2012

Quantitative analysis of neuropilin-2 expression in pigmented lesions and melanoma

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

QUANTITATIVE ANALYSIS OF NEUROPILIN-2 EXPRESSION IN PIGMENTED LESIONS AND MELANOMA

by

JACLYN TUCK

B.S., Brandeis University, 2010

Submitted in partial fulfillment of the

requirements for the degree of

Master of Arts

2012

Approved by

First Reader [redacted]

Theresa A. Davies, Ph.D.
Assistant Director, M.A. in Medical Sciences Program
Adjunct Assistant Professor of Biochemistry

Second Reader [redacted]

Rhoda M. Alani, M.D.
Herbert Mescon Professor and Chair of Dermatology
Dermatologist-in-Chief
Boston Medical Center

ACKNOWLEDGEMENTS

I would like to thank everyone in the Alani lab that contributed to this study. Specifically I would like to thank Dr. Rhoda Alani, Dr. Byungwoo Ryu, and Izabela Panova for providing all of their guidance, knowledge, and support during my time in the laboratory. Without them, this project would not have been possible. I would also like to express my sincere gratitude to my advisor, Dr. Theresa Davies for all of her encouragement and assistance throughout my time as a master's student. Finally, I would like to thank my family for always being there and believing in me.

QUANTITATIVE ANALYSIS OF NEUROPILIN-2 EXPRESSION IN PIGMENTED LESIONS AND MELANOMA

JACLYN TUCK

Boston University School of Medicine, 2012

Major Professor: Theresa A. Davies, Ph.D., Assistant Director, M.A. Medical Sciences Program and Adjunct Assistant Professor of Biochemistry

ABSTRACT

The prevalence of skin cancer has been continuously increasing in the American population over the last 3 decades. Melanoma, the most serious and dangerous form, causes the most skin cancer related deaths. If melanoma is found early enough, it can be treated and cured. Current therapy for melanoma is based on the stage of the disease. It is crucial to identify and treat the melanoma before metastasis occurs as the risk of death greatly increases once the disease becomes widespread. The processes of angiogenesis and lymphangiogenesis as well as changes in the extracellular environment all play significant roles in the expansion of a primary melanoma and ultimately metastasis. Neuropilins, specifically Neuropilin-2 (NRP2), have been found to be associated with both angiogenesis and lymphangiogenesis and thus metastasis. Since it is critical to identify and treat melanoma before it advances to metastasis, the relationship between NRP2 and tumor progression may prove to be extremely valuable as a

prognostic marker for primary melanoma tumors. In addition, it may serve to identify subsets of early stage tumors that would benefit from adjuvant therapy.

This study aimed to demonstrate if there is a relationship between the level of expression of NRP2 in primary melanocytic lesions and the prognosis of melanoma, and determine the potential utility of NRP2 as a biomarker for melanoma. To do so, the levels of expression of NRP2 were analyzed in formalin-fixed, paraffin-embedded, tissue samples of nevi, primary melanomas, and metastatic melanomas. Sample RNA was extracted and converted to cDNA. Quantitative RT-PCR was then used to amplify cDNA and appropriate normalizations were performed. Luciferase RNA was added at the start of processing as an exogenous control and was used to correct for procedural variation between samples. Furthermore, Actin, beta (ACTB) and Melan-A were used as reference genes to correct for differences in tissue and lesion size respectively. To analyze the data, standard curves were developed for NRP2, ACTB, and Melan-A to calculate the copy numbers for each gene in each sample.

When normalized by both ACTB and Melan-A, the differences in the level of expression of NRP2 between nevi and primary melanomas ($p=0.94422$), nevi and metastatic melanomas ($p=0.29538$), and primary melanomas and metastatic melanomas ($p=0.32827$) all failed to show statistical significance. In addition when normalized by only ACTB, there is no statistical significant difference in the level of expression of NRP2 between nevi and primary melanomas ($p=0.21001$),

or primary and metastatic melanomas (p=0.30874). However, there is a difference between nevi and metastatic melanomas (p=0.00472) that is statistically significant when just normalized by ACTB. Furthermore, when just normalized by Melan-A, there is no significant difference in the level of expression of NRP2 between nevi and primary melanomas (p=0.3152) or primary melanomas and metastatic melanomas (p=0.14179). However, there is a statistically significant difference in the level of expression of NRP2 between nevi and metastatic melanomas (p=0.01736). The fact that there is a statistically significant difference ($p<0.05$) in the level of expression of NRP2 between nevi and primary melanomas demonstrates its potential ability to be used as a biomarker. However, the inability to establish statistical significance between sample groups may be due to the small sample size. Further studies with an increased sample size may better show the relationship between the levels of NRP2 and the stage of melanoma. Still, NRP2 shows promise as both a diagnostic and prognostic biomarker for melanoma.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACTB	Actin, beta
AJCC	American Joint Comittee on Cancer
FGF-2	Fibroblast Growth Factor-2
gp100	Premelanosome protein
HPF	High Power Field
IL-2	Interleukin- 2
LYVE-1	Lymphatic Endothelial Hyaluronal Receptor-1
MAPK	Mitogen Activated Protein Kinase
Mitf	Microphthalmia-associated Transcription Factor
MMPs	Matrix Metalloproteinases
NRP1	Neuropilin-1
NRP2	Neuropilin-2
PGF	Placental Growth Factor
SEER	Surveillance, Epidemiology, and End Results Program
VEGF	Vascular Endothelial Growth Factor

