

2014

The chemokine receptor 4 (CXCR4) in
primary cutaneous
melanoma--correlation with established
histopathologic prognosticators, BRAF
status and expression of its ligand CXCL12

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**THE CHEMOKINE RECEPTOR 4 (CXCR4) IN PRIMARY CUTANEOUS
MELANOMA—CORRELATION WITH ESTABLISHED HISTOPATHOLOGIC
PROGNOSTICATORS, BRAF STATUS AND EXPRESSION OF ITS LIGAND
CXCL12**

by

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B.A., Colgate University, 2011

Submitted in partial fulfillment of the
requirements for the degree of
Master of Arts

2014

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ACKNOWLEDGMENTS

I would like to sincerely thank Dr. Meera Mahalingam and Dr. Shi Yang for their diligence and commitment to this project and my professional growth. I am grateful to have had the opportunity to work with them. I would like to thank Dr. Hee-Young Park for her exceptional advising and kind support. I would also like to thank Kyle Feller for his patience and assistance in helping me to succeed in my research.

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CXCL12

BRENDON C. MITCHELL

ABSTRACT

Dysregulation of the chemokine receptor 4 (CXCR4) and its primary ligand CXCL12 (SDF-1, stromal cell-derived factor-1), has been implicated in the progression of melanoma and the mechanisms by which the CXCR4/CXCL12 axis has been shown to activate cell cycle progression is via stimulation of the mitogen-activated protein kinase (MAPK) pathway. Given this, we sought to ascertain the potential cooperativity of CXCR4 with established histopathologic prognosticators including the BRAF status in primary cutaneous melanoma.

In this IRB approved study, archived tissue samples with diagnosis of primary cutaneous melanoma were retrieved from the Skin Pathology Laboratory at BUSM, Boston, MA and a total of 107 cases identified as meeting criteria for inclusion. Protein expression of CXCR4 and CXCL12 were assessed using commercially available rabbit polyclonal antibodies (Ab2074 and, ab9797 respectively, Abcam, Cambridge, MA, USA). CXCR4 gene expression (mRNA) was measured by semiquantitative RT-PCR with appropriate controls. For IHC, a semi-quantitative scoring (ranging from 0-3) was used and cases with a score of

≥ 2 (>10%) were considered positive. Molecular analysis for CXCR4 gene expression and BRAF exon 15 mutation status was performed using mRNA semi-quantitative RT-PCR and DNA Sanger sequencing respectively.

Univariate analyses of CXCR4 mRNA expression revealed a statistically significant correlation between elevated CXCR4 expression (low Δ Ct value) and presence of the BRAF mutation and absence of a host response ($p=0.03$ and $p=0.0003$ respectively). Univariate analyses revealed a significant correlation between elevated CXCR4 mRNA (low Δ Ct value) and the following: presence of BRAF mutation and absence of a host response ($p=0.03$ and 0.0003 respectively). CXCR4 mRNA was significantly higher among both AJCC stage 1 and stage 3 compared to stage 2 ($p=0.01$). Compared with CXCR4 negative samples, univariate analyses of CXCR4 protein showed that the proportion of CXCR4 positives was significantly greater in melanomas with absence of mitoses ($p<0.0001$), ulceration ($p=0.0008$) and regression ($p=0.02$). Patients presenting at shallower stages (AJCC 1-2) exhibited a larger proportion of CXCR4 positives (76.9%, $p<0.0001$ and 69.0%, $p=0.004$), while those at deeper stages (AJCC 3-4) exhibited a larger proportion of negatives (75.0%, $p=0.002$ and 66.7%, $p=0.10$). In a multivariable analysis, lower odds of CXCR4 protein expression were associated with AJCC stage-3 (OR=0.16, $p=0.01$), stage-4 (OR=0.17, $p=0.04$), and mitoses (OR=0.21, $p=0.01$).

Lack of correlation between CXCR4 mRNA and protein expression suggests that further study is required for a more precise understanding of

mRNA-protein interaction for CXCR4 in order to identify factors contributing to the lack of concordance. CXCR4 protein appears to be associated with established prognosticators of good clinical outcome as its expression is less frequently observed in melanomas with mitoses, ulceration and depth >2 mm. The association between CXCR4 mRNA and a brisk host response suggests that it may serve as a biomarker for recruiting melanoma patients for immunotherapy. Higher CXCR4 mRNA in patients with a BRAF mutation suggests its utility as a putative therapeutic target.

TABLE OF CONTENTS

Title	i
Copyright Page	ii
Reader's Approval Page	iii
Acknowledgements	iv
Abstract	v
Table of Contents	viii
List of Tables	xi
List of Figures	xii
List of Abbreviations	xiii
Introduction	1
1.1 Chemokine receptors and ligands—overview	1
1.2 CXCR4 and CXCL12	2
1.3 Pathways stimulated downstream of CXCR4	3
1.4 Function of the CXCR4/CXCL12 axis	5
1.5 CXCR4/CXCL12 in cancer	7
1.5.1 CXCR4/CXCL12 in hematopoietic malignancies	8
1.5.2 CXCR4/CXCL12 in other non-cutaneous malignancies	12
1.5.3 CXCR4/CXCL12 in cutaneous non-melanoma malignancies	29
1.5.4 CXCR4/CXCL12 in melanoma	32
1.6 Aims/Objectives	38

Materials and Methods	40
2.1 Sample selection	40
2.2 DNA analyses	42
2.3 RNA analyses	43
2.4 Immunohistochemistry	45
2.5 Statistical analyses	46
Results	48
3.1 DNA extraction and BRAF status	48
3.2 RNA extraction and CXCR4 mRNA expression <i>via</i> RT-PCR	51
3.3 CXCR4 mRNA expression correlates with select histopathologic prognosticators and BRAF mutation in primary cutaneous melanoma	53
3.4 Immunohistochemical analyses of CXCR4 and CXCL12 protein expressions	55
3.5 CXCR4 protein expression correlates with select histopathologic prognosticators and BRAF mutation in primary cutaneous melanoma	57
3.6 CXCL12 protein expression does not correlate with select histopathologic prognostications and BRAF mutation in primary cutaneous melanoma	58
3.7 CXCR4 protein expression does not correlate with expression of CXCR4 mRNA	60

Discussion	61
References	67
Vita	79

LIST OF TABLES

Table	Title	Page
1	Chronologic historical overview of studies on CXCR4 in non-cutaneous malignancies	21
2	Chronologic historical overview of studies on CXCR4 in non-cutaneous malignancies	31
3	Chronologic historical overview of studies on CXCR4 in non-cutaneous malignancies	36
4	Summary of patient demographics	41
5	DNA extraction and BRAF status	49
6	RNA extraction and Δ Ct values (CXCR4 mRNA)	52
7	Immunohistochemical staining for CXCR4 and CXCL12	56
8	Univariate analyses of CXCR4 protein and mRNA expression	59
9	Multivariate analyses of CXCR4 and CXCL12 protein expression profiles	60

LIST OF FIGURES

Figure	Title	Page
1	Binding interaction of CXCR4 and CXCL12	3
2	DNA Sanger sequencing	50
3	Representative examples of cases from study	54
4	Immunohistochemical staining for CXCR4 and CXCL12	57

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ALL	Acute Lymphoblastic Leukemia
AKT	Protein Kinase B
AML	Acute Myeloid Leukemia
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CXCL12	Chemokine XC Ligand 12
CXCR4	Chemokine XC Receptor 4
ERK	Extracellular Signal Regulated Kinase
IHC	Immunohistochemistry
MAPK	Mitogen-Activated Protein Kinase
MMT-1 MMP	Membrane Type-1 Matrix Metalloproteinase
NMR	Nuclear Magnetic Resonance
PI3K	Phosphoinositide 3-Kinase
PTEN	Phosphate and Tensin Homolog
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDF-1	Stromal Cell-Derived Factor-1

INTRODUCTION

1.1: Chemokine receptors and ligands—overview

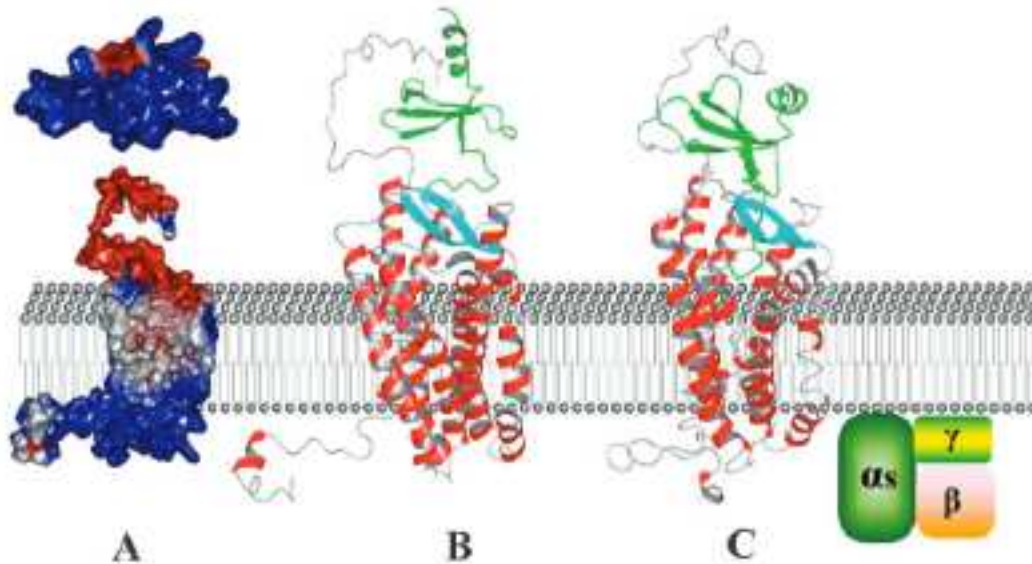
Members of the chemokine superfamily are small molecular weight signaling proteins (Payne & Cornelius 2002). Chemokines are classified into four groups based on the position of conserved cysteine residues—CC (2 adjacent N-terminal cysteines) CXC (2 N-terminal cysteines separated by 1 amino acid), C (1 N-terminal cysteine), and CX₃C (two N-terminal cysteines separated by 3 amino acids)—and for each family of ligand there is a corresponding receptor (Payne & Cornelius 2002). In total, there are seventeen known chemokine receptors—CCR1-CCR11 and CXCR1-CXCR6 (Wu 2010). The class of CXC ligands stimulates the CXC receptors (CXCR), which are a class of receptors consisting of seven-transmembrane domains and a G-coupled protein (Torregrossa 2012).

Chemokines and their receptors play an integral role in the immune system, directing leukocyte migration and regulating cellular proliferation (Balkwill 2004b). Chemokine receptor-ligand binding induces cytoskeletal rearrangement, which depending upon ligand concentration and quaternary structure can result in either firm adhesion or directional migration (Veldkamp 2008). Chemokines and their receptors—in particular CXCL12 and CXCR4—are believed to play a key role in cell migration and proliferation—two processes relevant in the progression and metastasis of cancer (Müller 2001; Sehgal 1998).

1.2 CXCR4 and CXCL12

The chemokine receptor CXCR4 is coupled to a G-protein and consists of seven transmembrane alpha helices with a thirty-four amino acid extracellular N-terminus (Wu 2010). Using X-ray crystallography it was determined that four domains of the receptor extend extracellularly and four domains extend intracellularly (Wu 2010). To date, known ligands for CXCR4 are CXCL12 (stromal derived factor-1, SDF-1) and ubiquitin (Saini 2011). Ubiquitin does not bind the same site as CXCL12 and its effects are poorly understood (Saini 2011). The structure of CXCL12 has been visualized in nuclear magnetic resonance (NMR) studies, showing a 67 residue peptide consisting of an N-terminal strand and a core globular domain (Crump 1997). With the use of NMR spectroscopy, migration assays, and calcium measurements Crump *et al.* demonstrated in 1997 that binding of CXCR4 and CXCL12 involves all extracellular domains of the receptor and both the core globular domain and N-terminus of the ligand resulting in a two site mechanism of binding for signaling transduction (Crump 1997). A recent study further elucidated these two steps and demonstrated that electrostatic interactions of the N-loops, B-sheet, and 40-s loop of CXCL12 with the extracellular loops and N-terminus of CXCR4 (step 1) induces movement of the CXCR4 transmembrane domains (step 2) (Xu 2013). This transmembrane shift of the CXCR4 molecule provides access to a deep binding pocket allowing for interactions with the CXCL12 N-terminus, particularly Lysine-1 and Proline-2, resulting in signal transduction (Gupta 2001; Xu 2013).

Figure 1. Binding interaction of CXCR4 and CXCL12



A) Inactive state B) Step 1: N-loops, B-sheet, and 40-s loop of CXCL12 interact with the extracellular loops and N-terminus of CXCR4 C) Step 2: CXCL12 N-terminus searches CXCR4 binding pocket and induces transmembrane movement for signal transduction (Xu 2013)

Of note, a recent *in vitro* study of Chinese hamster ovarian cells showed that the tyrosine residues located on the CXCR4 N-terminus at positions 7, 12, and 21 can be post-translationally sulfated to enhance ligand binding affinity, highlighting the important of all extracellular components of the CXCR4 molecule to signal transduction (Ziarek 2013).

1.3 Pathways stimulated downstream of CXCR4

Stimulation of CXCR4 by CXCL12 can activate four downstream signaling pathways leading to four distinct cellular responses (Drury 2011). The phospholipase C pathway can be induced by CXCR4, which results in the release of intracellular calcium stores and cellular migration (Kremer 2011). Using human T-cells from the Jurkat cell line researchers showed that the

binding of CXCR4 to CXCL12 results in an increase of intracellular calcium and cellular migration *in vitro* (Kremer 2011). Another pathway that can be stimulated through CXCR4 is the RhoA pathway, which leads to an increase in the production of the collagenase membrane type-1 matrix metalloproteinase (MMT-1 MMP) (Bartolomé 2004). Cross-talk between CXCR4 and MMT-1 MMP increases the invasiveness of malignant cells and enables them to migrate through membranes and extracellular matrices, thus enhancing metastatic potential (Bartolomé 2009). The PI3K/AKT pathway can also be activated by CXCR4 leading to cell survival (Luo 2013). In 2013, Luo *et al.* demonstrated that nasopharyngeal carcinoma-derived non-metastatic 6-10B and metastatic 5-8F cell lines displayed enhanced survival in the presence of CXCL12, while addition of PI3K/AKT pathway inhibitors blocked the survival promoting effects of CXCL12 (Luo 2013). Lastly, the MAPK pathway may be stimulated resulting in cellular proliferation (Robledo 2001; Sun 2002; Alsayed 2007; Heinrich 2012). A study on the BLM melanoma cell line demonstrated that phosphorylation of the MAP kinases p44/42 (ERK1/2) and p38 (downstream of the Raf protein) upon *in vitro* CXCL12 administration, indicating enhanced MAPK pathway activation (Robledo 2001). In summary, CXCR4 binding CXCL12 can induce migration, invasion, proliferation, and survival. These functions help delineate the contribution of the CXCR4/CXCL12 axis to the progression and metastasis of cancer.

