.44

(standard deviation of 1371.74). When plotted on a frequency histogram, the distribution of CXCR4 AQUA scores was right skewed and ranged from a minimum of 536.00 to a maximum of 8317.73 (median 2227.31).

In order to divide the patients into high and low CXCR4 expressing groups an AQUA score cutpoint 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.(Therneau T 2000) Twenty nine (17.1%) patients had an AQUA score above 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 3.2.

FIGURE 3.2. CXCR4 IHC Staining: Interrogation of stage IV non small cell lung cancer (NSCLC) samples on tissue micro array for CXCR4 expression by immunohistochemistry (IHC) using the HistoRx/AQUA platform. A) CXCR4 staining in positive control Hela cells, and tonsil tissue with and without the primary antibody. B) Interrogation of normal and non small cell lung cancer (NSCLC) samples.The two stage IV samples included represent a low and very high CXCR4 expressing tumor. CXCR4 is expressed in normal lung tissue only in the endothelial cells and is frequently expressed in NSCLC tumor cells with a cyto-membranous distribution. The first column of images, DAPI, shows nuclear staining. The second column, PCK, are the pan cytokeratin positive tumor cells. The third column demonstrates positive CXCR4 staining. The pink fluorescent staining in the fourth column indicates positive CXCR4 expression.

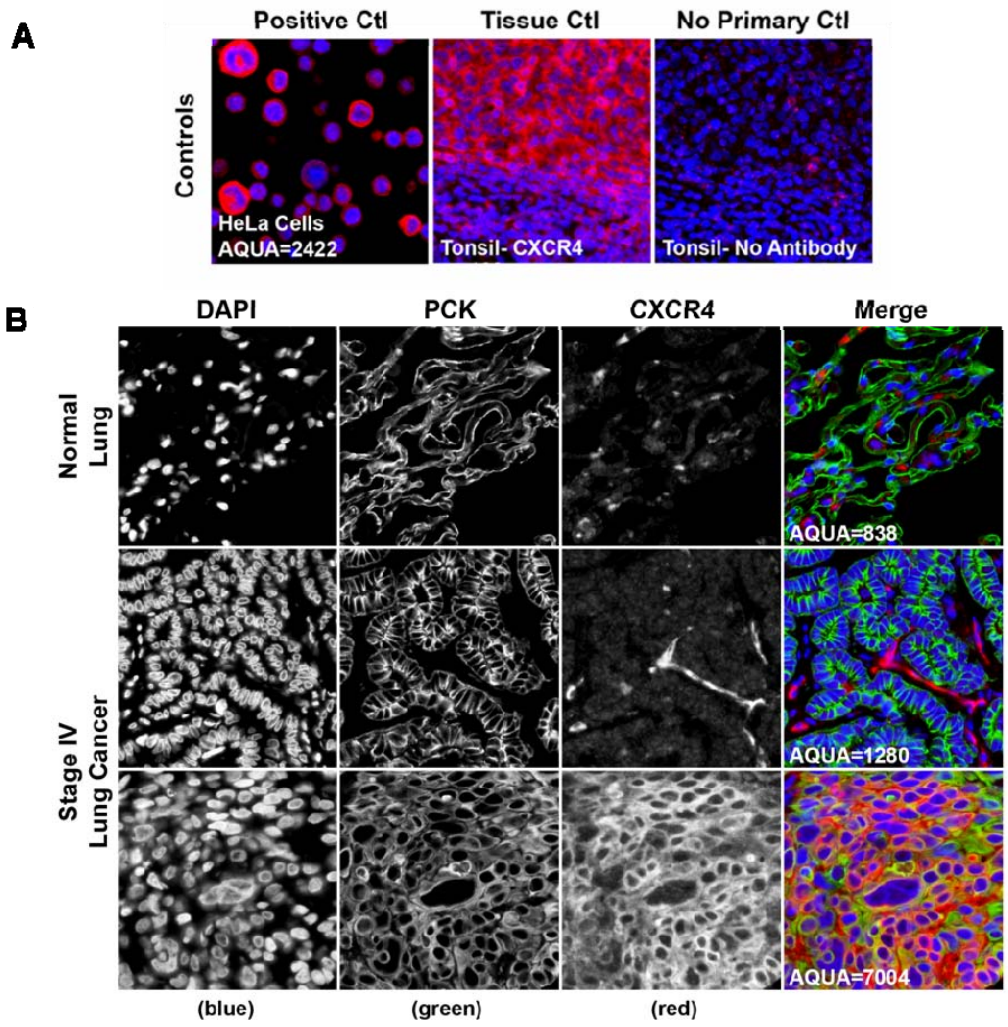

A
Positive Ctl
Tissue Ctl
No Primary Ctl
Controls
HeLa Cells
AQUA=2422
Tonsil- CXCR4
Tonsil- No Antibody
B
DAPI
PCK
CXCR4
Merge
Normal Lung
AQUA=838
Stage IV Lung Cancer
AQUA=1280
AQUA=7004
(blue)
(green)
(red)

TABLE 3.2. Demographic details of stage IV patients with high and low CXCR4 expression, stratified by gender

	Females (n=84)					Males (n=86)				
	Low (n=74)		High (n=10)			Low (n=67)		High (n=19)		
	No.	%	No.	%	p *	No.	%	No.	%	p *
Histology					0.37					**0.0074**
Adenocarcinoma	45	60.8	4	40.0		38	56.7	4	21.1	
Squamous Cell	16	21.6	5	50.0		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	10.0		9	13.4	2	10.5	
Radiotherapy					0.11					0.64
No	10	13.5	3	30.0		15	22.4	3	15.8	
Yes	64	86.5	7	70.0		52	77.6	16	84.2	
Systemic therapy					0.35					0.77
No	54	72.9	9	90.0		50	74.6	15	78.9	
Yes	20	27	1	10.0		17	25.4	4	21	
Metastases					0.86					0.67
Distant	52	70.3	7	70.0		49	73.1	13	68.4	
Local	22	29.7	3	30.0		18	26.9	6	31.6	
Smoker					0.23					0.60
Current	19	2.7	4	40.0		21	31.3	9	47.4	
Ex	36	48.7	5	50.0		37	55.2	9	47.4	
Never	16	21.6	0	0		6	8.9	1	5.3	
Unknown	3	4.1	1	10.0		3	4.5	0	0	
Ethnicity					0.44					0.22
North American	51	68.9	10	100.0		45	67.2	18	94.7	
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					0.18					0.73
Mean (SD)	66.6 (11.9)		71.6 (10.3)			65.7 (9.9)		64.9 (8.4)		
Median	68.5		75.5			65		67		
Range	32 - 88		48 - 83			43 - 85		46 - 76		

p * based on Fisher's 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; SD, standard deviation

3.3.3 Association of CXCR4 Expression and Overall Survival in Stage IV NSCLC

Overall survival was the main outcome of interest, with only one patient censored at the study end date of 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, 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.

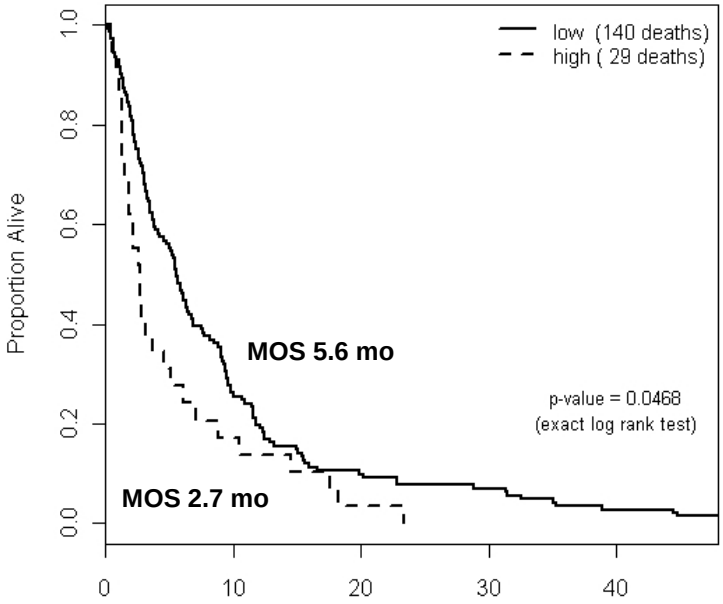
Two factors associated with overall survival in the final Cox PH regression model included: 1) EGFR TKI treatment and 2) CXCR4 AQUA score status – gender interaction. Receiving EGFR TKI treatment reduced the risk of dying by 0.48 (95% CI = (0.22, 1.01)), which was marginally above the statistical significance 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 over a five-fold 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 (see Figure 3.3a). The high expression group (AQUA score ≥ 3371.00) had a significantly poorer survival experience compared to the low expression group (p = 0.047 from exact log rank test statistic), with a median overall survival (MOS) of 2.7 months (95% CI = (1.9,5.2)) compared to 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 3.3b and 3.3c, respectively. In females, the patients with high CXCR4 expression have a significantly decreased overall survival when compared to 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 (95% CI = (2.6, 6.0)) months for the low expressors and 3.7 months (95% CI = (1.9,8.8)) for the high expressors.