The pathways stimulated in response to CXCR4 binding CXCL12 are dependent upon the concentration and structure of CXCL12 (Veldkamp 2008;

Drury 2011). In 2008, *in vitro* studies of the human leukemic monocyte THP-1 cell line demonstrated that the chemotactic response to CXCL12 was biphasic and occurred within a narrow concentration window (Veldkamp 2008). This same study showed that the monomeric form of CXCL12 stimulates cellular migration, whereas the dimeric form inhibits it (Veldkamp 2008). A 2011 study showed that *in vitro* administration of a monomeric CXCL12 slowly activated ERK1/2, whereas dimeric CXCL12 induced rapid ERK1/2 activation in human colorectal carcinoma cell lines, helping to explain the observed difference in response to monomeric and dimeric CXCL12 (Drury 2011). This study also elucidates how the extent of MAPK pathway stimulation, as influenced by CXCL12 concentration and quaternary structure, contributes to cellular phenotype (Drury 2011).

The aforementioned studies have clarified the role of CXCR4 and CXCL12 in controlling the cell cycle and chemotaxis. Most importantly, these findings have allowed researchers to identify this chemokine-receptor pair as a clinically relevant participant of cancer development and progression.

1.4 Function of the CXCR4/CXCL12 axis

Beginning as early as embryogenesis, CXCR4 and CXCL12 play a role in directing cellular migration in all mammals (Domanska 2013). In 1996 Nagasawa *et al.* demonstrated that loss of function of the CXCR4/CXCL12 axis results in defects in lymphopoiesis and bone marrow myelopoiesis (Nagasawa 1996).

Knockout studies of CXCR4 and CXCL12 in mice demonstrated a loss in stem cell migration and perinatal death due to failed embryonic development of the hematopoietic, renal, cardiovascular and nervous systems (Sierro et al., 2007; Takabatake 2009; Bonig & Papayannopoulou 2013; Mithal 2013). In addition to its contributions to embryonic development the CXCR4/CXCL12 axis is important to adult mammals as it directs immune responses, induces neovascularization and promotes cellular proliferation after injury (Fu 2013) (Liu 2013) (Bollag & Hill, 2013). Varied expression of CXCL12 throughout the body allows for the establishment of CXCL12 gradients, which help to direct migration of CXCR4 expressing cells (Loetscher et al., 2000). In 2000 Loetscher *et al.* demonstrated that constitutive CXCL12 expression at sites of inflammation results in the establishment of a CXCL12 gradient, which directs CXCR4 expressing phagocytic cells of the innate immune system (Loetscher 2000). A 2001 study showed that elevated CXCL12 expression was not limited to sites of inflammation, but was also be observed at the lymph nodes, lung, liver and bone marrow, whereas comparatively lower expression was demonstrated in the small intestine, kidney, skin, brain and skeletal muscle (Müller 2001). This pattern of expression is necessary for normal function of the immune system, directing lymphocyte trafficking (Stein & Nombela-Arrieta 2005). As will be discussed, anatomical sites of higher relative CXCL12 expression have been identified as common sites for metastatic development (Müller 2001).

1.5 CXCR4/CXCL12 in cancer

To date, CXCR4 expression has been identified in 37 different primary tumors, in addition to many tumor metastases, of epithelial, mesenchymal, and hematopoietic origin (Balkwill 2004a). Expression of CXCR4 is not typically uniform throughout a neoplasm, but rather contained to a subpopulation of malignant cells (Balkwill 2004a). In 2001 Müller *et al.* performed *in vitro* migration and invasion assays confirming that murine CXCL12 enhances migration and invasion in human breast carcinoma MDA-MB-231 cells and showed that *in vivo* inhibition of the CXCR4/CXCL12 axis can block the development of metastasis, demonstrating for the first time the contribution of this axis to the metastatic cascade (Müller 2001). Briefly, Muller *et al.* co-injected breast carcinoma MDA-MB-231 cells and a CXCR4 monoclonal antibody into severe combined immunodeficient mice and observed a 61-68% suppression of pulmonary metastasis formation compared to mice injected with MDA-MB-231 cells only (Müller 2001). This groundbreaking study set the template for future research of the roll of the CXCR4/CXCL12 axis in cancer progression and metastasis.

1.5.1 CXCR4/CXCL12 in hematopoietic malignancies

The first study to implicate the CXCR4/CXCL12 axis in the progression of hematopoietic malignancies was published in 1999 by Möhle *et al.*, who demonstrated a CXCR4 fluorescence intensity four times greater in B-lymphocytes from patients with B-cell chronic lymphocytic leukemia (B-CLL) compared to normal B-lymphocytes and postulated that constitutive CXCL12 expression in the bone marrow may promote the clinically observed infiltration and accumulation of lymphocytic blasts in the bone marrow of B-CLL patients (Möhle 1999). This was confirmed by *in vitro* migration assays performed in a conditioned medium of human bone marrow-derived stromal cells—high CXCL12—and showed that CXCL12 stimulated transendothelial migration to a greater extent in leukemic B-lymphocytes compared to normal B lymphocytes, implicating the CXCL12/CXCR4 axis as a contributor to the invasive capacity of malignant cells (Möhle 1999). In 2002 Ishibe *et al.* demonstrated a correlation between CXCR4 expression on B-cells from chronic lymphocytic leukemia patients and patient morbidity, indicating CXCR4 expression as a prognosticator for poor outcome in chronic lymphocytic leukemia (Ishibe 2002). Similar findings were published in a 2003 paper by Barretina *et al.* who used flow cytometry to quantify CXCR4 expression in B lymphocytes derived from B-CLL patients and identified a correlation between CXCR4 expression and lymphocyte counts, effectively associating CXCR4 expression with disease progression (Barretina 2003). The same study also observed reduced CXCL12 levels in B-CLL patient

blood samples suggesting that low CXCL12 concentrations in blood may contribute to the propensity of B-CLL cells to migrate towards sites of greater CXCL12 expression for metastatic development (Barretina 2003). Möhle *et al.* followed up their 1999 findings with a study that demonstrated functional CXCR4 protein expression on blast cells from patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) by using flow cytometry and an *in vitro* CXCL12 induced calcium flux assay (Möhle 2000). This same study showed that CXCL12 induced transmembrane migration of AML and ALL cells (Möhle 2000). The chemoattraction of CXCR4 expressing cells for CXCL12 allowed Möhle *et al.* to conclude that CXCR4 expression contributes to bone marrow compartmentalization—a site of high CXCL12 expression—often observed in ALL and AML patients (Möhle 2000). This same year a study by Crazzolara *et al.* identified a correlation between CXCR4 expression and extramedullary organ infiltration in patients with acute lymphoblastic leukemia, linking CXCR4 expression to progression and metastasis (Crazzolara 2001). Seven years later, Spoo *et al.* showed that acute myeloid leukemia patients exhibiting low CXCR4 expression, as measured by flow cytometry, displayed significantly longer survival compared to patients with medium and high expression, helping to classify CXCR4 protein expression as a prognosticator of poor patient outcome in acute myeloid leukemia (Spoo 2007). The same year, these findings were confirmed using immunohistochemistry to correlate CXCR4 expression to patient morbidity (Konoplev 2007). Yet another B-cell malignancy in which CXCR4

expression may contribute to metastatic development is follicular center lymphoma (Corcione 2000). Corcione *et al.* demonstrated CXCR4 protein upregulation in follicular center lymphoma B-cells as compared to normal germinal center B-cells by using flow cytometry (Corcione 2000). This same study compared *in vitro* CXCL12 induced migration of normal germinal center B-cells and follicular center lymphoma B-cells and showed migration was enhanced in malignant B-cells, implicating the CXCR4/CXCL12 axis as a potential contributor to malignant cell migration away from the primary tumor site and the eventual development of metastasis (Corcione 2000). In 2003 Möller *et al.* demonstrated CXCR4 expression in multiple myeloma biopsies and revealed a potential role for the CXCR4/CXCL12 axis in directing multiple myeloma B-cells to compartmentalize in the bone marrow (Möller 2003). Briefly, the authors demonstrated that multiple myeloma cells and multiple myeloma cell lines express CXCR4 protein and exhibit a chemoattraction for CXCL12 through the use of flow cytometry, *in vitro* CXCL12 induced calcium influx measurements, and an *in vitro* migration assay (Möller 2003). Möller *et al.* concluded that, much like in B-CLL, the high concentration of CXCL12 in the bone marrow causes compartmentalization of the multiple myeloma cells (Möller 2003). Interestingly, a 2013 study by Bao *et al.* demonstrated that CXCR4 protein expression on multiple myeloma cells is correlated to longer patient survival, indicating CXCR4 protein expression may be a prognosticator of good clinical outcome in multiple myeloma patients (Bao 2013).

In addition to B-cell malignancies, the CXCR4/CXCL12 axis has been implicated in the metastatic cascade of precursor myeloid cell and T-cell malignancies (Peled 2002; Weng 2003). In 2002 Peled *et al.* demonstrated that loss of function of CXCR4 in precursor myeloid cells from patients with chronic myelogenous leukemia (CML) allows for detachment and migration away from their primary tumor site in the bone marrow (Peled 2002). Briefly, flow cytometry and calcium flux assays performed on CML samples showed the presence of functional CXCR4 protein expression on pluripotent, immature CML cells (Peled 2002). As mentioned previously, CXCL12 is constitutively expressed in the bone marrow and one would expect compartmentalization of CXCR4 expressing CML cells in the bone marrow; however, this is not observed (Peled 2002). Peled *et al.* attributed this unexpected behavior to a *bcr-abl* mutation common to CML cells, which inhibits stimulation of the CXCR4 pathway and allows for migration away from the bone marrow (Peled 2002). In contrast, normal blood cord cells lacking the *bcr-abl* mutation remain within the bone marrow until further maturation (Peled 2002). Peled *et al.* confirmed this hypothesis by demonstrating a weaker chemotactic response to CXCL12 and a weaker binding affinity for fibronectin in *bcr-abl* mutated CML cells as compared to normal cord blood cells, indicating that the CML cells were more likely to detach from their surroundings, thus initiating the metastatic cascade (Peled 2002). Lastly, CXCR4 expression has been linked to T-cell malignancies as demonstrated by a study of T_H1 and T_H2 lymphocyte subsets from patients with non-Hodgkin's Lymphoma (Weng 2003).

Weng *et al.* showed that CXCR4 protein is preferentially expressed on malignant T_H2 cells—a phenotypically aggressive subtype—compared to the “less” malignant T_H1 cells and concluded that CXCR4 expression may contribute to the metastatic capacity of non-Hodgkin’s Lymphoma cells (Weng 2003).

1.5.2 CXCR4/CXCL12 in other non-cutaneous malignancies

Expression of CXCR4 has been studied in squamous cell carcinoma of the oral cavity, esophagus, cervix, and larynx (Uchida 2003; Gockel 2006; L. Zhang 2012; Huang 2013). Of these, CXCR4 and CXCL12 expression was first studied in oral squamous cell carcinoma (Uchida 2003). In 2003 Uchida *et al.* demonstrated enhanced expression of CXCR4 mRNA and protein in primary oral squamous cell carcinoma and metastases as compared to adjacent normal tissues; however, CXCR4 expression could not be correlated to patient prognosis or tumor size (Uchida 2003). In a 2007 follow-up study Uchida *et al.* noted a statistically significant decrease in the 5-year survival of oral squamous cell carcinoma patients exhibiting high CXCR4 and/or CXCL12 protein expression at primary tumor sites, linking the CXCR4/CXCL12 axis to poor patient outcome (Uchida 2007). In 2011 Uchida *et al.* demonstrated that metastatic development in nude mice injected with B88 oral squamous cell carcinoma cells could be inhibited by inoculation with the CXCR4 antagonist AMD3100, thus elucidating the potential therapeutic value of targeting the CXCR4/CXCL12 axis (Uchida 2011).

In contrast to oral squamous cell carcinoma, studies of the CXCR4/CXCL12 axis in esophageal carcinoma have yielded conflicting results (Gockel 2006; Sasaki 2009; Wang 2009; Lu 2013). Briefly, in 2006 Gockel *et al.*, using immunohistochemistry, showed CXCR4 protein expression in esophageal squamous cell carcinoma biopsies and noted an association between high expression and patient morbidity; although these findings did not achieve statistical significance (Gockel 2006). Following this, Sasaki *et al.* found that expression of CXCR4 protein did not correlate with clinicopathologic prognosticators nor patient survival, whereas Wang *et al.* demonstrated a correlation between expression and American Joint Committee on Cancer (AJCC) staging (Sasaki 2009; Wang 2009). Although these three studies fail to conclusively establish CXCR4 expression as a prognosticator for patient outcome, a recent study by Lu *et al.* highlighted the potential relevance of the CXCR4/CXCL12 axis in progression of esophageal squamous cell carcinoma (Lu 2013). Briefly, Lu *et al.* demonstrated that CXCR4 expression at sites of esophageal squamous cell metastasis was significantly greater than primary tumor sites, implying that the upregulation of CXCR4 may occur with metastatic development (Lu 2013). The same study showed that high CXCR4 expressing cells, determined by flow cytometry, from the esophageal squamous cell carcinoma Ec109 cell line displayed enhanced migratory potential *in vitro*, providing further evidence that the CXCR4/CXCL12 axis may play a role in the metastatic cascade (Lu 2013).

Upregulation of CXCR4 and consequently stimulation of the CXCR4/CXCL12 axis has been implicated in the progression laryngeal squamous cell carcinoma (Tan 2008; Zheng 2011; Zhang 2012). In response to earlier findings implicating the CXCR4/CXCL12 axis in the progression of esophageal squamous cell carcinoma Tan *et al.* evaluated CXCR4 mRNA and protein expression, using RT-PCR and immunohistochemistry respectively, in laryngeal squamous cell carcinoma and demonstrated a correlation between expression and the development of metastasis (Tan 2008). A follow up study evaluated CXCR4 mRNA and protein expression and correlated expression to AJCC stage, presence of metastasis, and micro-vascularization, all of which identified CXCR4 expression as a prognosticator of poor patient outcome (Zheng 2011). In 2012 Zhang *et al.* demonstrated that CXCR4 protein expression in laryngeal squamous cell carcinoma correlated with clinical stage and presence of metastasis, confirming the relevance of the CXCR4/CXCL12 axis in the progression and metastatic cascade of laryngeal squamous cell carcinoma (Zhang 2012).

The role of the CXCR4/CXCL12 axis in cervical squamous cell carcinoma has been studied in three papers, all of which highlight its contribution to cancer progression and potential prognosticative value (Kodama 2007; Zhang 2007; Huang 2013). Briefly, in 2007 Kodama *et al.* showed that CXCR4 protein expression, as determined by immunohistochemistry, correlated with advanced tumor staging, tumor size ≥ 4 mm, stromal invasion, absence of a brisk host

response, and lymph node metastasis, thus identifying CXCR4 expression as an indicator for poor patient outcome (Kodama 2007). Later that year Zhang *et al.* demonstrated CXCR4 protein expression in cervical squamous cell carcinoma HeLa cells and showed HeLa cell migration along a CXCL12 gradient *in vitro*, which implies that the CXCR4/CXCL12 axis may contribute to the migratory capacity of malignant cells, potentiating the metastatic cascade (Zhang 2007). In the same study, immunohistochemical analysis of cervical squamous cell carcinoma and corresponding lymph node biopsies revealed CXCR4 expression at the primary site and CXCL12 expression at the lymph node, confirming the conclusion of Zhang *et al.* that a CXCL12 gradient originating at the lymph node may attract primary tumor cells for metastasis (Zhang 2007). A more recent study confirms the prognostic value of CXCR4 by demonstrating a correlation between protein expression in primary cervical squamous cell carcinoma biopsies and histologic grade (Huang 2013).

In addition to squamous cell carcinoma, the CXCR4/CXCL12 axis has been studied, although on a more limited basis, in adenocarcinoma of the pancreas, esophagus, cervix, and lung (Koshiba 2000; Gockel 2006; Yang 2007; Wagner 2009). Immunohistochemical analyses of pancreatic invasive ductal adenocarcinoma biopsies demonstrated CXCR4 protein expression in lesional tissue, but the absence of expression in adjacent normal tissue (Koshiba 2000). In the same study, although Koshiba *et al.* failed to correlate protein expression with clinicopathological features, *in vitro* chemotactic assays on pancreatic

cancer cell lines showed that CXCL12 enhances migration, favoring a role for the CXCR4/CXCL12 axis in the metastatic cascade of pancreatic adenocarcinoma (Koshiba 2000). In a 2009 study of pancreatic adenocarcinoma, high CXCR4 expression, as established by semi-quantitative immunohistochemical scoring, correlated with the presence of lymph node metastasis and liver recurrence, indicating that CXCR4 expression may be a prognosticator for poor patient outcome (Maréchal 2009). Although limited to one study, and needing further investigation, CXCR4 protein expression in esophageal adenocarcinoma correlated with patient morbidity, but did not correlate with other established prognosticators (Gockel 2006). The following year Yang *et al.* determined that CXCR4 protein expression in cervical adenocarcinoma correlated with metastatic development and patient morbidity, indicating that expression may be a prognosticator of poor patient outcome (Yang 2007). A similar study on non-small cell lung adenocarcinoma demonstrated a correlation between CXCR4 protein expression and the following: presence of lymph node metastasis and shorter disease-free survival (Wagner 2009). These studies indicate that the CXCR4/CXCL12 axis may contribute to the progression of adenocarcinoma and serve as a valuable prognosticator for identifying advanced and/or phenotypically aggressive adenocarcinomas.

The chemokine receptor CXCR4 and its ligand CXCL12 have been studied in other non-cutaneous carcinomas as well. A study mentioned earlier by Müller *et al.* elucidated the relevance of CXCR4 and CXCL12 in breast

carcinoma (Müller 2001). Briefly, using RT-PCR and immunohistochemistry Müller *et al.* showed that CXCR4 mRNA and protein expression was absent in normal human mammary epithelial and ductal cells, but markedly upregulated in breast cancer cell lines and epithelial breast carcinoma metastasis biopsies (Müller 2001). In the same study, it was determined that the organs exhibiting the highest CXCL12 expression—lymph nodes, lungs, liver, and bone marrow—were also the most common sites of breast carcinoma metastasis, implicating the CXCR4/CXCL12 axis in the directional migration of malignant cells (Müller 2001). Two years later a study of the colon carcinoma cell line CT-26 demonstrated the importance of tumor microenvironment on CXCR4 expression (Zeelenberg 2003). Zeelenberg *et al.* showed that CXCR4 protein expression in CT-26 cells was low *in vitro*, but significantly upregulated after injection and metastasis to murine spleen, liver, or lung (Zeelenberg 2003). In the same study, CXCR4-deficient colonic carcinoma cells colonized the lung, but failed to proliferate at the same rate as CXCR4 positive colonic carcinoma cells (Zeelenberg 2003). Interestingly, Zeelenberg *et al.* showed that, in contrast to breast carcinoma, the CXCR4/CXCL12 axis appeared to contribute to malignant cell proliferation, but not migration in colon carcinoma (Zeelenberg 2003). These two early studies delineate the unique contribution of the CXCR4/CXCL12 axis in the progression of different malignancies (Müller 2001; Zeelenberg 2003). Despite these differences two recent studies demonstrated the prognostic value of CXCR4 expression in both breast carcinoma and colon carcinoma (Xu 2013; Lv 2014).

Other carcinomas in which CXCR4 expression has been identified as a prognosticator of poor patient outcomes are clear cell renal carcinoma and non-small cell lung carcinoma (Spano 2004; Wang 2012). In these studies immunohistochemical analyses demonstrated a correlation between CXCR4 expression and shorter survival (Spano 2004; Wang 2012; Xu 2013; Lv 2014).

Studies pertaining to the CXCR4/CXCL12 axis in thyroid carcinoma have elucidated its role in malignant progression as well as the potential cooperativity of this axis with mutated proteins downstream of its signaling cascade (Hwang 2003; He 2010; Torregrossa 2012). In a study of anaplastic thyroid carcinoma cell lines, using flow cytometry and *in vitro* migration assays, researchers demonstrated CXCR4 protein expression and a chemotactic response to CXCL12 reiterating the potential contribution of the CXCR4/CXCL12 axis to the metastatic cascade (Hwang 2003). In a study of benign and malignant thyroid lesions, researchers noted that CXCR4 expression was greater in malignant lesions suggesting the importance of CXCR4 in malignant progression (He 2010). In the same study, researchers compared CXCR4 expression in “more” malignant (poorly differentiated and medullary) *versus* “less” malignant (papillary and follicular) thyroid carcinomas and noted a significantly greater expression in the former (He 2010). More recently, in a study of papillary thyroid carcinoma, CXCR4 expression in primary biopsies correlated with tumoral infiltration, supporting the utility of CXCR4 expression as a prognosticator for poor patient outcome (Torregrossa 2012). In addition, this study noted a correlation between

CXCR4 expression and presence of a mutation in the MAPK BRAF protein (Torregrossa 2012).

Studies of CXCR4 expression have elucidated the potential contribution of the CXCR4/CXCL12 axis in the progression of various sarcomas, but have been limited in number. In 2002 expression of CXCR4 was first identified on cell lines derived from rhabdomyosarcoma and it was demonstrated that *in vitro* administration of CXCL12 induced migration and membrane invasion, implying that dysregulation of the CXCR4/CXCL12 axis may potentiate the metastatic cascade (Libura 2002). Similarly, a 2010 study demonstrated CXCR4 protein expression in chondrosarcoma biopsies and showed that CXCL12 enhanced invasion *in vitro* (Sun 2010). This study also demonstrated that CXCR4 antagonists and MAPK inhibitors negated the observed effects of CXCL12, highlighting the potential therapeutic value of targeting CXCR4 (Sun 2010). To date, the prognostic value of CXCR4 in rhabdomyosarcoma and chondrosarcoma has not been evaluated. A 2012 study using flow cytometry demonstrated CXCR4 expression in Ewing sarcoma biopsies and *in vitro* migration and proliferation assays with Ewing sarcoma cell lines showed that CXCL12 has a proliferative effect, but no effect on migratory, leading the authors to conclude that the CXCR4/CXCL12 axis may be relevant in the growth of this malignancy (Berghuis 2012). Of note, this study failed to correlate CXCR4 expression with metastatic development, but identified a significant correlation with patient morbidity indicating that CXCR4 may be a predictive of poor patient

outcome (Berghuis 2012). The same year, a study demonstrated a similar prognostic value for CXCR4 in osteosarcoma by showing that the intensity of immunohistochemical staining for CXCR4 protein correlated with patient morbidity (Ma 2012).

The CXCR4/CXCL12 axis has been implicated in a variety of cancers derived from the nervous system as well. A 2001 study demonstrated expression of CXCR4 protein on neuroblastoma cell lines and showed that *in vitro* administration of CXCL12 resulted in downregulation of CXCR4 (Geminder 2001). A common site for neuroblastoma metastasis is the bone marrow (Geminder 2001). Given this, Geminder *et al.* hypothesized that high concentrations of CXCL12 in the bone marrow may induce CXCR4 downregulation on malignant neuroblastoma cells causing compartmentalization within the bone marrow and resulting in metastatic development (Geminder 2001). This was confirmed in a follow up study by Russell *et al.* who demonstrated that CXCR4 protein expression correlated with tumor staging and the presence of bone marrow metastasis, confirming the importance of the CXCR4/CXCL12 axis in disease progression and the potential prognostic utility of CXCR4 (Russell 2004). In two other tumors, astrocytoma and glioblastoma, researchers demonstrated CXCR4 mRNA expression using RT-PCR and showed that *in vitro* CXCL12 administration to rat astrocytes induced cellular proliferation (Barbero 2002). Although the findings of Barbero *et al.* revealed a potential contribution to tumor progression, to date the prognostic value of

CXCR4 expression in astrocytoma and glioblastoma has not been evaluated.

Table 1. Chronologic historical overview of studies on CXCR4 in non-cutaneous malignancies

REFERENCE	STUDY DESIGN	FINDINGS	CONCLUSION
Möhle <i>et al.</i> 1999	Samples studied: B-cell chronic lymphocytic leukemia and normal B-lymphocytes Methods: Flow cytometry for CXCR4 protein and <i>in vitro</i> migration assay	CXCR4 fluorescence intensity four times greater in B-lymphocytes from patients with B-cell chronic lymphocytic leukemia, as compared to normal B-lymphocytes; CXCL12 stimulates transendothelial migration to a greater extent in leukemic B-lymphocytes compared to normal B lymphocytes	The CXCR4/CXCL12 axis may enhance the invasive capacity of chronic lymphocytic leukemia B-lymphocytes contributing to disease progression and metastatic development.
Möhle <i>et al.</i> 2000	Samples studied: Acute myeloid leukemia and acute lymphoblastic leukemia Methods: Flow cytometry for CXCR4 protein, <i>in vitro</i> calcium flux assay and transmembrane migration assay	CXCR4 protein expressed on acute myeloid leukemia blasts and acute lymphoblastic leukemia blasts; CXCL12 induces intracellular calcium influx and transmembrane migration	The CXCR4/CXCL12 axis may contribute to bone marrow accumulation of acute myeloid leukemia blasts and acute lymphoblastic leukemia blasts.
Corcione <i>et al.</i> 2000	Samples studied: Follicular center lymphoma B-cells and normal germinal center B-cells Methods: Flow cytometry for CXCR4 and <i>in vitro</i> migration assay	CXCR4 protein upregulation in follicular center lymphoma B-cells as compared to normal germinal center B-cells; CXCL12 induces greater migration of malignant B-cells as compared to normal germinal center B-cells	The CXCR4/CXCL12 axis may contribute to the metastatic cascade by directing malignant cell migration along a CXCL12 concentration gradient.
Geminder <i>et al.</i> 2001	Sample studied: Neuroblastoma cell lines Methods: Flow	CXCL12 induces downregulation of CXCR4	High concentrations of CXCL12 in the bone marrow may induce CXCR4 downregulation on

	cytometry for CXCR4 and <i>in vitro</i> migration assay		malignant neuroblastoma cells causing compartmentalization within the bone marrow and metastatic development
Koshiba <i>et al.</i> 2000	Samples studied: Pancreatic invasive ductal adenocarcinoma and cell lines Methods: IHC for CXCR4 protein and <i>in vitro</i> migration assay	CXCR4 protein expression in lesional tissue, but absent in adjacent normal tissue; CXCR4 protein expression does not correlate with clinicopathological features; CXCL12 enhances migration	The CXCR4/CXCL12 axis may contribute to progression and metastasis of pancreatic invasive ductal adenocarcinoma.
Crazzolara <i>et al.</i> 2001	Sample studied: Acute lymphoblastic leukemia Method: Flow cytometry for CXCR4 protein	CXCR4 protein expression correlates with extramedullary organ infiltration	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with acute lymphoblastic leukemia.
Müller <i>et al.</i> 2001	Sample studied: Breast carcinoma metastasis and cell line Method: RT-PCR for CXCR4 mRNA and IHC for CXCR4 protein	CXCR4 mRNA and protein expression absent in normal human mammary epithelial and ductal cells, but markedly upregulated in breast carcinoma cell lines and epithelial breast carcinoma metastasis; CXCL12 expression greatest at common sites of breast carcinoma metastasis.	The CXCR4/CXCL12 axis may contribute to progression and perhaps be involved in directing sites at which metastasis of breast carcinoma may occur.
Libura <i>et al.</i> 2002	Sample studied: Rhabdomyosarcoma cell lines Methods: Flow cytometry for CXCR4 protein, <i>in vitro</i> migration and transmembrane invasion assays	CXCR4 protein expression is present; CXCL12 induces migration and membrane invasion	The CXCR4/CXCL12 axis may potentiate the metastatic cascade in rhabdomyosarcoma.
Ishibe <i>et al.</i>	Sample studied:	CXCR4 protein	CXCR4 protein expression

2002	B-cell chronic lymphocytic leukemia Method: Flow cytometry for CXCR4 protein	expression correlates with patient morbidity	may be of utility as a prognosticator for poor outcome in patients with B-cell chronic lymphocytic leukemia.
Peled <i>et al.</i> 2002	Samples studied: Chronic myelogenous leukemia and normal cord blood cells Methods: Flow cytometry for CXCR4 protein, <i>in vitro</i> calcium flux assay, migration assay, and cell adhesion assay	CXCR4 protein expression present on chronic myelogenous leukemia cells; CXCL12 induces intracellular calcium flux and migration of normal cord blood cells but not chronic myelogenous leukemia cells	Inhibition of the CXCR4/CXCL12 axis by the presence of a <i>bcr-abl</i> mutation may initiate chronic myelogenous leukemia cell migration away from the bone marrow, potentiating the metastatic cascade.
Barbero <i>et al.</i> 2002	Sample studied: Astrocytoma, glioblastoma and rat astrocytes Methods: RT-PCR for CXCR4 mRNA and <i>in vitro</i> proliferation assay	CXCR4 mRNA expression is present; CXCL12 induces proliferation of rat astrocytes.	The CXCR4/CXCL12 axis may contribute to the growth and development of astrocytoma and glioblastoma.
Barretina <i>et al.</i> 2003	Sample studied: Chronic lymphocytic leukemia B-lymphocytes and patient plasma samples Methods: Flow cytometry for CXCR4 and CXCL12 protein	CXCR4 protein expression correlates with lymphocyte counts; CXCL12 expression significantly lower in B-cell chronic lymphocytic leukemia patients as compared to disease free patients	CXCR4 and CXCL12 protein expression may be of utility as a prognosticator for poor outcome in patients with B-cell chronic lymphocytic leukemia. The CXCR4/CXCL12 axis may contribute to the metastatic cascade by directing malignant cell migration along a CXCL12 concentration gradient.
Möller <i>et al.</i> 2003	Samples studied: Multiple myeloma cell lines and multiple myeloma	CXCR4 protein expression present on multiple myeloma cell lines and patient biopsies; CXCL12 induces calcium	The CXCR4/CXCL12 axis may be implicated in bone marrow compartmentalization of multiple myeloma cells