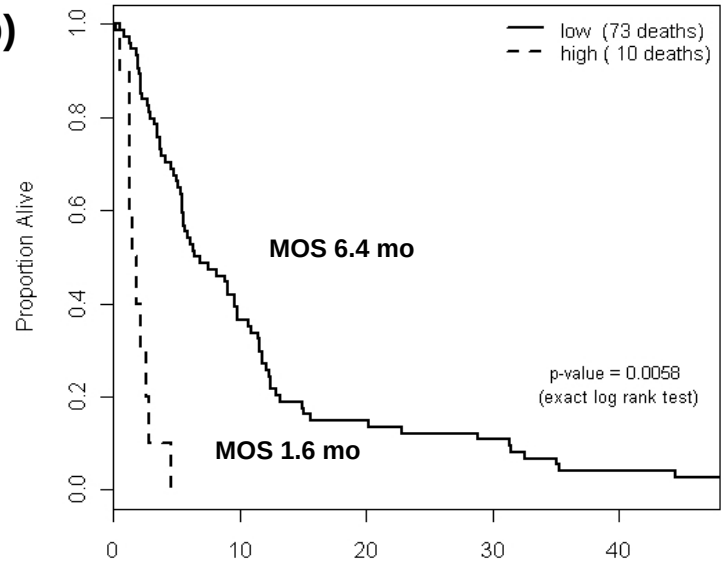
Since systemic therapy and radiotherapy failed to meet the proportional hazards assumption, they were incorporated as stratifying factors into the model. Diagnostic plots assessed the functional form of age and the presence of influential or outlying observations. Internal model validation was carried 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) (Harrell 2001).

FIGURE 3.3. 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 hazard ratios for high and low expressing groups stratified on gender. High expressing females have a 5.4 times increased risk of death compared to the low expressors, and a median overall survival (MOS) of only 1.6 months.

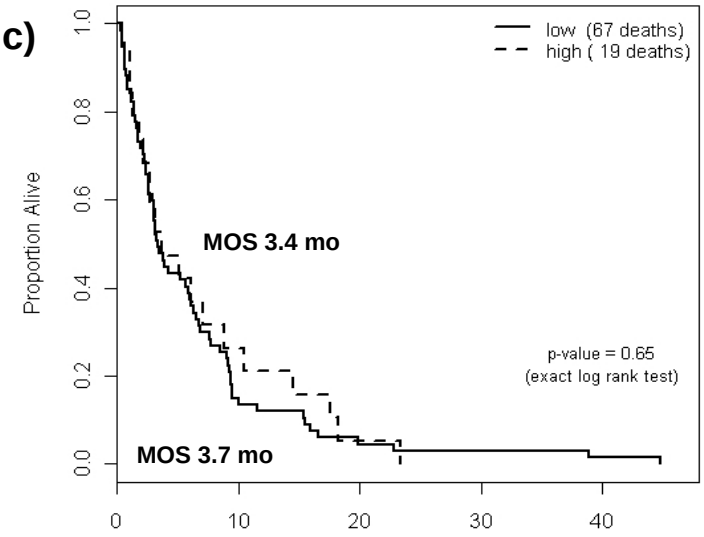
a)


low (140 deaths)
high (29 deaths)
Proportion Alive
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
MOS 5.6 mo
MOS 2.7 mo
p-value = 0.0468
(exact log rank test)

b)


low (73 deaths)
high (10 deaths)
Proportion Alive
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
MOS 6.4 mo
MOS 1.6 mo
p-value = 0.0058
(exact log rank test)

c)



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)

3.3.4 Association of CXCR4 Expression and Histology in Stage IV NSCLC

The relationship between CXCR4 AQUA score status and histology type was investigated to determine if 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 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 amongst 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 above analyses show that histology differences are not solely driving the observed gender – CXCR4 group interaction.

3.4 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 is little data on CXCR4 in advanced disease. The previous 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 cyto-membranous 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 HIV (Donzella et al. 1998), thus allowing rapid transition into clinical trials.

Additionally, the results also suggest that CXCR4 expression appears to be a prognostic biomarker in stage IV NSCLC. It is reported here, that in the cohort of patients studied, 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, since only the females are negatively affected by high CXCR4 receptor expression with a 5 times greater risk of death compared to 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. This 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 (Cerfolio et al. 2006; Ferguson et al. 2000; Minami et al. 2000), as well as in more advanced disease (Hsu et al. 2009; Radzikowska et al. 2002; Visbal et al. 2004; Wakelee et al. 2006). More recently, clinical experience (Kris et al. 2003; Lynch et al.

2004; Shepherd et al. 2005), and now molecular analyses (Paez et al. 2004; Rosell et al. 2009), have 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 this 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 influences both ER and CXCR4 dependent gene expression and ultimately tumor cell growth in vitro (Sauve et al. 2009). Some studies have demonstrated that a significant proportion of NSCLC tumors express ERs (Beattie et al. 1985; Cagle et al. 1990), and that there may be a gender dependent difference in ER expression (Fasco et al. 2002; Kaiser et al. 1996). Despite this, there does not seem to be a consensus on whether ER expression has any bearing on clinical outcome in NSCLC (Kawai et al. 2005; Schwartz et al. 2005). It can be postulated that if ERs were also present in tumors which 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 that 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 (Lee et al.; Ugocsai et al. 2005). 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 (UMB2), 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, as well as cytoplasm/membrane of NSCLC tumor cells (Minamiya et al. 2009; Na et al. 2008; Spano et al. 2004; Wagner et al. 2009). 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 (Spano et al. 2004), has had no effect on outcome (Wagner et al. 2009), and has also been associated with lymph node metastasis (Na et al. 2008). On the other hand, total mRNA expression has been associated with a better clinical outcome (Minamiya et al. 2009), while cytomembraneous CXCR4 expression has been shown to confer a worse prognosis (Wagner et al. 2009).

Much of this inconsistency may be due to the use of under-characterized mouse monoclonal antibodies in these studies, which have not been thoroughly tested for specificity in FFPE tissues (Fischer et al. 2008), as well as the potential subjectivity in the

analysis of semi-quantitative 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 cyto-membranous. The UMB2 rabbit monoclonal antibody used was extensively characterized and shown to accurately detect membrane receptors while showing little staining in the cell nucleus (Fischer et al. 2008), which is more compatible with the known function and signaling of this receptor (Ganju et al. 1998).

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 (Kang et al. 2005; Sutton et al. 2007), while 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 (Pituch-Noworolska et al. 2003). The role played by the CXCR4/SDF-1 axis in leukocyte trafficking and homing of stem cells (Lapidot et al. 2005; Wang et al. 2006), is likely analogous to organ selective metastasis of cancer stem cells (Geminder et al. 2001). In this model, a cell's metastatic potential will be determined by surface CXCR4 expression while 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 since women whose tumors over express this receptor, appear 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 estrogen receptor function in NSCLC.

Chapter Four: Influence of the CXCR4 Chemokine Receptor on Clinical Outcome in Resected Early Stage NSCLC Patients

4.1 Introduction

The experiments described in chapter 3 were designed to assess the association between CXCR4, as assessed by novel quantitative immunohistochemistry technology, and clinical outcome in stage IV non small cell lung cancer, a study which hasn't been published to date. On the other hand, multiple studies have attempted to examine the role of CXCR4 and/or SDF-1 on clinical outcome in early stage NSCLC, resulting in many conflicting findings. In a study by Su *et al*, it was demonstrated that resected patients with high CXCR4 expressing tumors, as detected by rtPCR and semi-quantitative IHC, were more prone to metastasis compared to patients with low expressing tumors, however no associations with survival were made (Su et al. 2005). CXCR4 staining was limited to the cytoplasm and/or the cell membrane, and was not associated with any other clinicopathological factors. Similarly, Wagner and colleagues found that high cyto-membranous CXCR4 expression (also as assessed by semi-quantitative IHC) was an independent prognostic marker of worse outcome, and a shorter disease free survival in patients with adenocarcinoma (Wagner et al. 2009).

On the other hand, Wagner *et al* also found and that high nuclear expression of CXCR4 conferred a survival benefit in patients with adenocarcinoma tumor histology. Similarly, Spano and colleagues found that in stage I resected NSCLC patients, there was CXCR4 staining (also by semi-quantitative IHC) seen in both the cytoplasm, but also in the nuclear compartment in a significant number of tumor specimens (Spano et al. 2004). They found that patients with high nuclear expressing tumors had a better 5 year overall survival (OS) than those patients with tumors with negative nuclear CXCR4 expression,

but they also failed to find an association between cytoplasmic CXCR4 expression and outcome like the previously described studies did.

In yet another study, Minamiya *et al* reported that high CXCR4 mRNA expression, as assessed by semi-quantitative real time reverse transcription PCR, was associated with a better clinical outcome and longer 5 year disease free survival (DFS) in early stage resected patients with adenocarcinoma tumor histology (Minamiya et al.). Lastly, Na and colleagues demonstrated that high nuclear staining of CXCR4 was actually associated with lymph node metastasis (Na et al. 2008).

Clearly, the evidence with regard to the role of CXCR4 in early stage NSCLC is confusing at best, and entirely conflicting at worst. Some studies observed CXCR4 expression in the cytoplasm/cell membrane alone, some in the nucleus alone, and still other observed CXCR4 protein in both the nuclear and non nuclear compartments. Nuclear localization of CXCR4 expression has been linked to both good as well as poor prognosis, and some studies have reported associations between CXCR4 expression and clinical outcome only in patients with adenocarcinoma tumor histology. Furthermore, many of these studies were carried out using a small number of patient tumor specimens (ie < 50 samples) which may have limited the power of these studies to reliably assess the influence on clinical outcome. Obviously, more work must be carried out in order to fully understand the association, if any, between CXCR4 expression and clinical outcome in early stage NSCLC.

This chapter describes investigations to determine the association between CXCR4 expression in ex vivo patient tumor specimens, as assessed by novel quantitative

immunohistochemical analysis, and both overall survival and disease free survival in patients with stage I, II or III resected non small cell lung cancer.

4.2 Materials and Methods

4.2.1 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 was 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 was 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 TNM system and reflected the recent 2009 revisions for NSCLC staging. Ethnicity was determined by the birthplace of the patient due to the absence of available ethnicity data. North American ethnicity included patients born in both Canada and the United States. Southeast Asian ethnicity included patients born in China, Japan, Malaysia and Vietnam. Ethnicity 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: non-smoking status was defined as having

smoked less than 100 cigarettes total, while a current smoking status was assigned if the patient smoked at time of diagnosis.

Resection type was divided into wedge resection, lobectomy and > lobectomy, which included those patients who received a bi-lobectomy, hemi-pneumonectomy, or a pneumonectomy during surgery. Adjuvant treatment included patients who received adjuvant chemotherapy alone, adjuvant chemoradiotherapy, and those that received neoadjuvant radiotherapy. Patients who were treated radically at the time of recurrence, included those who received a resection of their recurrent lung nodule, those who received radical radiotherapy alone and those who received radical chemoradiotherapy to treat their recurrence.

4.2.2 Tissue Array Generation

All archived formalin fixed paraffin embedded (FFPE) tumor samples from the early stage resected NSCLC patients included in the clinical database were retrieved from Calgary Lab Services (CLS). Hematoxylin and eosin stained slides were reviewed by a pathologist to confirm diagnosis and were marked for sampling and inclusion into the tissue micro array (TMA). Representative cores (0.6 mm) from each specimen were assembled in triplicate into each TMA (25-45 specimens per TMA) using a Beecher Manual Tissue Microarrayer (Beecher Instruments Inc. Sun Prairie, WI, USA). Normal lung tissue specimens were also included in each TMA as controls.

4.2.3 Immunohistochemical Staining

Detailed description of the immunohistochemical staining and image analysis can be found in the Materials and Methods section of Chapter 2. Briefly, sections were cut from the tissue array block, deparaffinized and rehydrated. Heat induced epitope retrieval was performed in a decloaking chamber (Biocare Medical, Concord, CA, USA). Slides were stained with Signal Stain protein block (Cell Signaling, Danvers, MA, USA) with a 1:500 dilution of anti-pan-cytokeratin mouse mAb (Dako) to identify tumor cells, combined with a 1:25 dilution of anti-CXCR4 rabbit mAb (Biotrend, Köln, Germany). The next day, corresponding secondary antibodies were applied. Slides were then washed with buffer (Dako) and incubated with the TSA-Plus Cy5 tyramide signal amplification reagent (PerkinElmer, Woodbridge, ON, Canada). The TMA slides were then mounted 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 (Camp et al. 2002), and in the Material and Methods section of Chapter 2. Briefly, high resolution images 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. Images were taken for each channel for future use with the AQUAsition® program, version 2.2.1.7 as previously described (Camp et al. 2002). Briefly, a tumor specific mask was generated to distinguish the NSCLC cells from normal tissue by thresholding the pan-cytokeratin images. 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 predetermined criteria.

4.2.4 Statistical Analysis

Descriptive statistics compared the frequencies of measured patient and pathological features between the full, stage I, stage II and stage III cohorts, as well as the high and low CXCR4 expressing groups. The relationships between CXCR4 scores and clinicopathological variables of interest were evaluated using Fisher's exact test for categorical data, and two-sample student's *t*-test for the age variable. Overall survival and disease free survival comparisons between groups were made using a log rank test of the Kaplan-Meier survival curves.

4.3 Results

4.3.1 Early Stage Patient Characteristics

Between January 2003 and December 2006 there were 1507 diagnoses of NSCLC at the Tom Baker Cancer Centre (TBCC), of which 166 patients had stage I, II or IIIA disease which was fully resected. The majority of patients were classified as stage I (n = 104), with 46 stage II patients and 16 stage III patients. Patient demographics and clinical characteristics for the whole cohort, are shown in columns 2 and 3 of table 4.1. In short, median age was 64 years, 53.0% were male, 86.8% were current or ex smokers, and 6.6% were of Southeast Asian ethnicity. In terms of tumor histology, 53.6% were

adenocarcinomas, 28.9% were squamous cell carcinomas, and 17.4% had other tumor histology (which included bronchioloalveolar carcinoma, large cell carcinoma, adenosquamous carcinoma and sarcomatid carcinoma).

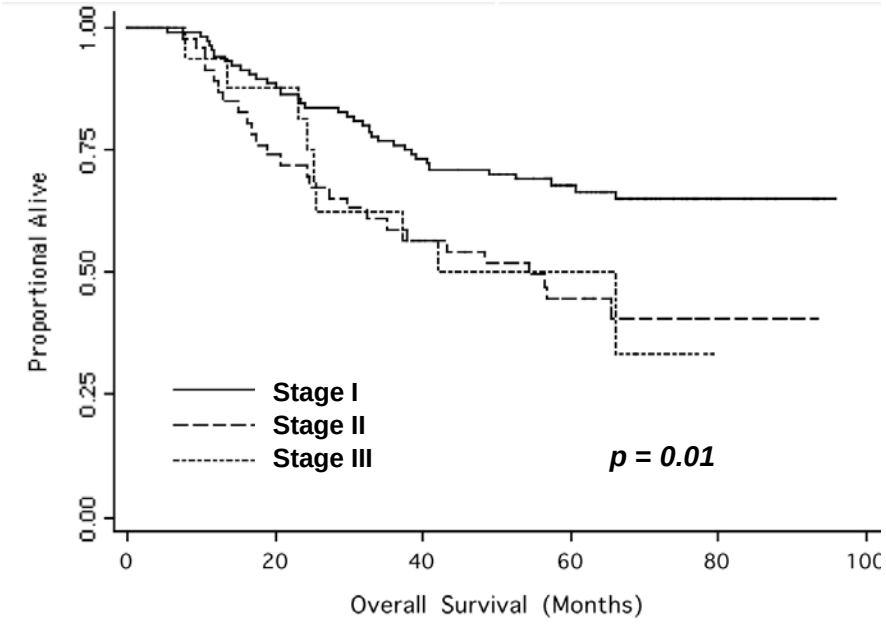
The majority of the patients (62.0%) underwent a lobectomy as their resection type, and 37.3% received adjuvant treatment following their surgery. Of the 166 patients, 66 (39.8%) patients recurred following surgery, of which 13 (7.8%) were treated radically (resection, radical chemoradiotherapy, or radical radiotherapy) for their recurrence. 20 (12.0%) patients were treated sometime throughout the course of their disease with palliative chemotherapy, and 43 (25.9%) with palliative radiotherapy. At the end of study date (January 1, 2011), 42.2% of the patients were still alive.

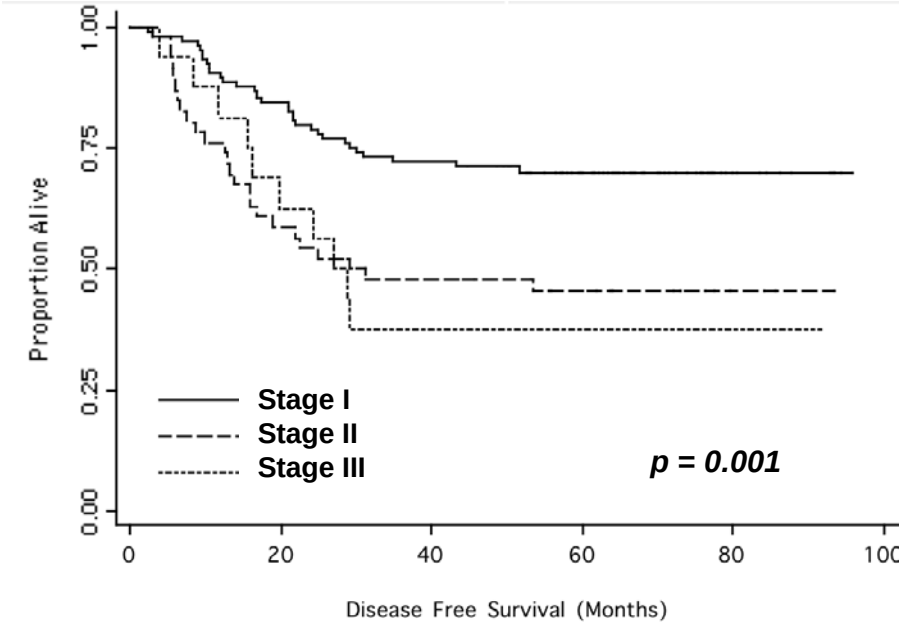
Demographic details and clinical characteristics of stage I, stage II and stage III patients are shown in columns 4-9 of Table 4.1. When comparing the survival of stage I, II and III patients, it can be seen that the stage I patients have a significantly improved overall (OS) (p = 0.01), and disease free survival (DFS) survival (p = 0.001) when compared with the stage II and III patients (Figure 4.1). The median overall survival (MOS) and median DFS for the stage I patients was not reached. The MOS and median DFS of the stage II patients was 54.5 and 31.2 months, respectively, and 42.1 and 26.9 months for the stage III patients. These findings were comparable to historical controls.