	Methods: Flow cytometry for CXCR4, <i>in vitro</i> calcium influx assay and migration assay	influx and migration of multiple myeloma cells	resulting from high CXCL12 concentrations in the bone marrow.
Weng <i>et al.</i> 2003	Sample studied: Non-Hodgkin's lymphoma Method: Flow cytometry for CXCR4 protein	CXCR4 protein expression greater on the more malignant T _H 2 cells as compared to T _H 1 cells	CXCR4 expression may contribute to the metastatic capacity of these of non-Hodgkin's lymphoma cells and is associated with a more malignant phenotype.
Uchida <i>et al.</i> 2003	Samples studied: Oral squamous cell carcinoma and metastasis Methods: RT-PCR for CXCR4 mRNA and IHC for CXCR4 protein	Enhanced expression of CXCR4 mRNA and protein in oral squamous cell carcinoma and metastases as compared to adjacent normal tissues; CXCR4 expression could not be correlated to patient prognosis or tumor size	The CXCR4/CXCL12 axis may be relevant in the development and progression of oral squamous cell carcinoma.
Zeelenberg <i>et al.</i> 2003	Sample studied: CT-26 colon carcinoma cell line Methods: Flow cytometry for CXCR4 and murine injection	CXCR4 protein expression in CT-26 cells low <i>in vitro</i> , but upregulated after injection and metastasis to murine spleen, liver, or lung; CXCR4-deficient CT-26 cells colonize the lung, but fail to proliferate at the same rate as CXCR4 positive CT-26 cells	The CXCR4/CXCL12 axis may be relevant in the growth of colon carcinoma. CXCR4 expression may be influenced by tumor microenvironment.
Hwang <i>et al.</i> 2003	Samples studied: Thyroid carcinoma cell lines Methods: Flow cytometry for CXCR4 protein and <i>in vitro</i> migration assay	CXCR4 protein expression present in cell lines; CXCL12 induces migration of cell lines	The CXCR4/CXCL12 axis may direct malignant cell migration, contributing to the metastatic cascade of thyroid carcinoma.
Spano <i>et al.</i> 2004	Sample studied: Non-small cell lung carcinoma Method: IHC for	CXCR4 protein expression correlates with patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with

	CXCR4 protein		non-small cell lung carcinoma.
Russell <i>et al.</i> 2004	Sample studied: Neuroblastoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with tumor staging and presence of bone marrow metastasis	The CXCR4/CXCL12 may contribute to the metastatic cascade, particularly metastasis at the bone marrow of neuroblastoma patients. CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with neuroblastoma.
Gockel <i>et al.</i> 2006	Samples studied: Esophageal squamous cell carcinoma and esophageal adenocarcinoma Method: IHC for CXCR4 protein	No significant association between CXCR4 protein expression and patient morbidity in esophageal squamous cell carcinoma; CXCR4 protein expression correlates with risk of morbidity in esophageal adenocarcinoma	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with esophageal squamous cell carcinoma or esophageal adenocarcinoma.
Yang <i>et al.</i> 2007	Sample studied: Cervical adenocarcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with metastatic development and patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with cervical adenocarcinoma.
Uchida <i>et al.</i> 2007	Sample studied: Oral squamous cell carcinoma Method: IHC for CXCR4 protein	CXCR4 and CXCL12 protein expressions correlate to patient morbidity	CXCR4 and CXCL12 protein expressions may be of utility as a prognosticator for poor outcome in patients with oral squamous cell carcinoma.
Spoo <i>et al.</i> 2007	Sample studied: Acute myeloid leukemia Method: Flow cytometry for CXCR4 protein	Patients with low CXCR4 expression displayed significantly longer survival compared to patients with medium and high expression	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with acute myeloid leukemia.
Konoplev <i>et al.</i> 2007	Sample studied: Acute myeloid leukemia Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with acute myeloid leukemia.
Kodama <i>et al.</i>	Sample studied:	CXCR4 protein	CXCR4 protein expression

2007	Cervical squamous cell carcinoma Method: IHC for CXCR4 protein	expression correlates with the following: tumor staging, tumor size ≥ 4 mm, stromal invasion, absence of a brisk host response, and presence lymph node metastasis	may be of utility as a prognosticator for poor outcome in patients with cervical squamous cell carcinoma.
Zhang <i>et al.</i> 2007	Samples studied: Cervical squamous cell carcinoma and Hela squamous cell carcinoma cell line Methods: IHC for CXCR4 and CXCL12 protein, flow cytometry for CXCR4 protein and <i>in vitro</i> migration assay	CXCR4 protein expression present in cervical squamous cell carcinoma and CXCL12 protein expression present in corresponding lymph nodes; Hela cells express CXCR4 protein and migrate along a CXCL12 gradient	The CXCR4/CXCL12 axis may contribute to the metastatic cascade of cervical squamous cell carcinoma.
Tan <i>et al.</i> 2008	Sample studied: Laryngeal squamous cell carcinoma Methods: RT-PCR for CXCR4 mRNA and IHC for CXCR4 protein	CXCR4 mRNA and protein correlates to the development of metastasis	CXCR4 mRNA and protein expressions may be of utility as a prognosticator for poor outcome in patients with laryngeal squamous cell carcinoma.
Maréchal <i>et al.</i> 2009	Sample studied: Pancreatic adenocarcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with the presence of lymph node metastasis and liver recurrence	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with pancreatic adenocarcinoma.
Sasaki <i>et al.</i> 2009	Sample studied: Esophageal squamous cell carcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression does not correlate with patient morbidity or established histopathologic prognosticators.	CXCR4 protein expression does not appear to be of utility as a prognosticator for poor outcome in patients with esophageal squamous cell carcinoma.
Wang <i>et al.</i> 2009	Sample studied: Esophageal squamous cell carcinoma Method: IHC for	CXCR4 protein expression correlates with tumor staging.	The CXCR4/CXCL12 axis may be relevant in the progression of esophageal squamous cell carcinoma. CXCR4 protein expression

	CXCR4 protein		may be of utility as a prognosticator for poor outcome in patients with esophageal squamous cell carcinoma.
Wagner <i>et al.</i> 2009	Sample studied: Non-small cell lung adenocarcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with metastatic development and patient morbidity.	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with non-small cell lung adenocarcinoma.
He <i>et al.</i> 2010	Samples studied: Benign and malignant thyroid lesions Method: IHC for CXCR4 protein	CXCR4 protein expression is upregulated in malignant lesions as compared to benign lesions; CXCR4 protein expression is greater in “more” malignant (poorly differentiated and medullary) than “less” malignant (papillary and follicular) thyroid carcinomas	The CXCR4/CXCL12 axis may be relevant to the development of a malignant phenotype in of thyroid lesions.
Sun <i>et al.</i> 2010	Sample studied: Chondrosarcoma Methods: Flow cytometry for CXCR4 protein and <i>in vitro</i> migration assay	CXCR4 protein expression is present; CXCL12 induces migration; CXCL12 migratory effects can be abrogated with CXCR4 antagonists and MAPK inhibitors.	The CXCR4/CXCL12 axis may be relevant to the metastatic cascade of chondrosarcoma. The CXCR4/CXCL12 may be a viable target for treatment of chondrosarcoma.
Uchida <i>et al.</i> 2011	Sample studied: B88 oral squamous cell carcinoma cell line Method: murine injection	Abrogation of metastatic development when co-injection with the CXCR4 antagonist AMD3100	The CXCR4/CXCL12 may be a viable target for treatment of oral squamous cell carcinoma.
Zheng <i>et al.</i> 2011	Sample studied: Laryngeal squamous cell carcinoma Methods: RT-PCR for CXCR4 mRNA and IHC for CXCR4 protein	CXCR4 mRNA and protein correlates with the following: tumor stage, presence of metastasis, and micro-vascularization	CXCR4 mRNA and protein expressions may be of utility as a prognosticator for poor outcome in patients with laryngeal squamous cell carcinoma.

Zhang <i>et al.</i> 2012	Sample studied: Laryngeal squamous cell carcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with clinical stage and presence of metastasis.	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with laryngeal squamous cell carcinoma.
Wang <i>et al.</i> 2012	Sample studied: Clear cell renal carcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with clear cell renal carcinoma.
Torregrossa <i>et al.</i> 2012	Sample studied: Papillary thyroid carcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with tumoral infiltration and presence of a BRAF mutation	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with papillary thyroid carcinoma.
Berghuis <i>et al.</i> 2012	Samples studied: Ewing carcinoma cell lines Methods: Flow cytometry for CXCR4 protein, <i>in vitro</i> migration assay and proliferation assay	CXCR4 protein expression is present; CXCL12 induces cell proliferation but not migration; CXCR4 protein expression correlates with patient morbidity	The CXCR4/CXCL12 axis may be relevant in the growth and development of Ewing sarcoma. CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with Ewing sarcoma.
Ma <i>et al.</i> 2012	Sample studied: Osteosarcoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with osteosarcoma.
Lu <i>et al.</i> 2013	Samples studied: Esophageal squamous cell carcinoma and metastasis, Ec109 esophageal squamous cell carcinoma cell line Methods: IHC for CXCR4 protein, flow cytometry for CXCR4 protein and <i>in vitro</i> migration assay	CXCR4 protein expression greater in metastasis than primary oral squamous cell carcinoma; higher CXCR4 protein expression correlates with enhanced migratory response to CXCL12	The CXCR4/CXCL12 axis may be relevant in the progression and metastasis of esophageal squamous cell carcinoma.

Bao <i>et al.</i> 2013	Sample studied: Multiple myeloma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with patient survival	CXCR4 protein expression may be of utility as a prognosticator for good outcome in patients with multiple myeloma.
Huang <i>et al.</i> 2013	Sample studied: Cervical squamous cell carcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with tumor histologic grade.	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with cervical squamous cell carcinoma.
Xu <i>et al.</i> 2013	Sample studied: Breast carcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with breast carcinoma.
Lv <i>et al.</i> 2014	Sample studied: Colon carcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor outcome in patients with colon carcinoma.

IHC=immunohistochemistry

1.5.3 CXCR4/CXCL12 in cutaneous non-melanoma malignancies

Both CXCR4 and its ligand CXCL12 appear to be involved in the progression and metastasis of cutaneous malignancies. In a study of the precancerous lesion actinic keratosis, basal cell carcinoma and cutaneous squamous cell carcinoma researchers demonstrated the presence of CXCR4 protein; however, compared to normal skin, expression appeared to be downregulated in actinic keratosis and basal cell carcinoma, while cutaneous squamous cell carcinoma exhibited upregulated expression (Basile 2008). Given the limited metastatic potential of basal cell carcinoma compared to squamous cell carcinoma, this study supports the notion that CXCR4 expression is

associated with neoplasms of more aggressive phenotype (Basile 2008). In the same year, a study showed the presence of CXCR4 protein expression in head and neck squamous cell carcinoma and noted the absence of expression in adjacent normal tissue (Ou 2008). This study demonstrated a correlation between CXCR4 protein expression and tumor staging, supporting the predictive value of CXCR4 for poor patient outcome (Ou 2008).

In a study of cells derived from malignant peripheral nerve sheath tumors (MPNST), researchers demonstrated that the CXCR4/CXCL12 axis promotes cell survival and metastasis (Mo 2013). Briefly, murine injection of CXCR4-negative MPNST cells resulted in impaired *in vivo* tumor cell proliferation and attenuated tumorigenesis compared to injection with CXCR4-positive MPNST cells (Mo 2013). Interestingly, co-injection of the CXCR4 antagonist AMD3100 and CXCR4-positive MPNST cells resulted in tumorigenesis similar to that of the CXCR4-negative MPNST cells (Mo et al., 2013). This same study demonstrated CXCR4 protein expression in human MPNSTs using immunohistochemistry (Mo 2013). Based upon these findings Mo *et al.* hypothesized that the CXCR4/CXCL12 axis may be relevant in the progression of MPNST and may be of utility as a therapeutic target (Mo 2013). In 2006 Tucci *et al.* failed to demonstrate CXCR4 protein expression in Merkel cell carcinoma; however, a 2012 study by Knapp *et al.* successfully demonstrated CXCR4 protein expression and noted statistically significant greater CXCR4 immunoreactivity in local nodal Merkel cell carcinoma metastasis biopsies compared to primary and

distant metastatic biopsies, leading the authors to conclude that CXCR4 is particularly crucial in the early stages of Merkel cell carcinoma progression (Tucci 2006; Knapp 2012). These studies implicate the CXCR4/CXCL12 axis in the progression and metastasis of non-melanoma cutaneous malignancies and indicate CXCR4 as a potential prognosticator for poor outcome and target for therapy.

Table 2. Chronologic historical overview of studies on CXCR4 in cutaneous non-melanoma malignancies

REFERENCE	STUDY DESIGN	FINDINGS	CONCLUSION
Tucci <i>et al.</i> 2006	Sample studied: Merkel cell carcinoma Method: IHC for CXCR4 protein	CXCR4 protein expression not detected.	The CXCR4/CXCL12 axis does not appear to be relevant in the etiopathogenesis of Merkel cell carcinoma.
Basile <i>et al.</i> 2008	Samples studied: Actinic keratosis, basal cell carcinoma, cutaneous squamous cell carcinoma Method: IHC for CXCR4 protein	Compared to normal skin, CXCR4 protein expression was downregulated in actinic keratosis and basal cell carcinoma, while expression was upregulated in cutaneous squamous cell carcinoma	CXCR4 protein expression may be associated with cutaneous malignancies with potential for metastasis.
Ou <i>et al.</i> 2008	Samples studied: squamous cell carcinoma (head and neck area) Method: IHC for CXCR4 protein	CXCR4 protein expression correlates with clinical stage and presence of lymph node metastasis.	CXCR4 protein expression may be of utility as a prognosticator for poor patient outcome in head and neck squamous cell carcinoma.
Toyozowa <i>et al.</i> 2010	Samples studied: dermatofibrosarcoma protuberance, malignant fibrous histiocytoma, dermatofibroma Method: IHC for CXCR4 protein	CXCR4 protein expression higher in dermatofibrosarcoma and malignant fibrous histiocytoma compared to dermatofibroma; CXCR4 protein expression higher in dematofibrosarcoma patients who relapsed patients	CXCR4 protein expression may be of utility as a predictor of phenotypically aggressive cutaneous fibrohistocytic tumors.