TABLE 4.1. Demographic details of early stage resected NSCLC patients

	Full Cohort (N = 166)		Stage I (N = 104)		Stage II (N = 46)		Stage III (N = 16)	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Gender								
Female	88	53.0	61	58.7	18	39.1	9	56.3
Male	78	47.0	43	41.3	28	60.9	7	43.7
Histology								
Adenocarcinoma	89	53.6	61	58.7	21	45.7	7	43.7
Squamous Cell	48	28.9	25	24.0	18	39.1	5	31.3
Large Cell	6	3.6	3	2.9	2	4.3	1	6.3
BAC	18	10.8	13	12.5	4	8.7	1	6.3
Adenosquamous	4	2.4	2	1.9	0	0.0	2	12.5
Sarcomatid	1	0.6	0	0.0	1	2.2	0	0.0
Smoking Status								
Current	40	24.1	23	22.1	12	26.1	5	31.3
Ex	104	62.7	66	63.5	30	65.2	8	50.0
Never	20	12.0	13	12.5	4	8.7	3	18.8
Unknown	2	1.2	2	1.9	0	0.0	0	0.0
Ethnicity								
North American	132	79.5	82	78.8	36	78.3	14	87.5
Southeast Asian	11	6.6	7	6.7	3	6.5	1	6.3
Other	23	13.9	15	14.4	7	15.2	1	6.3
Resection Type								
Wedge	35	21.1	26	25.0	6	13.0	3	18.8
Lobectomy	103	62.0	76	73.1	27	58.7	0	0.0
> Lobectomy	28	16.9	2	1.9	13	28.3	13	81.3
Adjuvant Treatment								
Yes	62	37.3	33	31.7	20	43.5	9	56.3
No	104	62.7	71	68.3	26	56.5	7	43.7
Recurred								
Yes	66	39.8	31	29.9	25	54.3	10	62.5
No	100	60.2	73	70.2	21	45.7	6	37.5
Radically Tx at Recurrence								
Yes	13	7.7	5	4.8	6	13.0	2	12.5
No	53	31.9	26	25.0	19	41.3	8	50.0
Palliative Chemo								
Yes	20	12.0	8	7.7	9	19.6	3	18.8
No	146	88.0	96	92.3	37	80.4	13	81.3
Palliative RT								
Yes	43	25.9	19	18.3	19	41.3	5	31.3
No	123	74.1	85	81.7	27	58.7	11	68.8
Patient Deceased								
Yes	96	57.8	35	33.7	26	56.5	9	56.3
No	70	42.2	69	66.3	20	43.5	7	43.7
Age								
Mean (SD)	64.1 (10.0)		65.4 (10.7)		60.7 (11.2)		65.6 (6.7)	
Median	64		65		61.5		64	
Range	41-84		43 - 84		41-77		55-79	

FIGURE 4.1. Kaplan-Meier survival curves comparing the overall survival (OS) and disease free survival (DFS) of stage I, II and III NSCLC patients with resected disease. Patients with stage I disease have a significantly better survival compared to those with stage II or III disease ($p = 0.01$). The median OS (MOS) and median DFS for the stage I patients was not reached. The MOS and median DFS of the stage II patients was 54.5 and 31.2 months, respectively, and 42.1 and 26.9 months for the stage III patients.


Proportional Alive
0.00
0.25
0.50
0.75
1.00
0
20
40
60
80
100
Overall Survival (Months)
Stage I
Stage II
Stage III
p = 0.01


Proportion Alive
0.00
0.25
0.50
0.75
1.00
0
20
40
60
80
100
Disease Free Survival (Months)
Stage I
Stage II
Stage III
p = 0.001

4.3.2 CXCR4 expression by quantitative IHC in early stage NSCLC patient specimens

Quantitative immunohistochemistry (IHC) was successfully completed for all patients included in the TMAs. AQUA analysis 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 only the epithelial cells of the cancer. 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 4.2). In all cases, there appeared to be strong CXCR4 expression in the associated endothelial cells of the capillaries. The mean AQUA score for the 166 patients was 1715.90 (standard deviation of 1098.83). When plotted on a frequency histogram, the distribution of CXCR4 AQUA scores was right skewed and ranged from a minimum of 565.83 to a maximum of 9012.05 (median 1422.84) (Figure 4.3.)

When compared to the AQUA scores obtained from the stage IV NSCLC previously tested, the range of scores was roughly the same (536.00 – 8313.73 for the stage IV patients), however the mean and median AQUA scores were significantly lower. The mean CXCR4 AQUA score for the stage IV patients was 2512.44 versus 1715.90 for the early stage patients ($p < 0.0001$), suggesting that CXCR4 expression is higher overall in stage IV patients than in early stage patients. Due to the significant difference in expression scores for the early stage patients, it was inappropriate to use the same AQUA

score cutoff as was used for the stage IV patients. For the early stage patients, it was found that an AQUA score cut-point based on the bottom quartile of patients effectively separated the patients into high and low CXCR4 expressing groups. Patients with AQUA scores above 1089.00 were considered to be high CXCR4 expressors and those with AQUA scores less than 1089.00 were considered low expressors. When divided this way, 42 (25.3%) patients fell into the low expressing group and 124 (74.7%) patients into the high expressing group. Patient demographics of the two CXCR4 expression groups are summarized in Table 4.2.

FIGURE 4.2. CXCR4 IHC Staining: Interrogation of resected early stage non small cell lung cancer (NSCLC) samples on tissue array for CXCR4 expression by immunohistochemistry (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 cyto-membranous distribution. The top row of images is from a low CXCR4 expressing tumor, the middle and bottom from a high and very high CXCR4 expressing tumor, respectively.

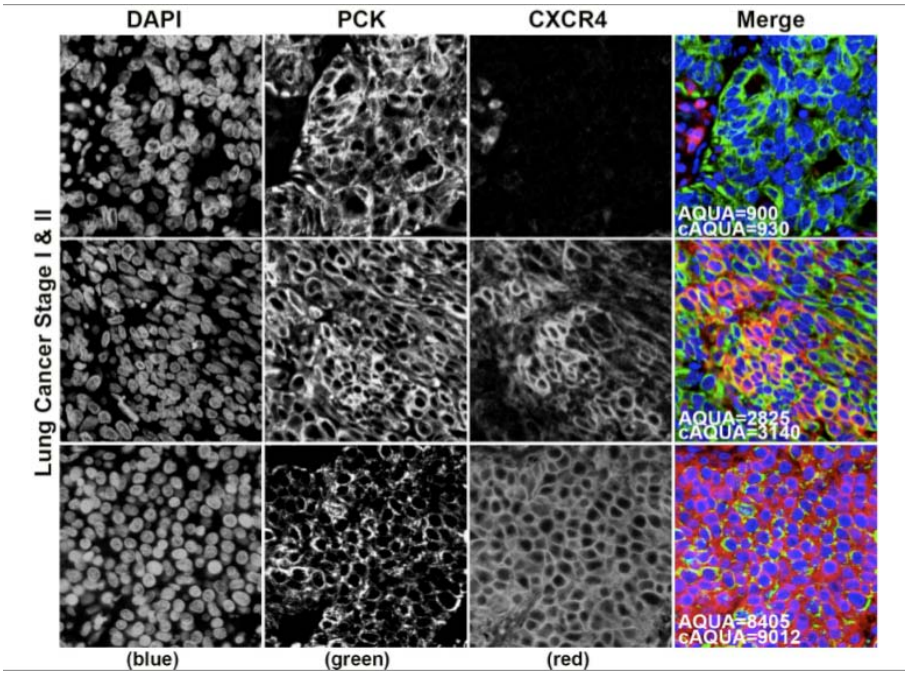
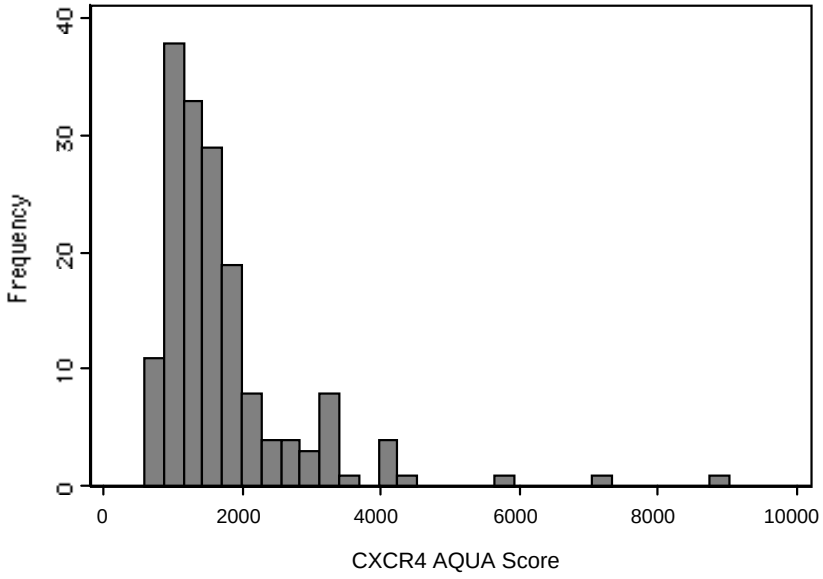


FIGURE 4.3. Frequency distribution of CXCR4 AQUA scores for the 166 early stage resected patients. The distribution is highly right skewed with a median of 1422.84, mean of 1715.90 (standard deviation = 1098.83) and ranges from 565.83 – 9012.05.