Knapp <i>et al.</i> 2012	Samples studied: Merkel cell carcinoma, local nodal metastasis and distant metastasis Method: IHC for CXCR4 protein	Higher expression in local nodal metastasis compared to primary and distant metastasis	CXCR4 expression may be associated with the progression of Merkel cell carcinoma, particularly early stages of metastasis.
Mo <i>et al.</i> 2013	Samples studied: malignant peripheral nerve sheath tumor (MPNST) cell lines and primary tumor Methods: <i>In vivo</i> murine injection of MPNST cells, RT-PCR for CXCL12 mRNA and IHC for CXCR4 protein	MPNST cells with CXCR4 knockdown displayed impaired <i>in</i> <i>vivo</i> tumor cell proliferation and attenuated tumorigenesis, as compared to injection with CXCR4 positive MPNST cells in murine models; CXCL12 highly expressed at sites of metastasis in murine models; administration of CXCR4 antagonist elicits abrogation of MPNST proliferation and metastasis in murine models; CXCR4 protein present in human MPNSTs	The CXCR4/CXCL12 axis may play a role in MPNST progression and metastasis. CXCR4 may be a putative target in treatment of MPNST.

IHC=immunohistochemistry

1.5.4 CXCR4/CXCL12 in melanoma

The highly metastatic and variable behavior of melanoma has accentuated the need for early detection and targeted therapy. Expression of CXCR4 in melanoma was initially demonstrated in biopsies of primary cutaneous melanoma, melanoma metastatic to the lymph node and melanoma cell lines using multiple techniques that included immunohistochemistry and flow cytometry (Robledo 2001). In this study CXCR4 protein expression was noted in 100% of biopsies of primary cutaneous melanoma as well as metastasis ($n= 7$ and 5 respectively) and the melanoma cell lines MeWo, A375 and BLM,

indicating that the CXCR4/CXCL12 axis may be relevant in melanoma. This same study showed that CXCL12 greatly enhanced MeWo cell adhesion to the extracellular matrix protein fibronectin, facilitating the invasion of normal tissue by malignant cells (Robledo 2001). In 2001 Robledo *et al.* also showed that *in vitro* administration of CXCL12 induced phosphorylation of the MAP kinases p44/42 and p38 (downstream of the Raf protein) indicating that CXCL12 could potentially be relevant in melanomagenesis (Robledo 2001). A year later Murakami *et al.* similarly demonstrated CXCR4 expression in 100% ($n=3$) of primary cutaneous melanoma biopsies and 40% ($n=5$) of pulmonary metastasis biopsies (Murakami 2002). In the same study Murakami *et al.* transduced CXCR4 cDNA into B16 melanoma cells and demonstrated that increasing CXCR4 protein expression resulted in significantly enhanced development of pulmonary metastasis in mice (Murakami 2002). Murakami *et al.* showed that administration of the CXCR4 antagonists T22 inhibited the formation of metastasis, highlighting the potential therapeutic value of targeting the CXCR4/CXCL12 axis (Murakami 2002). This study also showed that *in vitro* administration of CXCL12 enhanced B16 melanoma cell adhesion to pulmonary endothelial cells and promoted cell growth, identifying the CXCR4/CXCL12 axis as a potential contributor to melanomagenesis and the development of metastasis (Murakami 2002).

Following the early findings of Robledo *et al.* and Murakami *et al.* were a number of studies demonstrating the prognostic utility of CXCR4 protein expression in primary cutaneous melanoma (Longo-Imedio 2005; Scala 2005;

Tucci 2007; Toyozawa 2012). Briefly, Longo-Imedio *et al.*, demonstrated CXCR4 protein expression in 35% ($n=40$) of primary cutaneous melanoma and showed that CXCR4 expression correlated with ulceration, increased tumor thickness, development of metastases, and patient morbidity (Longo-Imedio 2005). This same year Scala *et al.*, correlated CXCR4 expression (56% positive, $n=71$) with shorter disease free survival and greater risk of morbidity in study patients with primary cutaneous melanoma (Scala 2005). Two years later Tucci *et al.* identified CXCR4 protein expression in 100% ($n=30$) of nodular melanoma biopsies and showed that expression correlated with greater Breslow depth and patient morbidity, supporting a role for the CXCR4/CXCL12 axis in melanoma growth and, the utility of CXCR4 as a prognosticator for poor patient outcome (Tucci 2007). Most recently, Toyozawa *et al.* demonstrated CXCR4 protein expression in 100% ($n=19$) of primary cutaneous melanoma cases and noted an association between expression and tumor thickness >2 mm as well as the development of distant metastasis, delineating the contribution of the CXCR4/CXCL12 axis to melanoma progression and development of metastases (Toyozawa 2012). Of note, a 2012 study by Kühnelt-Leddihn *et al.* noted CXCR4 protein expression in 82% ($n=38$) of primary cutaneous melanoma, but failed to demonstrate a correlation with established histopathologic prognosticators or patient morbidity (Kühnelt-Leddihn 2012). In addition to primary cutaneous melanoma, a 2012 study highlighted the prognostic value of CXCR4 protein expression in uveal melanoma by correlating expression with the presence of liver metastasis

(Dobner 2012).

Higher expression of CXCR4 mRNA, like that of the protein, has also been identified as a prognosticator of poor outcome in melanoma in two studies (Franco 2010; Monteagudo 2012). In 2010 Franco *et al.* demonstrated high CXCR4 mRNA expression in 91% ($n=23$) of melanoma metastases to the lymph node and demonstrated an association between expression and shorter disease free survival (Franco 2010). Of note, this same study evaluated CXCR4 protein expression in all cases and observed a perfect overlap of mRNA and protein expressions (Franco 2010). More recently, Monteagudo *et al.* found that lower expression of CXCL12 mRNA compared to that of CXCR4 was a valuable prognosticator for the development of metastasis in primary cutaneous melanoma (Monteagudo 2012). The same study showed that CXCL12 mRNA expression, as compared to that of CXCR4 was four times greater in thin (≤ 1 mm) than thick (>1 mm) primary cutaneous melanomas (Monteagudo 2012). Of note, a 2006 study by Kim *et al.* demonstrated CXCR4 mRNA expression in 89% ($n=27$) of melanoma biopsies, but noted the absence of a correlation with patient outcome (Kim 2006).

The aforementioned studies highlight the relevance of the CXCR4/CXCL12 axis in the progression of melanoma as well as its prognostic value. The therapeutic value of targeting this chemokine axis has also been observed in a three studies (Scala 2006; Liang 2012; O'Boyle 2013). In 2006 Scala *et al.* demonstrated abrogation of *in vitro* CXCL12 induced melanoma cell

proliferation by administering the CXCR4 antagonist AMD3100 (Scala 2006). In 2012 Liang *et al.* administered the small molecule CXCR4 inhibitor MSX-122 and observed significant inhibition in uveal melanoma liver metastasis in mice, supporting that the previously observed *in vitro* effects of blocking the CXCR4/CXCL12 axis were maintained in an *in vivo* model (Liang 2012). A year later O’Boyle *et al.* compared the migratory response to CXCL12 of CHL-1 melanoma cells transfected with either *BRAF*^{WT} or *BRAF*^{V600E} and found that migration was enhanced by *BRAF*^{V600E}, but could be inhibited by the newly synthesized CXCR4 antagonist AMD11070, indicating that targeting of CXCR4/CXCL12 axis may be therapeutically relevant (O’Boyle 2013). Further highlighting the clinical relevance of the CXCR4/CXCL12 axis is a 2010 paper by Kim *et al.* which demonstrated that concomitant use of a CXCR4 antagonist and dacarbazine treatment on chemoresistant CD133⁺ melanoma cells effectively blocked tumor metastasis in mice (Kim 2010).

Table 3. Chronologic historical overview of studies on CXCR4 in melanoma

REFERENCE	STUDY DESIGN	FINDINGS	CONCLUSION
Robledo <i>et al.</i> 2001	<p>Samples studied: Melanoma cell lines, primary cutaneous melanoma and metastasis</p> <p>Method: Flow cytometry for CXCR4 protein, IHC for CXCR4 protein, <i>in vitro</i> cell adhesion assay and proliferation assay</p>	CXCR4 protein is present on melanoma cell lines and melanoma metastases; CXCL12 induces enhanced fibronectin adhesion and phosphorylation of MAPK kinases.	The CXCR4/CXCL12 axis may stimulate melanoma cell proliferation and invasion, contributing to melanoma progression and the development of metastases.

Murakami <i>et al.</i> 2002	<p>Samples studied: B16 melanoma cell line, primary cutaneous melanoma and pulmonary metastases</p> <p>Methods: Murine injection with CXCR4 positive and CXCR4 negative melanoma cells and IHC for CXCR4 protein</p>	CXCR4 expression enhances pulmonary metastatic potential of melanoma cells in a murine model; CXCR4 is expressed on primary cutaneous melanoma and pulmonary metastasis	The CXCR4/CXCL12 axis contributes to the metastatic capacity of melanoma cells in mice and expression is present in human melanoma.
Longo-Imedio <i>et al.</i> 2005	<p>Sample studied: Primary cutaneous melanoma</p> <p>Method: IHC for CXCR4 protein</p>	CXCR4 protein expression correlates to the following: presence of ulceration, tumor thickness, development of lymph node metastasis, presence of distant metastasis, patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor patient outcome.
Scala <i>et al.</i> 2005	<p>Sample studied: Primary cutaneous melanoma with Breslow thickness >1 mm</p> <p>Method: IHC for CXCR4 protein</p>	CXCR4 protein expression correlates to presence of sentinel lymph node metastasis and patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor patient outcome.
Scala <i>et al.</i> 2006	<p>Sample studied: Melanoma cell lines and melanoma metastases</p> <p>Methods: RT-PCR for CXCR4 mRNA, <i>in vitro</i> proliferation assays and IHC for CXCR4 protein</p>	CXCR4 mRNA expressed in melanoma cell lines; CXCL12 induces melanoma cell proliferation and these effects can be abrogated by AMD3100; CXCR4 protein expressed in melanoma metastasis	The CXCR4/CXCL12 axis may stimulate melanoma growth and metastasis. CXCR4 may be a putative target for therapeutic treatment.
Kim <i>et al.</i> 2006	<p>Sample studied: primary cutaneous melanoma</p> <p>Method: RT-PCR for CXCR4 mRNA</p>	No significant correlation observed between overexpression of CXCR4 protein and survival	CXCR4 protein expression does not appear to have prognostic utility in primary cutaneous melanoma.
Tucci <i>et al.</i> 2007	<p>Sample studied: Primary cutaneous melanoma</p> <p>Method: IHC for CXCR4 protein</p>	CXCR4 protein expression correlates to Breslow thickness and patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor patient outcome.

Dobner <i>et al.</i> 2009	Sample studied: Primary uveal melanoma Methods: IHC for CXCR4 and CXCL12 protein	CXCR4 protein expression substantially greater than CXCL12 expression in primary tumor; correlation between CXCR4 expression and development of hepatic metastasis	The CXCR4/CXCL12 axis directs uveal melanoma cells to site of metastasis by a CXCL12 concentration gradient. CXCR4 may have prognostic value.
Franco <i>et al.</i> 2010	Sample studied: Primary cutaneous melanoma Methods: RT-PCR for CXCR4 mRNA and IHC for CXCR4 protein	CXCR4 mRNA and protein expression correlate to patient morbidity	CXCR4 protein expression may be of utility as a prognosticator for poor patient outcome.
Kühnelt-Leddihn <i>et al.</i> 2012	Sample studied: Primary cutaneous melanoma Method: IHC for CXCR4	CXCR4 protein expression does not correlate with prognosis or survival.	CXCR4 protein expression does not appear to have prognostic utility.
Monteagudo <i>et al.</i> 2012	Samples studied: Primary cutaneous melanoma and melanoma metastasis Methods: RT-PCR for CXCR4 and CXCL12 mRNA	Low CXCL12/CXCR4 ratio correlates with tumor thickness >1 mm and the development of metastasis	The CXCR4/CXCL12 axis may contribute to melanoma progression and metastasis. CXCL12/CXCR4 mRNA ratio expression may be of utility as a prognosticator for poor patient outcome.
Toyozawa <i>et al.</i> 2012	Sample studied: Primary cutaneous melanoma Method: IHC for CXCR4 and CXCL12 protein	CXCR4 protein expression correlates to the following: tumor thickness, development of distant metastasis, and CXCL12 protein expression.	CXCR4 and CXCL12 protein expressions may be of utility as prognosticators for poor patient outcome.
O'Boyle <i>et al.</i> 2013	Samples studied: BRAFWT and BRAF V600E melanoma cell lines Method: <i>In vitro</i> migration assay	Cell migration enhanced by BRAF V600E transfection and inhibited by the CXCR4 antagonist AMD11070	CXCR4 may be a putative target for therapeutic treatment.

IHC=immunohistochemistry

1.6 Aims/Objectives

As mentioned previously, one of the mechanisms by which the

CXCR4/CXCL12 axis has been shown to activate melanoma cell cycle progression is *via* stimulation of the MAPK pathway. While previous studies have delineated a role for the CXCR4/CXCL12 axis in melanoma metastasis and patient survival, the potential cooperativity with other known prognosticators has yet to be explored. Given this, in the current study, questions we sought answers to were the following:

- Is there a correlation between CXCR4 and established histopathologic prognosticators and/or the mutational status of the MAPK BRAF protein in primary cutaneous melanoma?
- Is there a correlation between CXCL12 and established histopathologic prognosticators and/or the mutational status of the MAPK BRAF protein in primary cutaneous melanoma?
- Is there a correlation between CXCR4 and its ligand CCL12?
- What is the correlation between CXCR4 expression by gene expression (mRNA RT-PCR) *versus* expression of the protein by immunohistochemistry?

MATERIALS AND METHODS

2.1 Sample Selection

This study was approved by the Boston University School of Medicine institutional review board (IRB # 478076). Formalin-fixed, paraffin-embedded tissues with a diagnosis of primary cutaneous melanoma ($n=107$) were retrieved from the archives of the Skin Pathology Laboratory, Boston University School of Medicine, Boston, MA, USA. Histopathologic sections of all cases were reviewed by two board-certified dermatopathologists (initial sign-out on all by a Board certified dermatopathologist; cases were then re-reviewed, and the diagnoses were confirmed by the senior author). All patient data were de-identified. Demographics of the patients included in the study are detailed in Table 4.