4.3.3 Association of CXCR4 expression and clinicopathological features

After dividing the patients into high and low CXCR4 expressing groups, the various clinicopathological features of the patients could be compared between the two groups. In univariate analysis, there was no significant difference between the high and low expressing groups in most clinical variables assessed, including gender, patient smoking status, ethnicity and recurrence rate. There were a couple of exceptions however. There were significantly fewer patients with squamous cell carcinoma in the low CXCR4 expressing groups compared to the high expressing group, where as there were significantly higher proportion of patients with adenocarcinoma and BAC in the low expressing group (p = 0.003). This trend towards a higher than normal proportion of squamous cell carcinoma in the high CXCR4 expressing groups was also observed in the stage IV patients, however tumor histology was not found to be significantly associated with survival in the Cox PH hazards model in Chapter 3.

It was also observed that there was a significantly higher proportion of stage II and III patients in the high CXCR4 expressing group and a higher proportion of stage I patients in the low expressing group (p = 0.03). This finding makes sense however, as it was found that the stage IV patients had significantly higher CXCR4 AQUA scores on average than the early stage patients, suggesting that perhaps overall mean CXCR4 AQUA scores increase with stage. The last clinicopathological variable which differed between the high and low CXCR4 expressing groups was adjuvant treatment. There was a significantly higher proportion of patients who received adjuvant treatment in the high CXCR4 expressing group compared with the low expressing group (p = 0.05). This finding can be easily explained, however, based on the previous finding that there were

more stage II and III patients in the high expressing group. Adjuvant treatment is traditionally not indicated for patients with stage I disease but is more so for stage II and III resected patients, so the higher proportion of stage II and II patients in the high expressing group would bring with it a higher proportion of patients treated adjuvantly.

TABLE 4.2. Demographic details of early stage resected patients with high and low CXCR4 expression

	High CXCR4 Expressors (N = 124)		Low CXCR4 Expressors (N = 42)		*p**
	No.	**%**	**No.**	**%**	
Gender					
Female	65	52.4	23	54.8	0.86
Male	59	47.6	19	45.2	
Histology					
Adenocarcinoma	63	50.8	26	61.9	**0.003**
Squamous Cell	43	34.7	5	11.9	
Large Cell	5	4.0	1	2.4	
BAC	9	7.3	9	21.4	
Adenosquamous	4	3.2	0	0.0	
Sarcomatid	0	0.0	1	2.4	
Stage					
I	71	57.3	33	78.6	**0.03**
II	38	30.6	8	19.0	
III	15	12.1	1	2.4	
Smoking Status					
Current	33	26.6	7	16.7	0.19
Ex	78	62.9	26	61.9	
Never	12	9.7	8	19	
Unknown	1	0.01	1	2.4	
Ethnicity					
North American	100	80.6	32	76.2	0.75
Southeast Asian	8	6.5	3	7.1	
Other	16	12.9	7	16.7	
Adjuvant Treatment					
Yes	56	45.2	11	26.2	**0.05**
No	68	54.8	31	73.8	
Recurred					
Yes	53	42.7	13	31.0	0.20
No	71	57.3	29	69.0	
Radically Tx at Recurrence					
Yes	8	6.5	5	11.9	0.11
No	45	36.3	8	19.0	
Palliative Chemo					
Yes	17	13.7	3	7.1	0.41
No	107	86.3	39	92.9	
Palliative RT					
Yes	35	28.2	8	19.0	0.31
No	89	71.8	34	81.0	
Patient Deceased					
Yes	58	46.8	12	28.6	
No	66	53.2	30	71.4	
Age					
Mean (SD)	64.2		64.0		0.90
Median	64		65		
Range	43 – 84		43 - 82		

*p** based on Fisher's exact test for categorical data, and a two-sample *t*-test for the age variable
BAC, Bronchioloalveolar carcinoma

4.3.4 Association of CXCR4 expression and survival

After dividing the patients into high and low CXCR4 expressing groups based on the chosen AQUA score cut-point, associations with overall survival (OS) and disease free survival (DFS) were made. Figure 4.4 shows a Kaplan-Meier survival curve comparing the OS of high and low CXCR4 expressing groups. It can be seen that the patients with low CXCR4 expression have a significantly better survival than the patients with high CXCR4 expression ($p = 0.022$). The median overall survival (MOS) of the low expressing group was not reached, while the MOS of the high expressers was 66.0 months. When comparing the DFS of the high and low CXCR4 expressing groups, it can be seen that there is a trend towards better survival for the low expressing group, however the results did not reach statistical significance ($p = 0.14$). The median DFS for both the high and low expressing groups was not reached.

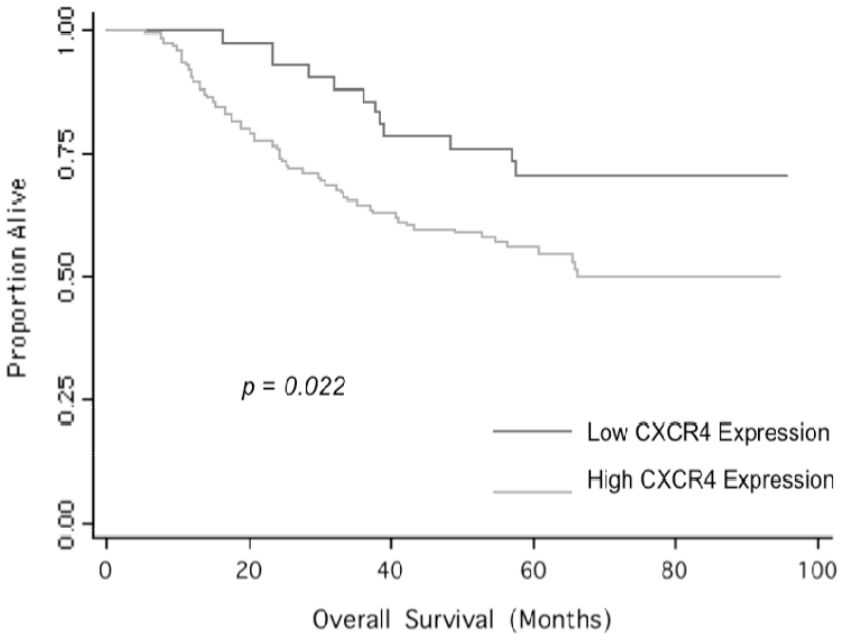
Due to the gender discrepancy observed in the association between CXCR4 expression and overall survival in the stage IV NSCLC patients analyzed in Chapter 3, Kaplan-Meier survival curves were also generated to assess the association between CXCR4 expression and both overall survival and disease free survival within genders, to see if this gender discrepancy persisted for the early stage patients. Interestingly, in contrast to the advanced NSCLC patients studied previously, it seems to be the male high expressing patients in this cohort which are driving the observed association between high CXCR4 expression and worse overall survival.

The high expressing males had a significantly worse OS compared to the low expressing males ($p = 0.02$) (high CXCR4 expressors MOS = 65.6 months; low CXCR4 expressors MOS = NR) (Figure 4.5), while there was no significant difference seen in OS

between high and low CXCR4 expressing female patients (p = 0.36) (MOS not reached for both high and low expressors) (Figure 4.6). This observed association between high CXCR4 expression and poor clinical outcome in only the male patients in this cohort was an unexpected finding, as the results were the complete opposite for the stage IV patients.

In terms of disease free survival (DFS), there was no significant difference between the high and low CXCR4 expression groups in both the males (p = 0.20) and females (p = 0.43), although a trend towards a worse outcome in the high expressing group can be seen (median disease free survivals were not reached in all groups).

FIGURE 4.4. Kaplan-Meier survival curves comparing the overall survival (OS) and disease free survival (DFS) of the high and low CXCR4 expressing patients. Patients with low expressing CXCR4 tumors (median overall survival not reached) had a significantly better OS (top graph) compared to the high expressing patients (median overall survival = 66.0 months) ($p = 0.022$). There was no significant difference in DFS (bottom graph) between the high and low expressing patients ($p = 0.14$).


Proportion Alive
0.00
0.25
0.50
0.75
1.00
p = 0.022
Low CXCR4 Expression
High CXCR4 Expression
0
20
40
60
80
100
Overall Survival (Months)