Table 4. Summary of patient demographics

Age	67 years (mean), range 19-103 years
Gender	
Male	72 (67%)
Female	35 (33%)
Site	
Upper extremities	31 (29%)
Lower extremities	22 (21%)
Trunk	27 (25%)
Head and Neck	27 (25%)
Thickness	1.75 mm (mean), range 0.2-10.4 mm
<1mm	44 (41%)
1-2mm	30 (28%)
>2-4mm	21 (20%)
>4mm	12 (11%)
Mitosis	
Present	69 (64%)
Absent	38 (36%)
Tumor Infiltrating Lymphocytes (TIL)	
Present	22 (21%)
Absent	85 (79%)
Ulceration	
Present	23 (21.5%)
Absent	84 (78.5%)
Regression	
Present	28 (26%)
Absent	79 (74%)
Vascular invasion (H&E)	
Present	2 (2%)
Absent	105 (98%)
AJCC Pathology Staging	
T1a	24 (22%)
T1b	18 (17%)
T2a	28 (26%)
T2b	7 (6.5%)
T3a	13 (12%)
T3b	7 (6.5%)
T4a	4 (4%)
T4b	6 (6%)

2.2 DNA Analyses

Five 10-um sections were cut from formalin-fixed, paraffin-embedded archival tissue blocks using a micrometer and placed in eppendorf tubes. For deparaffinization samples were incubated at 60°C in 750 μ l xylene for 5 minutes, centrifuged at 14000 rotations per minute for 2 minutes, the supernatant was discarded, incubated at 60°C in 750 μ l ethanol for 5 minutes, centrifuged at 14000 rotations per minute for 2 minutes and the supernatant was discarded. For separation of DNA and RNA from this mixture samples were resuspended in a mixture of 150 μ l Buffer PKD (Qiagen, Valencia, CA, USA) and 10 μ l proteinase K, incubated at 56°C for 15 minutes, placed on ice for 3 minutes, and centrifuged at 14000 rotations per minute for 15 minutes. The RNA containing supernatant was removed for PCR analysis (see section 2.3 RNA Analyses). Depending on sample size 15-25 μ l Proteinase K were added to the remaining pellet and digested at 55°C overnight. The crude DNA containing pellet was boiled for 10 minutes, quantified by spectrophotometer (Table 2) and diluted to the appropriate concentration (100 ng/ μ l).

For determination of BRAF mutational status 1-2 μ l sample DNA was amplified in a volume of 12.5 μ l containing 200 μ M dNTP, 100-200 nM of primers, 1.5-2.0 mM MgCl₂, 10 mM Tris HCL (pH 8.3), 50 mM KCL and 0.25 μ l of TaqGold polymerase (Applied Biosystems, CA, USA). Cycling was carried out in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, CA USA) as follows: 5 minutes at 94°C; 45 cycles of 94°C for 30 seconds, 55°C for 30

seconds, 72°C for 30 seconds with a final extension step of 5 minutes at 72°C. PCR products were visualized on a 2-3% agarose gel. Sanger sequencing was performed on *BRAF* gene exon 15 with forward primer BRAF_X15F 5'-TGCTCTGATAGGAAAATGAGATC-3' and reverse primer BRAF_X15R 5'-CTAGTAACTCAGCAGCATCTCAG-3' and analyzed by the Genetic Analyzer 3130XL. The sequencing results were analyzed with ABI DNA Sequencing Analysis Software version 6. Appropriate positive, negative and no-DNA controls were included in each batch of PCR and sequencing reactions.

2.3 RNA Analyses

Deparaffinization and a simple proteinase K digestion were performed as described in section 2.2 DNA Analyses. RNA extraction was carried out per the AllPrep DNA/RNA Mini Kit (Qiagen). Briefly, the RNA containing supernatant obtained from 2.2 DNA Analyses and was incubated at 80°C for 15 minutes, centrifuged at 14,000 rotations per minute, and mixed with 320 μ l Buffer RLT and 1120 μ l ethanol. Samples were centrifuged through an RNeasy MinElute spin column at 10,000 rotations per minute, the flow-through was discarded and these steps were repeated with 350 μ l Buffer FRN. 80 μ l DNase I incubation mix (10 μ l DNase I and 70 μ l Buffer RDD) were added to the RNeasy column membrane and after 15 minutes 500 μ l Buffer FRN were added to each column. Each MinElute spin column was centrifuged at 10,000 rotations per minute for 15 seconds and placed in a new 2 ml collection tube for a second centrifugation.

Buffer RPE was added and centrifuged through twice in 500 μ l aliquots and the RNeasy MinElute spin column was placed in a new 1.5 mL collection tube. 25 μ l RNase-free water were added to the spin column membrane and incubated for 1 minute at room temperature before being centrifuged at full speed for 1 minute. A nanodrop was used to quantify RNA content in each sample (Table 3).

Based upon total highest RNA content 96 samples were selected for cDNA synthesis and RT-PCR analysis. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, Massachusetts, USA) was used for converting RNA to cDNA. Briefly, approximately 2 μ g RNA were added to a nuclease-free tube along with 1 μ l random hexamer primer and the appropriate volume of nuclease-free water to bring the total volume to 12 μ l. Added to this solution were 4 μ l 5X Reaction Buffer, 1 μ l RiboLock RNase Inhibitor, 2 μ l 10 mM dNTP Mix, and 1 μ l RevertAid M-MuLV Reverse Transcriptase. Samples were incubated as follows: 5 minutes at 25°C, 60 minutes at 42°C, and 5 minutes at 70°C.

Real time-PCR was performed using the ABI 7900HT Fast Real-Time PCR instrument. Levels of CXCR4 mRNA were assessed in reference to the internal positive control beta actin. Each sample was run in duplicates for both CXCR4 (Qiagen) and beta actin (Qiagen). All samples were analyzed in the RT² SYBR Green ROX qPCR Mastermix (Qiagen). In order to normalize CXCR4 mRNA expression across each sample a Δ Ct was calculated by using the equation $Ct_{CXCR4} - Ct_{\text{beta actin}}$. Briefly, higher Δ Ct values are indicative of lower

mRNA expression.

2.4 Immunohistochemistry

Immunohistochemistry was performed on 4 μ m formalin-fixed, paraffin-embedded sections using commercially available rabbit polyclonal antibodies anti-CXCR4 (ab2074 Abcam, Cambridge, MA, USA) and anti-SDF-1 alpha (anti-CXCL12) (ab9797 Abcam, Cambridge, MA, USA) at a dilution of 1:500. Target retrieval using low pH Target Retrieval solution (DAKO, Carpinteria, CA, USA) was performed at 97°C for 20 min. The slides were treated with dual endogenous enzyme block (DAKO) before primary antibody staining and overnight incubation at 4°C. The remaining steps were carried out using the DAKO Autostainer Plus (DAKO). Color development and contrast were achieved using DAB and hematoxylin, respectively. For all immunohistochemical stains used in the study appropriate positive and negative controls were included with each run. All stained slides were reviewed and scored by the first author (BM) and the senior author (MM) in a blinded fashion with respect to each other's scores. Any disagreements were reviewed together to achieve a consensus score.

For CXCR4, cytoplasmic staining of suprabasal keratinocytes, membrane staining of eccrine glands, and cytoplasmic staining of lymphocytes served as the positive internal controls in each case where they could be visualized. A normal spleen sample was used as a positive control for each staining batch. For CXCL12, cytoplasmic staining of suprabasal keratinocytes in the epidermis,

cytoplasmic staining of endothelial cells and cytoplasmic staining of mature sebocytes were used as internal positive controls for CXCL12 in each case where they could be visualized. A rectal carcinoma sample was used as a positive control for each staining batch.

For both stains, a semi-quantitative scoring system was utilized with the following cut-offs: 0= \leq 1%, 1=1-10%, 2= \geq 10-50% and 3= \geq 50%. Cells were considered positive when the intensity of staining was equal to or greater than staining of the internal positive controls. For purposes of statistical analyses cases with \geq 10% or a score of 2 or more were considered positive.

2.5 Statistical analyses

Univariate analysis of CXCR4 at the protein level (immunohistochemical staining) and RNA level (RT-PCR) was used to inform variable selection for multivariable analysis and to identify potential confounders. Significant differences among subgroups for each clinical factor were examined using a difference of proportions for protein expression and t-test for difference of mean RNA expression. Differences in RNA expression among AJCC stages were analyzed using a Welch adjustment to ANOVA with stage 2 as the reference.

Clinical factors that showed a significant difference in the presence of CXCR4 on either the protein or RNA level were then analyzed by multivariable logistic regression to assess for the association with prognosticators. Depth of the tumor was fully captured by AJCC stage 1 and dropped from the

multivariable model.

Adjusting for multiple comparisons in the analyses of both protein and RNA expression did not alter covariate selection for logistic regression at the 0.20 level for BRAF, AJCC staging, mitosis, host response, ulceration, or regression. Both Hochberg, and the more conservative Bonferroni method, were used to adjust p-values for the 14 immunohistochemical and 7 mRNA expression analyses. The results were comparable, except for stage 2 AJCC and Regression variables, for which Hochberg but not Bonferroni were <0.20 . Therefore to reduce the probability of a type II error, we relied on Hochberg's method to adjust p-values and guide multivariable selection.

RESULTS

3.1 DNA extraction and BRAF status

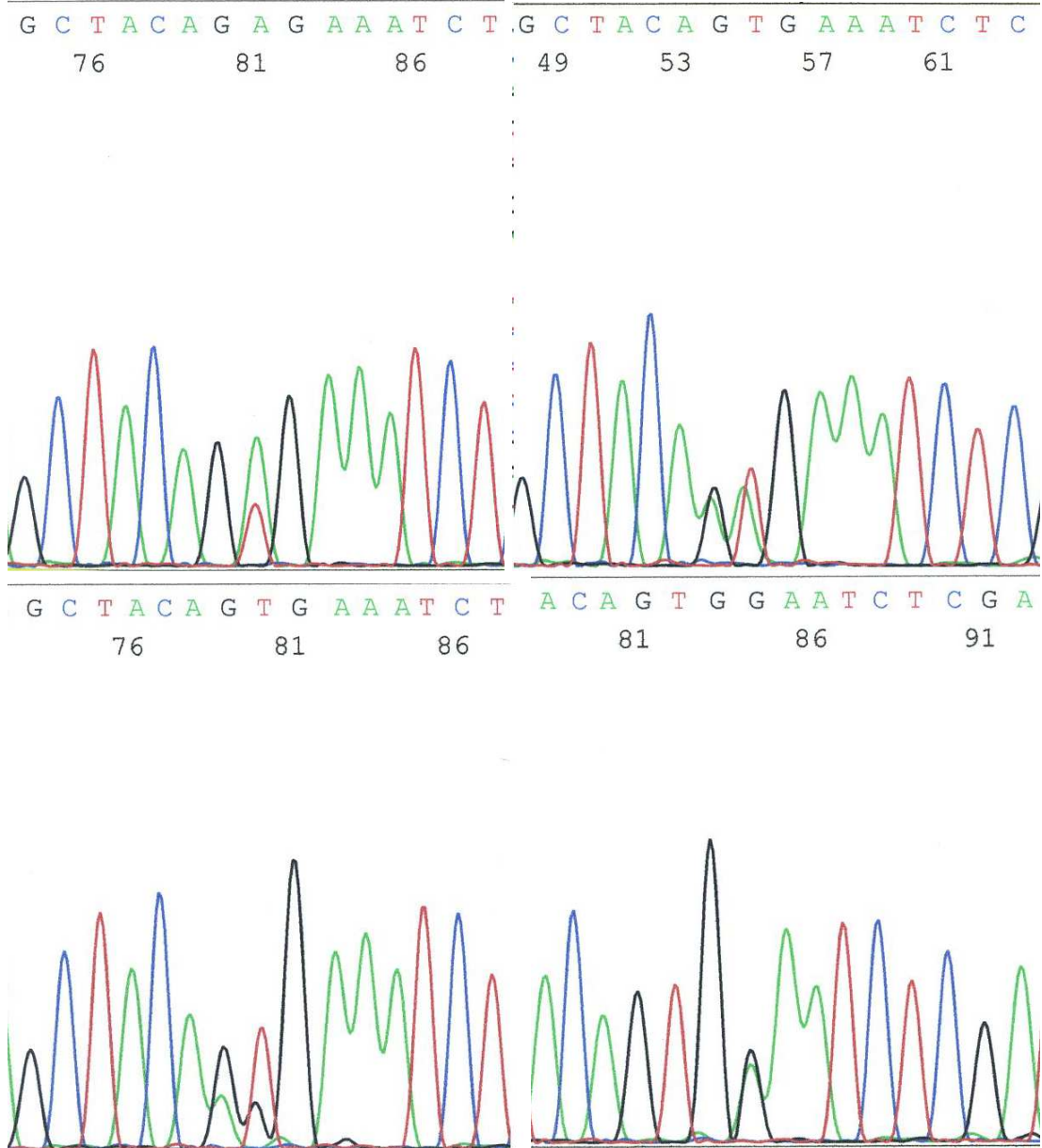
DNA extraction and determination of BRAF mutation status were successful in all 107 samples analyzed. A *BRAF* mutation was observed in 26/107 (24.3%) of cases. These included mutations in *BRAF*^{V600E} ($n=18$; cases K3, K5, K9, K10, K11, K16, K36, K44, K45, K57, K60, K65, K68, K71, K73, K80, K87 and K89), *L597S* ($n=2$; cases K6 and K67), *S607F* ($n=1$; case K13), *K601E* ($n=1$; case K85), *V600K* ($n=3$; cases K51, 79 and K81), and *V600R* ($n=1$; case 93) (Table 5 and Figure 2).

Table 5. DNA extraction and BRAF status

Case	DNA (ng/ μ l)	BRAF Status	Case	DNA (ng/ μ l)	BRAF Status	Case	DNA (ng/ μ l)	BRAF Status
K1	165	WT	K38	170	WT	K74	1355	WT
K2	1640	WT	K39	435	WT	K75	595	WT
K3	2030	V600E	K40	305	WT	K76	865	WT
K4	540	WT	K41	210	WT	K77	255	WT
K5	1376	V600E	K42	950	WT	K78	500	WT
K6	12100	L597S	K43	320	WT	K79	480	V600K
K7	285	WT	K44	1620	V600E	K80	1155	V600E
K8	155	WT	K45	475	V600E	K81	1455	V600K
K9	210	V600E	K46	150	WT	K82	2575	WT
K10	124	V600E	K47	325	WT	K83	1190	WT
K11	355	V600E	K48	230	WT	K84	310	WT
K12	195	WT	K49	500	WT	K85	1125	K601E
K13	380	S607F	K50	590	WT	K86	135	WT
K14	855	WT	K51	185	V600K	K87	180	V600E
K15	125	WT	K52	630	WT	K88	270	WT
K16	205	V600E	K53	1190	WT	K89	955	V600E
K17	1565	WT	K54	1325	WT	K90	335	WT
K18	330	WT	K55	2950	WT	K91	850	WT
K19	340	WT	K56	655	WT	K92	1110	WT
K20	425	WT	K57	1575	V600E	K93	750	V600R
K21	2550	WT	K58	365	WT	K94	670	WT
K23	625	WT	K59	410	WT	K95	400	WT
K24	370	WT	K60	225	V600E	K96	320	WT
K25	3430	WT	K61	165	WT	K97	195	WT
K26	120	WT	K62	120	WT	K98	290	WT
K27	190	WT	K63	290	WT	K99	315	WT
K28	160	WT	K64	80	WT	K100	445	WT
K29	60	WT	K65	80	V600E	K101	330	WT
K30	100	WT	K66	130	WT	K102	115	WT
K31	575	WT	K67	165	L597S	K103	170	WT
K32	110	WT	K68	180	V600E	M40	180	WT
K33	230	WT	K69	1245	WT	M41	275	WT
K34	200	WT	K70	690	WT	M42	165	WT
K35	275	WT	K71	645	V600E	M43	240	WT
K36	390	V600E	K72	1295	WT	M44	3470	WT
K37	230	WT	K73	780	V600E			

DNA concentrations determined by spectrophotometry. BRAF status determined by DNA Sanger sequencing. Blue=BRAF wild type. Green=BRAF mutant.

Figure 2: DNA Sanger sequencing



Clockwise from top left: *BRAFV600E*; *BRAFV600K*; *BRAFV600R*; *BRAFV601E*.

3.2 RNA extraction and CXCR4 mRNA expression *via* RT-PCR

RNA extraction was performed on 100 samples and RT-PCR for CXCR4 was performed on the 96 samples yielding the highest RNA content. Of these, 89 samples were successfully analyzed for CXCR4 mRNA content. The mean ΔC_t value for the 89 samples successfully analyzed was 6.37 ± 1.68 (Table 6).

Table 6. RNA extraction and Δ Ct values (CXCR4 mRNA)

Case	RNA (ng/ μ l)	Δ Ct (mean)	Case	RNA (ng/ μ l)	Δ Ct (mean)	Case	RNA (ng/ μ l)	Δ Ct (mean)
K1	75	9.54	K38	43	2.29	K74	794	5.99
K2	1337	4.53	K39	179	6.50	K75	414	6.82
K3	1850	5.37	K40	105	6.31	K76	90	5.73
K4	115	5.32	K41	30	7.20	K77	316	8.84
K5	955	N/A	K42	197	5.17	K78	567	8.36
K6	75	N/A	K43	108	N/A	K79	449	6.37
K7	27	5.28	K44	186	3.28	K80	305	5.48
K8	40	6.13	K45	70	6.48	K81	824	5.09
K9	165	5.00	K46	7	N/A	K82	588	N/A
K10	863	5.85	K47	133	6.44	K83	647	5.51
K11	168	5.42	K48	241	7.48	K84	162	5.17
K12	239	5.46	K49	116	5.72	K85	458	5.63
K13	142	5.63	K50	198	9.91	K86	119	8.21
K14	269	5.86	K51	33	5.57	K87	277	4.59
K15	16	N/A	K52	384	7.46	K88	128	6.11
K16	27	5.13	K53	1147	7.62	K89	644	5.64
K17	331	5.16	K54	986	N/A	K90	76	9.76
K18	127	6.17	K55	270	4.55	K91	149	8.03
K19	35	6.62	K56	600	4.06	K92	348	8.87
K20	106	5.96	K57	1347	N/A	K93	467	7.92
K21	383	5.38	K58	72	6.65	K94	124	8.83
K23	72	7.65	K59	584	N/A	K95	241	6.72
K24	127	6.08	K60	74	5.49	K96	58	6.62
K25	1860	6.48	K61	91	6.55	K97	49	9.82
K26	93	9.04	K62	30	5.64	K98	103	7.43
K27	147	3.91	K63	101	2.90	K99	46	7.79
K28	73	5.98	K64	75	5.46	K100	371	8.10
K29	18	N/A	K65	76	9.86	K101	82	8.14
K30	40	5.75	K66	34	10.69	K102	N/A	N/A
K31	390	6.26	K67	17	N/A	K103	N/A	N/A
K32	35	7.51	K68	143	7.44	M40	N/A	N/A
K33	67	N/A	K69	969	N/A	M41	N/A	N/A
K34	390	6.75	K70	528	7.26	M42	N/A	N/A
K35	286	5.14	K71	565	3.82	M43	N/A	N/A
K36	165	4.09	K72	501	7.14	M44	N/A	N/A
K37	114	3.20	K73	146	6.11			

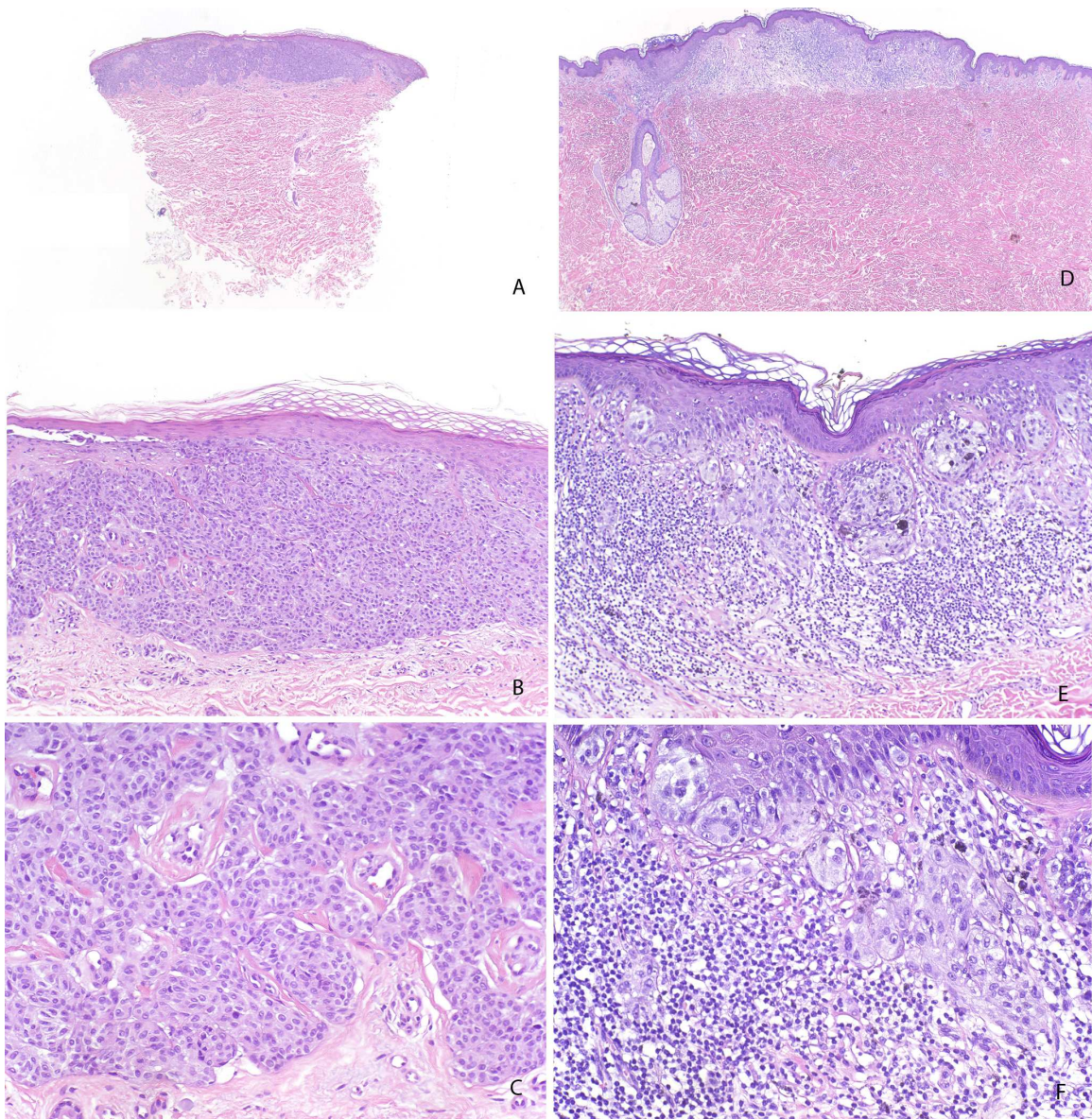
RNA concentrations determined by nanodrop spectrophotometry. Higher Δ Ct values are indicative of lower CXCR4 mRNA expression.

3.3 CXCR4 mRNA expression correlates with select histopathologic prognosticators and BRAF mutational in primary cutaneous melanoma.

Expression of CXCR4 mRNA (mean Δ Ct values) was significantly different among those exhibiting a host response (present=6.63, absent=5.39, $p=0.0003$) (Figure 3) and BRAF mutation (mutant=5.69, wild-type=6.60, $p=0.03$).

Expression of CXCR4 mRNA was also significantly different among the AJCC stages (T1=6.13, T2=7.28, T3=5.94, T4=5.87, $p=0.01$) (Table 7).

Figure 3. Representative examples of cases from study



A-C=Melanoma with absent host response and low CXCR4 mRNA expression ($\Delta\text{Ct}=9.04$). D-F=Melanoma with brisk host response and high CXCR4 mRNA expression ($\Delta\text{Ct}=5.00$).

3.4 Immunohistochemical analyses of CXCR4 and CXCL12 protein expressions

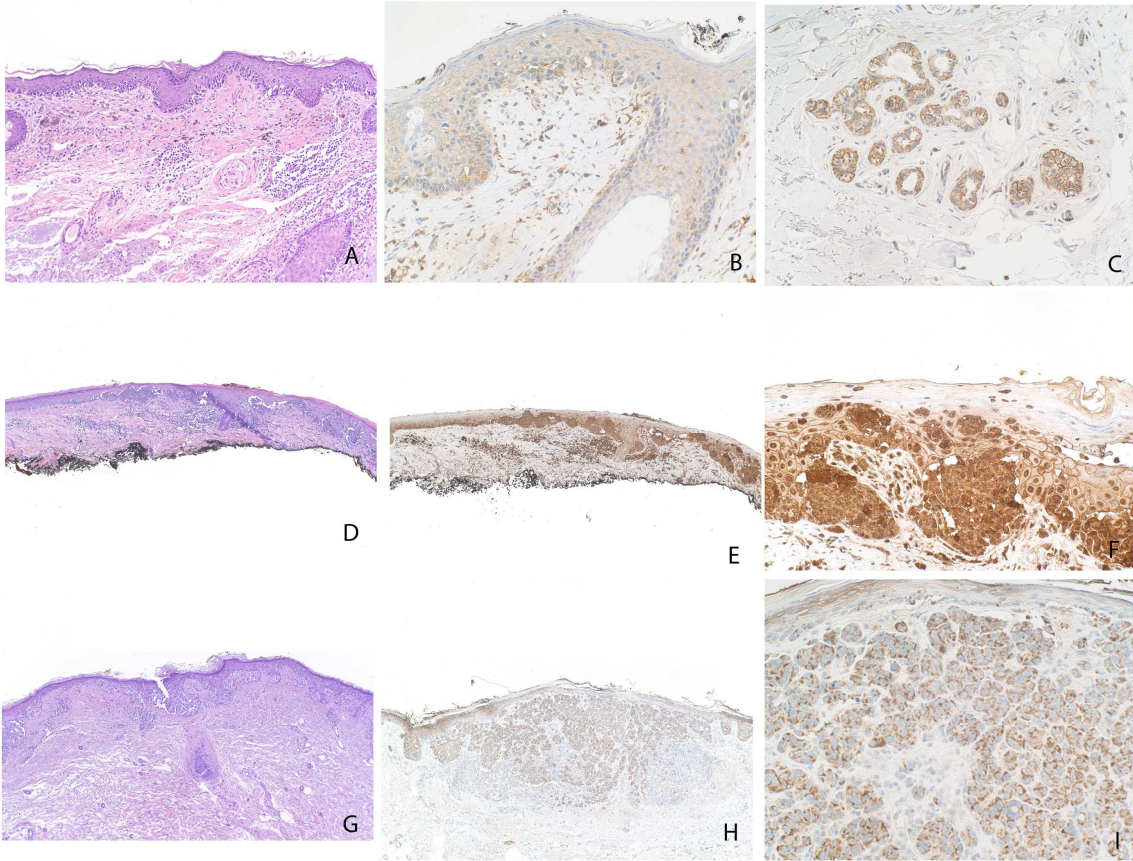
Overall, positive staining for CXCR4 was noted in 59/100 (59%) (7 failed) cases with the following scores: 0=12/100 (12%), 1=29/100 (29%), 2=30/100 (30%), and 3=29/100 (29%). Positive staining for CXCL12 was noted in 35/103 (34%) (4 failed) cases with the following scores: 0=47/103 (46%), 1=21/103 (20%), 2=22/103 (21%), and 3=13/103 (12%) (Table 7, Figure 4).

Table 7. Immunohistochemical staining for CXCR4 and CXCL12

Case	CXCR4	CXCL12	Case	CXCR4	CXCL12	Case	CXCR4	CXCL12
K1	+	+	K38	-	-	K74	+	-
K2	-	-	K39	+	+	K75	-	-
K3	-	+	K40	-	-	K76	-	-
K4	+	-	K41	-	-	K77	+	-
K5	+	+	K42	+	-	K78	-	-
K6	+	+	K43	-	+	K79	-	+
K7	+	+	K44	+	-	K80	-	-
K8	+	-	K45	+	-	K81	+	-
K9	+	-	K46	+	+	K82	-	-
K10	+	+	K47	+	-	K83	-	-
K11	+	-	K48	-	-	K84	+	-
K12	+	-	K49	+	-	K85	-	-
K13	-	-	K50	-	-	K86	+	-
K14	+	+	K51	-	+	K87	-	-
K15	N/A	N/A	K52	-	+	K88	-	-
K16	+	-	K53	-	-	K89	-	-
K17	-	-	K54	+	-	K90	-	+
K18	N/A	N/A	K55	+	+	K91	+	+
K19	N/A	N/A	K56	+	+	K92	+	-
K20	+	+	K57	-	-	K93	+	-
K21	+	+	K58	-	+	K94	+	-
K23	-	+	K59	-	-	K95	-	-
K24	N/A	N/A	K60	+	-	K96	+	-
K25	-	+	K61	+	+	K97	+	+
K26	+	+	K62	N/A	-	K98	-	-
K27	+	-	K63	N/A	-	K99	+	+
K28	+	-	K64	-	-	K100	+	-
K29	+	+	K65	+	-	K101	+	-
K30	+	-	K66	N/A	-	K102	+	-
K31	+	-	K67	+	-	K103	-	-
K32	+	-	K68	+	+	M40	+	-
K33	+	-	K69	-	+	M41	+	-
K34	-	-	K70	-	+	M42	+	-
K35	+	+	K71	+	-	M43	+	-
K36	-	+	K72	-	-	M44	-	+
K37	+	+	K73	-	+			

Blue=CXCR4+/CXCL12+. Green=CXCR4-/CXCL12-. Orange=CXCR4+/CXCL12-. Red=CXCR4-/CXCL12+. N/A=not scored due to absence of lesional area in tissue studied.

Figure 4: Immunohistochemical staining for CXCR4 and CXCL12



A-C=Case 23; A=H&E, 4x, B=CXCR4 20x (score 0), C=eccrine glands (positive internal control). D-F=Case 94; D=H&E, 4x, E=CXCR4 4x (score 3), F=CXCR4 20x. G-I=Case 90; G=H&E, 4x, H=CXC12 4x (score 3), I=CXCL12 20x.