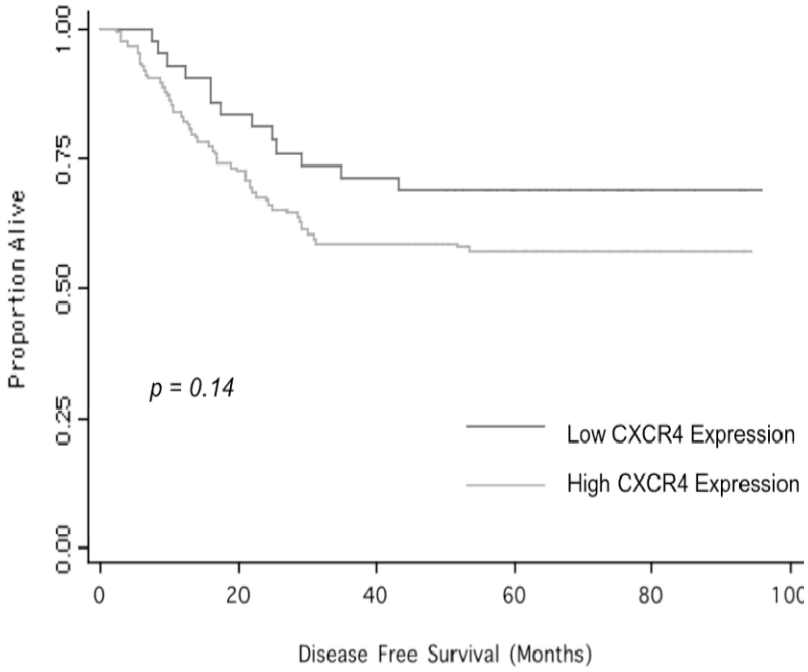
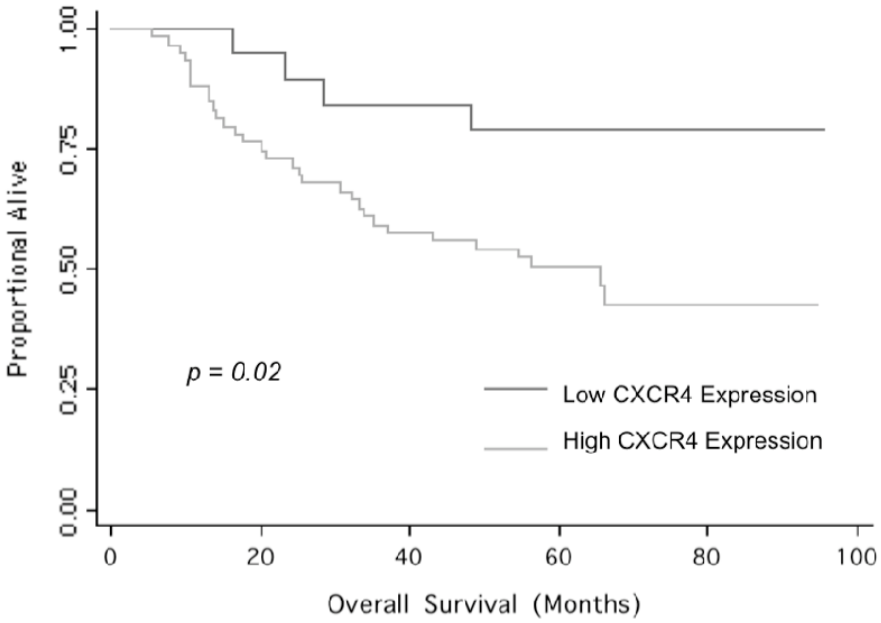

Proportion Alive
0.00
0.25
0.50
0.75
1.00
p = 0.14
Low CXCR4 Expression
High CXCR4 Expression
0
20
40
60
80
100
Disease Free Survival (Months)

FIGURE 4.5. Kaplan-Meier survival curve comparing the overall survival (OS) and disease free survival (DFS) of high and low CXCR4 expressing males. The high expressing males had a significantly worse OS compared to the low expressing males (*p* = 0.02) (high CXCR4 expressors MOS = 65.6 months; low CXCR4 expressors MOS = NR). There was no significant difference in the DFS of the high and low expressing patients.


Proportional Alive
0.00
0.25
0.50
0.75
1.00
p = 0.02
Low CXCR4 Expression
High CXCR4 Expression
0
20
40
60
80
100
Overall Survival (Months)

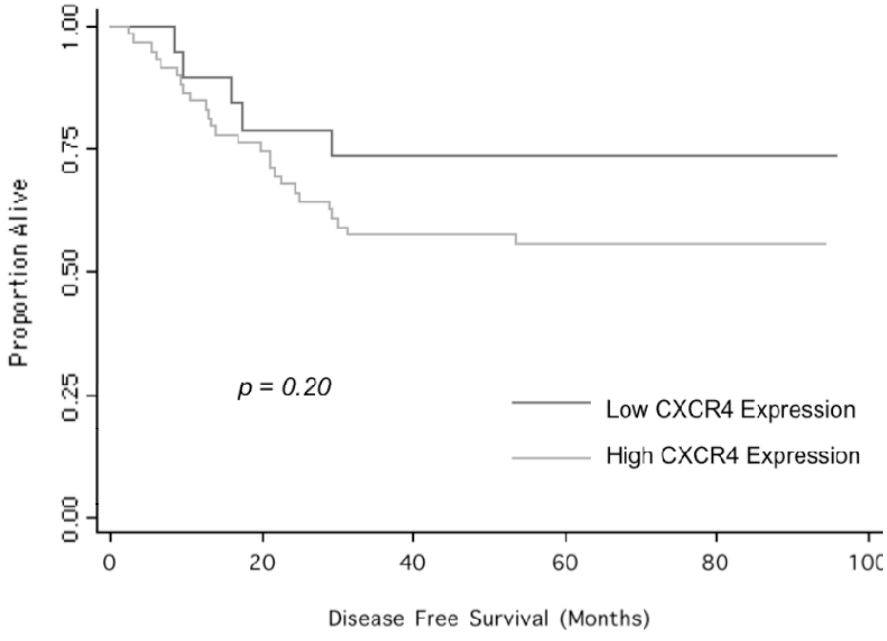
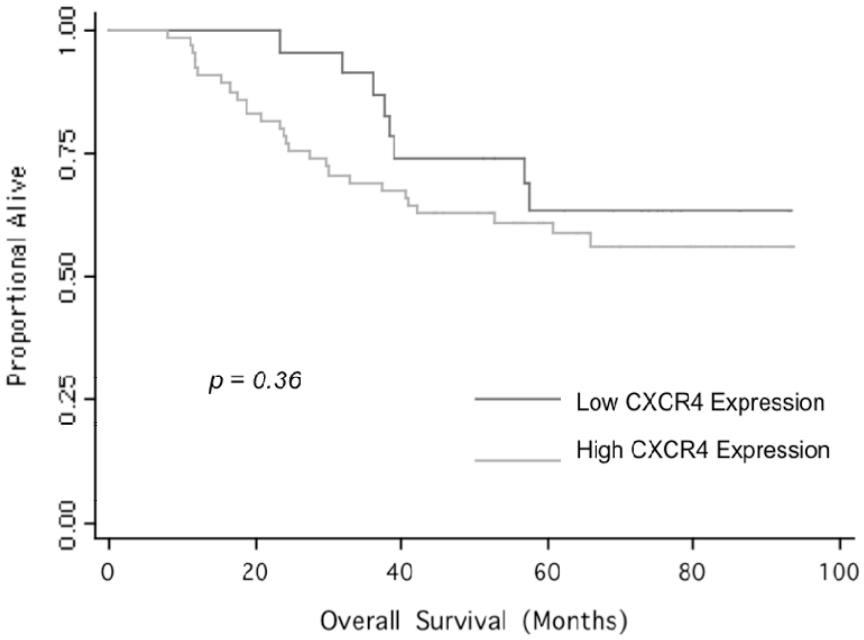
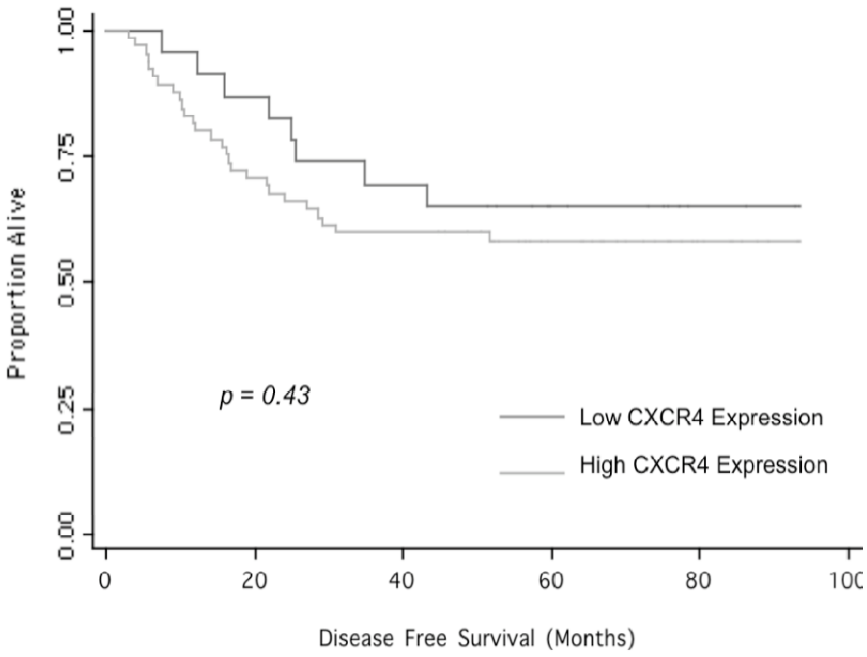

Proportion Alive
0.00
0.25
0.50
0.75
1.00
p = 0.20
Low CXCR4 Expression
High CXCR4 Expression
0
20
40
60
80
100
Disease Free Survival (Months)

FIGURE 4.6. Kaplan-Meier survival curve comparing the overall survival and disease free survival of high and low CXCR4 expressing females. There was no significant difference seen in OS between high and low CXCR4 expressing female patients ($p = 0.36$) (MOS not reached for both high and low expressors). There was also no significant difference in the DFS of the high and low expressing patients.


Proportional Alive
0.00
0.25
0.50
0.75
1.00
p = 0.36
Low CXCR4 Expression
High CXCR4 Expression
0
20
40
60
80
100
Overall Survival (Months)


Proportion Alive
0.00
0.25
0.50
0.75
1.00
p = 0.43
Low CXCR4 Expression
High CXCR4 Expression
0
20
40
60
80
100
Disease Free Survival (Months)

4.4 Discussion

In the majority of studies to date, CXCR4 expression in human *ex vivo* tumor specimens has been shown to be associated with a poorer clinical outcome (Chu et al. 2010; Hu et al. 2005; Jiang et al. 2006; Kang et al. 2005). The findings in NSCLC are less clear. Many studies in early stage NSCLC have actually reported that when high CXCR4 expression is confined to the nucleus, a better prognosis is seen (Spano et al. 2004; Wagner et al. 2009). From a mechanistic point of view, this finding could make sense because if the receptor is sequestered in the nucleus, it is prevented from functioning in its normal signalling capacity on the cell membrane. But some of these same studies also failed to find a correlation between cytoplasmic/cell membrane expression and worse clinical outcome, or did not report finding any CXCR4 expression in the cytoplasm/cell membrane at all, which is not in concordance with the majority of the literature on the subject.

On the other hand, the investigations described in this chapter have demonstrated that in the 166 early stage resected NSCLC patients tested, CXCR4 was localized to the cell membrane and/or cytoplasm of the cells, with very little if any staining seen in the cell nuclei. This was the first study in published literature which utilized the HistoRx platform for the quantitative analysis of CXCR4 immunohistochemical staining. This system utilizes “compartment masking” which enables accurate determination of CXCR4 expression within specific cells and cellular compartments, ie. only in the nucleus of the epithelial cells of the cancer. In this way, a measure of the tumor specific subcellular expression and localization of the CXCR4 receptor was obtained.

It was also demonstrated that the overall survival (OS) of the NSCLC patients differed based on their level of expression of CXCR4. High CXCR4 expressors had a significantly worse OS compared to their low expressing counterparts, and a trend towards a worse disease free survival in the high expressors was also seen, although the results were not statistically significant. This finding of a significantly worse overall survival in patients with tumors expressing high CXCR4 is similar to that seen in the stage IV patients tested in the previous chapter, however a more rigorous and multivariate analysis of the early stage patients was needed in order to ensure that the association between CXCR4 expression and survival is still significant when the other covariates are taken into consideration.

The association between CXCR4 expression and clinical outcome was also examined within genders as the influence of CXCR4 expression on poor survival was found to be a gender dependent phenomenon in the stage IV NSCLC patients studied. In the early stage patients it was found that high CXCR4 expression correlated with a worse overall survival only in the males, while CXCR4 expression was not significantly associated with either overall survival or disease free survival in the female patients. This finding is contrary to what was found in the stage IV patients where high CXCR4 expression conferred a worse survival in the female patients. Again, a more detailed multivariate analyses was required to determine if this gender discrepancy in the influence of CXCR4 on survival is statistically significant. Furthermore, if this conflicting gender difference holds up, further analyses of the whole cohort of patients (ie. early stage and stage IV patients), with a specific focus on the interaction between stage, gender and CXCR4 expression will need to be carried out.

Intrestingly, further statistical analysis utilizing a Cox PH regression model was completed for the purpose of a future publication, and it was found that there was no significant association between CXCR4 expression in the resected early stage NSCLC tissue samples, and both overall and disease free survival. This was also the case when the patients were divided by gender, so unlike what was seen for the Stage IV patients in Chapter 3, there was no gender dependent effect seen in the influence of CXCR4 on survival. It was, however, found that males on average had significantly higher CXCR4 AQUA expression scores than females and that CXCR4 AQUA expression scores increased significantly with stage, from stage I-IV.

In summary, it is reported here that CXCR4 expression, as assessed by quantitative immunohistochemisty, may be associated with overall survival in stage I, II and III resected NSCLC. CXCR4 expression may also be associated with some clinicopathological features such as disease stage and tumor histology, however further multivariate analyses are needed to fully assess the impact of high CXCR4 expression on clinical outcome in early stage resected NSCLC.

Chapter Five: Concluding Remarks and Future Directions

Lung cancer is a devastating disease which is diagnosed more than any other malignancy in the developed world, has a staggering rate of mortality (85%), and a dismal overall 5 year survival rate (15%) (Coate et al. 2009; Jemal et al. 2009; NCI 2011). The primary cause of lung cancer, cigarette smoking, remains extremely prevalent in many developing nations, and as a result, lung cancer is going to continue to be a significant health issue for the better part of the next century (Molina et al. 2008; Zhang & Cai 2003). The high mortality associated with lung cancer can mainly be attributed to metastatic spread, which is highly characteristic of lung cancer in general, and non small cell lung cancer (NSCLC) in particular.

Chemokines, and specifically the CXCR4/SDF-1 chemokine axis, have been shown to play a significant role in many aspects of the metastatic process including migration, invasion, adhesion and angiogenesis (Burger et al. 2005; Hart et al. 2005; Jiang et al. 2006; Ohira et al. 2006; Scotton et al. 2002; Su et al. 2005). CXCR4 has also been shown to be associated with a poor clinical outcome in a variety of tumor types such as breast, colon, prostate and lung cancer. This thesis aimed to further explore the role of CXCR4 in NSCLC using two different approaches. First, the role of CXCR4 in *in vitro* cellular migration was investigated by assessing the migrational capacity of NSCLC cell lines after CXCR4/SDF-1 axis inhibition utilizing a variety of methods. Second, clinical outcome in stage IV and resected early stage NSCLC patients was examined in relation to the expression of CXCR4 in *ex vivo* tumor specimens, to determine if CXCR4 was associated with worse survival. Together, the results of these investigations suggest that CXCR4 plays an important, yet complex role in the metastasis of NSCLC.

The findings from Chapter 2 confirmed that NSCLC cell line cells express the CXCR4 receptor in the cell membrane and/or cytoplasm and that NSCLC cells migrate in response to SDF-1. It was also demonstrated that inhibition of either CXCR4 or SDF-1 was capable of significantly decreasing SDF-1 induced NSCLC cell migration, though most inhibitors were unable to completely eliminate SDF-1 induced migration. The CXCR4 inhibitor CTCE 9908 appeared to be the most consistent and effective in its abilty to decrease cell migration, while SDF-1 neutralization was not nearly as effective in these investigations. Lastly, SDF-1 and CXCR4 combination inhibition was not more effective than CXCR4 inhibition alone, however more extensive titration experiments could be carried out to determine if there is utility in this approach.

These findings suggest that CXCR4 could be a therapeutic target for anti-metastatic therapies in non small cell lung cancer. However, further studies must be carried out in order to validate the *in vitro* findings mentioned above. First, CXCR4 null cell lines could be generated utilizing CXCR4 siRNA techniques to assess what impact this has on NSCLC cell migration. This technique would help deal with the uncertainties with regard to the how effective the CXCR4 inhibitors were at binding to and inhibiting the CXCR4 receptor, and further confirm the role of CXCR4 in NSCLC cell migration.

Second, the CXCR4 and SDF-1 inhibitors must be tested in an *in vivo* animal model to test their efficacy as anti-cancer agents. The Bebb laboratory is currently in the process of designing animal experiments in order to test the anti-metastatic capability of CTCE 9908 and the anti-SDF-1 antibody in NSCLC. Designing a clinically relevant lung cancer metastatic mouse model poses many challenges however. The xenograft models utilizing hind flank tumors traditionally used in the laboratory in order to test new

therapies for lung cancer, would not suffice as a metastatic model as hind flank tumors rarely metastasize to other locations. Similarly, tail vein administration of tumor cells – which is one possible pseudo-metastatic model - would also not be an ideal model to test anti-CXCR4 drugs. In this model, the tumor cells start out circulating in the blood, but initially flow to the lungs, where the majority of cells become trapped and initiate lung metastases. This model however, would not accurately reflect the natural course of lung cancer metastasis as the cells are not given the opportunity to metastasize to other organs. The most ideal xenograft model would utilize intracardiac injections of NSCLC cells, which are circulated throughout the whole body and are therefore given an opportunity to develop metastases in multiple organs. Alternatively, a murine model that spontaneously generates CXCR4 expressing lung tumors could also be used, in which anti-CXCR4 drugs could be administered prior to the evolution of metastatic disease.

The clinical studies outlined in Chapter 3 demonstrated for the first time that CXCR4 expression is an independent biomarker of poor prognosis in advanced NSCLC. High expression of CXCR4, as assessed by novel quantitative fluorescent IHC, was associated with significantly worse overall survival. Furthermore, a gender dependent effect was seen, with only females being negatively affected by high CXCR4 expression. It was postulated that estrogen receptors (ERs) may be playing a role in this observed gender difference as recent research demonstraed a link between ER and CXCR4 signalling. To assess the influence of ERs on the association between CXCR4 expression and clinical outcome, the Bebb lab is currently in the process of staining the same 170 tissue samples in the TMAs for ER expression to assess if there is any co expression of ERs and CXCR4 in the samples and what, if any effect this has on outcome.

Further analyses will also need to be carried out with regard to the role of CXCR4 expression in early stage resected disease. The studies described in Chapter 4 suggested that CXCR4 may also be a prognostic biomarker for overall survival, but not disease free survival, in stage I, II and III resected disease. More rigorous analyses must be undertaken to determine if CXCR4 is an independent prognostic marker, and if gender plays a role in the association between CXCR4 and outcome. Additionally, analyses comparing the CXCR4 AQUA expression scores between tumor samples from patients with stage I, II, III and IV disease would be an interesting exercise to determine if CXCR4 expression increased with stage.