3.5 CXCR4 protein expression correlates with select histopathologic prognosticators and BRAF mutation in primary cutaneous melanoma.

In a univariate analysis, compared with CXCR4 negative samples, the proportion of CXCR4 positive samples was significantly greater in melanomas with absence of the following: mitotic figures ($p=<0.0001$), ulceration ($p=0.0008$), regression ($p=0.02$), vascular invasion ($p=0.01$), and the BRAF mutation ($p=0.02$). A trend towards statistical significance was noted in melanomas with

absence of CXCL12 expression ($p=0.05$). Among samples from patients presenting at shallower AJCC stages (1-2), we found a significantly larger CXCR4 positive proportion compared to patients presenting at deeper stages (3-4) (Table 8, Figure 4).

In a multivariable logistic regression analyses, compared to stage 1, stage 3 (OR=0.16, 95%CI: 0.04–0.68, $p=0.013$) and stage 4 (OR=0.21, 95%CI: 0.03–0.92, $p=0.040$) were significantly associated with lower odds of staining CXCR4 positive. Mitotic figures were also significantly associated with lower odds of staining CXCR4 positive (OR=0.21, 95%CI: 0.06–0.73, $p=0.14$). Although not significant, BRAF mutation (OR=2.31, 95%CI: 0.67–8.01) and host response (OR=2.84, 95%CI: 0.72–11.20) are suggestive of an association (Table 9). Vascular invasion was not analyzed due to the limited number of samples ($n=2$).

3.6 CXCL12 protein expression does not correlate with select histopathologic prognosticators and BRAF mutation in primary cutaneous melanoma

No association was observed between CXCL12, histopathologic prognosticators, and BRAF mutation in primary cutaneous melanoma (Table 9). CXCL12 was not globally associated with the clinical values as demonstrated in a separate logistic model ($X^2=8.61$, $df=8$, $p=0.376$).

Table 8. Univariate analyses of CXCR4 protein and mRNA expression

Demographic and Clinical Correlates	n	Immunohistochemical		p-value	Significance	RNA Expression		Effect Estimate	p-value	Significance
		%CXCR4+	%CXCR4-			n	Mean Δ CT CXCR4			
<u>Gender</u>										
Male	66	60.6 (40)	39.4 (26)	0.0148	**	58	6.21	-0.49	0.1985	
Female	34	55.9 (19)	44.1 (15)	0.332		29	6.70			
<u>BRAF</u>										
Mutant	26	57.7 (15)	42.3 (11)	0.2673		22	5.69	-0.91	0.0275	*
Wild-type	74	59.5 (44)	40.5 (30)	0.0214	*	65	6.60			
<u>Depth</u>										
<1mm	26	76.9 (30)	23.1 (9)	<0.0001	***	37	6.13	-0.42	0.2549	
≥ 1mm	74	47.5 (29)	52.5 (32)	0.587		50	6.55			
<u>AJCC</u>										
Stage 1	39	76.9 (30)	23.1 (9)	<0.0001	***	37	6.13	-1.15	0.0122	⊙ *
Stage 2	29	69.0 (20)	31.0 (9)	0.0039	**	23	7.28	REF		
Stage 3	20	25.0 (5)	75.0 (15)	0.0016	**	19	5.94	-1.34		*
Stage 4	12	33.3 (4)	66.7 (8)	0.1025		8	5.87	-1.41		
<u>Mitotic Figures</u>										
Present	66	45.5 (30)	54.5 (36)	0.2963		56	6.18	-0.53	0.1564	
Absent	34	85.3 (29)	14.7 (5)	<0.0001	***	31	6.71			
<u>Host Response</u>										
Present	18	61.1 (11)	38.9 (7)	0.1824		18	6.63	1.24	0.0003	***
Absent	82	58.5 (48)	41.5 (34)	0.0288	*	69	5.39			
<u>Ulceration</u>										
Present	21	42.9 (9)	57.1 (12)	0.3545		16	6.06	-0.37	0.4260	
Absent	79	63.6 (50)	36.7 (29)	0.0008	***	71	6.44			
<u>Regression</u>										
Present	26	57.7 (15)	42.3 (11)	0.2673		25	6.14	-0.33	0.4161	
Absent	74	59.5 (44)	40.5 (30)	0.0214	*	62	6.46			
<u>Vascular Invasion</u>										
Present	2	50.0 (1)	50.0 (1)	1		1	5.63			
Absent	98	59.2 (58)	40.8 (40)	0.0101	*	86	6.38	0.75	§	
<u>CXCL12 ⊙</u>										
Positive	35	60.0 (21)	40.0 (14)	0.0943		28	6.53	0.23	0.5633	
Negative	65	58.5 (38)	41.5 (27)	0.0537		56	6.30			

* Marginal significance, ** Significant, *** Highly significant, ⊙ Welch's adjustment for unequal variance (ANOVA), § Not calculated due to only one sample of vascular invasion, □ Kappa Agreement between CXCR4 and CXCL12: K=0.1133, p=0.8814

Table 9. Multivariate analyses of CXCR4 and CXCL12 protein expression profiles

Clinical Factor (n=100)	CXCR4				CXCL12			
	OR	95%CI	p-value	Significance	OR	95%CI	p-value	Significance
<u>BRAF</u> mutant	2.31	0.67-8.01	0.1871		0.99	0.33-2.91	0.9883	
<u>AJCC</u>								
Ref: Stage1	---	---	---		---	---	---	
Stage2	0.99	0.29-3.39	0.9828		0.41	0.13-1.25	0.1169	
Stage3	0.16	0.04-0.68	0.0127	**	0.15	0.03-0.69	0.0149	
Stage4	0.17	0.03-0.92	0.0398	*	0.6	0.13-2.83	0.5165	
<u>Mitotic</u> Figures	0.21	0.06-0.73	0.0141	**	1.49	0.54-4.15	0.4439	
<u>Host</u> Response	2.84	0.72-11.20	0.1358		0.54	0.16-1.88	0.335	
<u>Ulceration</u>	0.7	0.22-2.29	0.5571		1.57	0.48-5.12	0.4577	
<u>Regression</u>	0.4	0.13-1.28	0.1234		0.93	0.34-2.56	0.8863	

3.7 CXCR4 protein expression does not correlate with expression of CXCR4 mRNA

The mean Δ Ct value for cases that were positive for expression of the CXCR4 protein was 6.38 ± 1.72 and the mean Δ Ct for negative cases was 6.36 ± 1.49 ($p=0.96$). Linear correlation analysis of Δ Ct (mRNA) and immunohistochemistry (protein) showed a low correlation coefficient ($r=0.1$).

DISCUSSION

Expression of CXCR4 mRNA in melanoma was initially reported in melanoma metastases in 2004 (Kim 2004). Since then, studies ascertaining the utility of CXCR4 mRNA as a prognosticator in primary cutaneous melanoma have revealed conflicting results. While Kim *et al.*, on a study of 23 patients, found that low *versus* high CXCR4 expression had no correlation with patient survival, Franco *et al.*, on a study of 47 patients, showed that expression in melanoma lymph node metastases correlated to shorter disease free survival and Monteagudo *et al.*, on a study of 51 patients, demonstrated the prognosticative value of the CXCL12/CXCR4 mRNA ratio by showing that a low ratio correlated to tumor thickness >1 mm and the development of metastases (Kim 2004; Franco 2010; Monteagudo 2012). Findings from the current study, the largest to date studying primary cutaneous melanomas, favor a role for CXCR4 mRNA expression as a prognosticator associated with poor clinical outcome as we found higher CXCR4 mRNA expression in tumors in AJCC stages 3 and 4 compared to stage 2.

Of note, we found significant differences in primary cutaneous melanomas with and without a host response with reduced CXCR4 mRNA expression in the former. An established cell non-autonomous tumor suppressive effect that has proven to be crucial for the development and progression of many different types of human cancers is the host immune response (Zitvogel 2006). It has been

previously shown that tumor cells develop strategies to escape immune surveillance (Mapara & Sykes 2004). However, tumors vary in their ability to evade the host immune response, which in some instances might explain their differential response to immune-based therapies. With the aim of identifying the genetic determinant of immune response in primary cutaneous melanoma, we have recently shown that the tumor suppressor, phosphatase and tensin homologue (PTEN), is an important regulator of the host immune response against melanoma cells (Dong 2013). Findings from the current study indicate that, like PTEN, CXCR4 may be of utility as a biomarker for recruiting melanoma patients for immunotherapy.

While the treatment of malignant melanoma with inhibitors targeting the *BRAFV600E* mutation has demonstrated dramatic clinical response with improved progression free and overall survival in the majority of melanoma patients receiving treatment, failed therapies are noted 6-8 months after initiation in approximately 47% patients (Filitis & Mahalingam 2013). The potential cooperativity between the mutational status of BRAF and the CXCR4/CXCL12 axis has been shown in a recent study (O'Boyle 2013). In comparing the migratory response of melanoma cells transfected with *BRAFWT* or *BRAFV600E* to CXCL12, O'Boyle *et al.* found that migration appeared to be enhanced by *BRAFV600E* transfection and inhibited by the CXCR4 antagonist AMD11070 (O'Boyle 2013). Given this, our findings of higher CXCR4 mRNA expression in patients with a BRAF mutation lends credence to the hypothesis that CXCR4

may be an ancillary molecule to explore as a putative target in primary cutaneous melanoma.

The samples we worked with were archival formalin-fixed paraffin-embedded tissue samples. Evers *et al.* showed that paraffin embedding of tissue samples can cause RNA aggregation and reductions in yield and quality (Evers 2011). As a result of the effects of paraffin embedding Evers *et al.* observed 10- to 160-fold decreases in amplifiable RNA compared to controls (Evers 2011). In addition to during paraffin embedding, Nolan *et al.* demonstrated that extracted RNA can be rendered unstable by the environment and storage time should be limited to prevent breakdown (Nolan 2006). As has been demonstrated previously, studies measuring target mRNA expression *via* RT-PCR are reliant upon high RNA yield as well as superior quality from tissue samples (Fleige & Pfaffl 2006). Perez-Novo *et al.* showed that RNA instability or low RNA concentrations can significantly alter RT-PCR measurements (Pérez-Novo 2005). In our study, to conserve RNA stability all extracted RNA samples were stored at -120°C and cDNA conversion and RT-PCR were performed within two weeks of extraction. To ensure proper RNA concentrations Nanodrop spectrophotometry was performed on all samples and those demonstrating the highest RNA concentration were selected for RT-PCR analysis.

Studies demonstrating the utility of CXCR4 protein as a prognosticator in primary cutaneous melanoma, like that of CXCR4 mRNA, have been limited by sample size and show no consensus. Favoring a role for CXCR4 protein as a

prognosticator are studies by Longo-Imedio *et al.*, on 40 patients with primary cutaneous melanoma, showing that CXCR4 expression correlates with ulceration, increased tumor thickness, development of metastases, and patient morbidity; by Tucci *et al.*, on 30 patients with nodular melanoma, demonstrating an association between CXCR4 protein expression and greater Breslow depth of the primary tumor as well as patient morbidity; and by Toyozawa *et al.*, on 19 patients with primary cutaneous melanoma, noting an association between CXCR4 protein expression and tumor thickness >2 mm as well as the development of distant metastases (Longo-Imedio 2005; Tucci 2007; Toyozawa 2012). Studies refuting an association to established histopathologic prognosticators are limited to two: one by Scala *et al.* on 71 patients with primary cutaneous melanoma and another by Kühnelt-Leddihn *et al.* on 38 patients (Scala 2005; Kühnelt-Leddihn 2012). Our findings support the fact that CXCR4 protein expression is not an adverse prognosticator by demonstrating in a multivariable logistic regression analysis that stages 3 and 4, compared to stage 1, were significantly associated with lower odds of staining CXCR4 positive. Also, mitoses were significantly associated with lower odds of staining CXCR4 positive.

In an effort to accurately demonstrate CXCR4 and CXCL12 protein expression we performed optimization of the immunohistochemistry protocol to ensure maximal target protein staining with minimal background. Control samples were stained with primary antibody at a range of dilutions (1:100, 1:250,

1:500, 1:1000) and incubated at room temperature or 4°C for varying lengths of time (2-12 hours). A common challenge to overcome when performing immunohistochemistry on formalin-fixed paraffin embedded samples is the presence of protein cross-linking, which can mask target proteins from being recognized by their corresponding antibody (Shi 1991). One way of overcoming this is using heat-induced antigen retrieval, which causes protein unfolding allowing for efficient recognition by the antibody (Shi 1991). Our study performed heat-induced antigen retrieval in low pH for 20 minutes at 97°C. During immunohistochemical optimization, we manipulated antigen retrieval temperature and incubation time to ascertain ideal staining. These steps allowed for accurate staining and efficient quantification of protein expression within lesional tissue.

In the only other study to evaluate an association between CXCR4 mRNA and protein expression in melanoma, unlike us, Franco *et al.* noted a perfect overlap of semi-quantitative mRNA and protein measurements (Franco 2010). Possible differences may relate to antibody used (polyclonal *versus* monoclonal) and cut-off for interpreting positivity (10 *versus* 30%). Of note, several previous studies have shown that non-transcriptional variance may be attributed to translation and/or protein degradation (Preiss 1998; Gebauer & Hentze 2004; Abreu 2009). For example, Abreu *et al.* noted that because transcription and translation do not occur simultaneously, there is greater opportunity for transcript modification and breakdown while Gebauer and Hentze showed that post transcriptional modification, particularly phosphorylation, can greatly impact

translational rate and efficiency (Gebauer & Hentze 2004). In addition to changes in translation, protein degradation in the form of lysosomal degradation and ubiquitin-mediated proteolysis has also been shown to contribute to variance in mRNA and protein expressions (Abreu 2009).

One previous study, on 19 patients, has shown that there appears to be a significant and positive correlation between immunohistochemical expression of CXCR4 and CXCL12 with 57% (4 of 7 cases) demonstrating “high” expression of both (Toyozawa 2012). Of the 59 cases that were CXCR4 positive, we noted CXCL12 positivity in only 21 (36%) and no statistically significant correlation between the two. The larger number of cases in the current study argues in favor of lack of an association between CXCR4 and CXCL12.

In conclusion, preliminary findings from the current study, the largest to date on primary cutaneous melanoma, suggest that CXCR4 may have multiple clinical uses. These include its potential utility as a prognosticator, given that expression of the protein is less frequently observed in melanomas with mitoses and depth >2 mm, its potential utility as a biomarker for recruiting melanoma patients for immunotherapy given the association between CXCR4 mRNA expression and a brisk host response and, its utility as a putative therapeutic target given our findings of higher CXCR4 mRNA expression in patients with a BRAF mutation. Longitudinal studies are required to confirm our findings.

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CURRICULUM VITAE