Lastly, the role of CXCR7 should be explored both *in vitro* and clinically to determine what, if any, influence CXCR7 has on the CXCR4/SDF-1 chemokine axis and its biological effects. Many have reported that CXCR7 functions as a ligand scavenger or 'molecular sink' for SDF-1, sequestering the ligand in order to regulate the levels of SDF-1 in the microenvironment and produce or strengthen SDF-1 chemotactic gradients (Boldajipour et al. 2008; Haraldsen & Rot 2006). If this is the case, then the role of SDF-1/CXCR4 in cell tumor cell migration may be much more complex than originally thought. Therefore, the role of CXCR7 in the migration of NSCLC cell lines *in vitro* should also be addressed to determine what, if any role it plays in the migration of NSCLC cells. This would entail assessing the expression of CXCR7 in the NSCLC cell lines, and using CXCR7 inhibition to determine if blocking CXCR7 has any effect on NSCLC cell migration.

Intrestingly, Torisawa and colleagues recently reported that they had designed a microfluidically engineered tumor microenvironment in order to test the role of CXCR7

in chemotaxis and to see how CXCR7 influences SDF-1 gradients (Torisawa et al. 2010). Utilization of an *in vitro* model such as this would be very useful as it allows the investigator to assess the influence of the tumor microenvironment on the tumor cells, and in the case of CXCR4/SDF-1, would allow a more comprehensive investigation into the mechanisms behind SDF-1 induced tumor cell migration and would be a very interesting future research endeavor to help elucidate the role of the CXCR4/SDF-1 chemokine axis in NSCLC.

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APPENDIX A: AMERICAN JOINT COMMITTEE ON CANCER NON-SMALL CELL LUNG CANCER TNM STAGING 6TH EDITION

NSCLC Stage	T	N	M
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T1	N1	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T1, T2	N2	M0
	T3	N1, N2	M0
Stage IIIB	T4	Any N	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1

TNM Descriptors *

T – Primary Tumor
- T1 Tumor 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without evidence of invasion more proximal than the main bronchus
- T2 Tumor more than 3 cm but not more than 7 cm; or tumor with any of the Following features – involves main bronchus (2 cm or more distal to the carina), invades visceral pleura
- T3 Tumor that directly invades any of the following: chest wall, diaphragm, phrenic nerve, medisastinal pleura, parietal pericardium; or tumor in main bronchus less than 2 cm distal to the carina but without involvement of the carina
- T4 Tumor of any size that invades any of the following: mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina; or a tumor associated with a malignant pleural or pericardial effusion; or tumor with satellite nodules in the same lobe as the primary tumor

N – Regional Lymph Nodes
- N0 No regional lymph node metasatsis
- N1 Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes
- N2 Metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes
- N3 Metastasis in contralateral mediastinal or hilar nodes, or involvement of the supraclavicular or cervical lymph nodes

M – Distance Metastasis
- M0 No distant metastasis
- M1 Distant metastasis

* Adopted from the American Joint Committee on Cancer (AJCC) 6th Edition.

APPENDIX B: ADDITIONAL ASSESSMENTS OF CXCR4 RECEPTOR EXPRESSION

B.1. FACS analysis

Fluorescent activated cell sorting (FACS) analysis was performed in the Bebb lab in order to assess the expression of the CXCR4 chemokine receptor expression on the membrane of NSCLC cell line cells. Exponentially grown cells were trypsinized and counted. 5x 10^5 cells were suspended in 500 ul of Phosphate Buffer Saline (PBS) containing 1% bovine serum albumin (BSA). The cells were pelleted and 1ug of mouse anti-human CXCR4 MAb (Sigma) in 4ul of PBS-BSA was added. Similarly, 1 ug of mouse anti-human IgG MAb (Sigma) in 4ul of PBS-BSA was added to another tube and was used as the negative control. For blank control, no cells and 4 ul of PBS-BSA was used. All samples were incubated at 4^0C for 1 hour with occasional mixing then washed in 1ml PBS-BSA twice. To all tubes, 1 μg of secondary antibody, goat anti-mouse IgG - FITC (BD BioSciences), was added and then samples were incubated at 4^0C for 1 hour in the dark. Cells were washed twice again in 1 ml PBS-BSA then resuspended in 300 ul PBS. Fluorescence intensity was measured by BD FACSCAN cytometer (Flow Lab, University of Calgary, Calgary, Alberta).

B.2. Cell fractionation followed by Western Blot

Cell fractionation followed by western blotting was also performed in the Bebb lab to determine the protein expression of the CXCR4 chemokine receptor in the membrane, cytosolic, nuclear, and cytoskeletal compartments. 1.5 x 10^7 exponentially grown cells were trypsinized and washed 2 times in cold PBS. Cells were re-suspended in

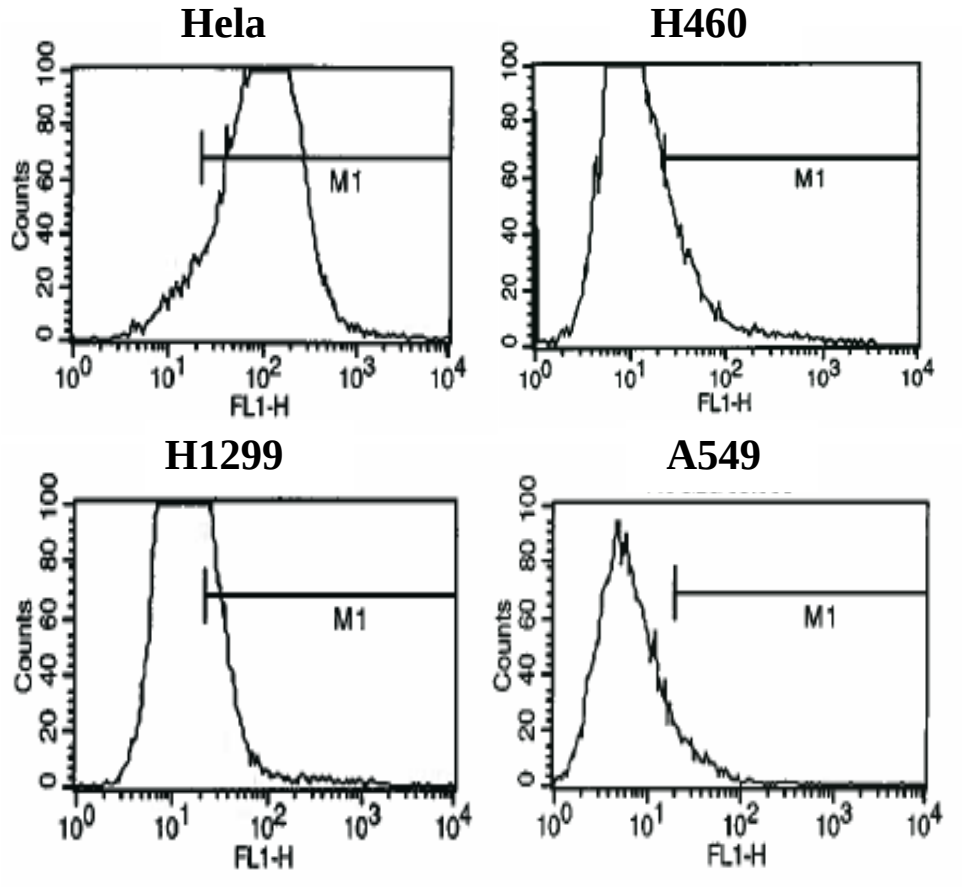
2 mls of cold PBS. Half of the cell suspension was pelleted and lysed in lysis buffer and was used as whole cell lysate. The other half of the cell suspension was used for fractionation as per manufacturer's protocol (FractionPrep Cell Fractionation Kit, Biovision Research Product, Mountain View, California USA). Cell lysates and fractions were stored at -80◦C until use.

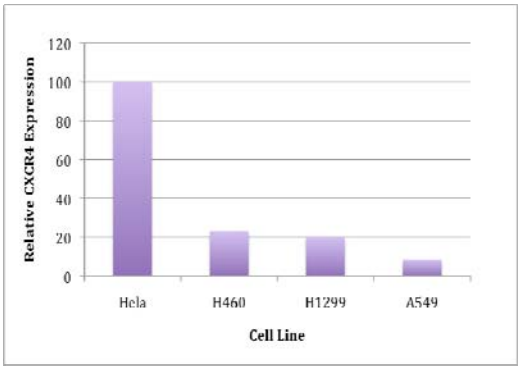
For western blotting, 20 μg of protein was separated using 12% SDS - polyacrylamide gels, transferred to nitro cellulose membrane, blocked in Tris- Buffered Saline containing 0.1% Tween and 5% skim milk for 1 hour. Blocked membranes were incubated in appropriate antibody solution overnight at 4◦C. The next day, after extensive washing in Tris-Buffered Saline Tween 20, membranes were incubated in secondary antibody, washed and then visualized with ECL Western detection system (GE Healthcare). Mouse anti-human CXCR4 monoclonal antibody (Sigma) was used to probe CXCR4.

To identify cell fractions the following antibodies were used: Rabbit anti-beta actin (Cell Signaling, New England Biolabs) for cytoskeleton; Rabbit anti-FAS (C-20) (Santa Cruz) for cytolsolic fraction; Rabbit anti-CD36 (H-300) (Santa Cruz) for membrane fraction; and rabbit anti-PCNA (Abcam) for nuclear fraction. Secondary antibodies were supplied by GE Healthcare, sheep anti-mouse HRP (Amersham) and donkey anti-rabbit HRP (Amersham).

B.3. Results

FACS Analysis of CXCR4 expression in NSCLC cell lines





Cell Line	Relative CXCR4 Expression (%)
H460	22.9
H1299	20.1
A549	8.4
Hela	100.0

Expression and localization of CXCR4 in NSCLC cell lines by cell fractionation followed by western blot