INTRODUCTION

Skin Cancer

The prevalence of skin cancer has been continuously increasing in the American population over the last 3 decades and is currently the most common type of cancer in the United States with over 2 million cases diagnosed annually. Melanoma, the most serious and dangerous form, causes the most skin cancer related deaths (American Cancer Society, 2012). The American Cancer Society approximates that each year there are 75,000 new cases of invasive melanoma diagnosed and that the disease kills over 8,000 people annually in the United States.

If found early enough melanoma can be treated and cured. As you get older, the risk of acquiring melanoma increases; still the disease can be detected in patients of any age (Miller and Mihm, 2006). A family history of melanoma, fair skin, the presence of multiple atypical nevi, and immunosuppression are all risk factors that increase a person's chance of developing melanoma. The cancer can occur anywhere on the skin, from the neck and face to the eyes and mouth (American Cancer Society, 2012). The most common site that melanoma is found in men is the trunk and in women is the legs.

Melanoma originates from particular cells in the skin called melanocytes. The skin has three layers: the epidermis, the dermis and the hypodermis. The epidermis is the outermost layer while the hypodermis is the innermost. The

epidermis is mainly comprised of keratinocytes. There are no blood vessels or lymphatics in the epidermis. The layer is nourished via diffusion from blood vessels located in the dermis and hypodermis. Melanocytes are found in the dermal-epidermal junction of the skin as well as in hair follicles (Hearing and Leong, 2006). They produce a substance known as melanin, which gives rise to skin and hair color. An external stimulus, such as ultraviolet light, can cause melanocytes to increase melanin production, which serves as a protective mechanism for the skin (Hearing and Leong, 2006, Merk, 2011). Unfortunately, if the stimulus causes DNA damage, the melanocyte may become abnormal and start to reproduce uncontrollably leading to melanoma (Merk, 2011). When these cancerous cells spread to other parts of the body such as the brain, lungs, liver, or bone, metastasis occurs. However, this is not a simple process. The cell needs to separate from the primary site, migrate into a blood or lymphatic vessel, survive as it travels through and exits the blood or lymphatic vessel, and then be able to replicate at the secondary site (Stine et al., 2011). It is crucial to identify and treat the melanoma before metastasis occurs as the risk of death greatly increases once the disease becomes widespread.

Once the melanoma becomes metastatic, it becomes extremely difficult to treat. This is because of the molecular heterogeneity of the tumor and the ability to resist apoptotic processes as well as chemotherapeutics (Soengas and Lowe, 2003). This is why it is critical to detect the disease as early as possible. Typically, a biopsy will be taken of the initial lesion and will be checked for

cancerous cells via histological examination (Garb et al., 2010). Once it is identified as melanoma, other factors are assessed such as thickness, ulcerations, microsatellites, and how far the cancer has spread.

Melanoma Staging

Melanoma diagnosis is done via staging based on the American Joint Committee on Cancer (AJCC) system. Staging the melanoma involves physical exams, imaging and laboratory tests, as well as pathology and surgical reports (National Cancer Institute, 2011). Stages range from 0-IV. Stage 0, melanoma in situ, refers to the presence of abnormal melanocytes in the epidermis. Stage I is subdivided into IA and IB. In stage IA, the lesion is <1mm thick with no ulcerations and a mitotic rate <1/HPF whereas in stage IB the lesion is either <1mm thick and shows ulcerations, possesses a mitotic rate $\geq$ 1/HPF, or is between 1mm and 2mm thick with no ulcerations. There are three sub-stages for stage II. In stage IIA the thickness is between 1 and 2 mm with ulcerations or between 2 and 4mm with no ulcerations. In stage IIB, the tumor is between 2 and 4 mm thick with ulcerations or more than 2mm thick with no ulcerations. Stage IIc refers to the tumor being more than 4mm thick and having ulcerations. In stage *III*, the thickness of the tumor varies and it may or may not have ulcerations. However, the cancer has begun to spread to other areas under the skin not more than 2 cm away, the lymph vessel between the primary site and the lymph nodes, or the actually lymph nodes (National Cancer Institute, 2011).

Stage IV is the most advanced stage of metastatic disease and is identified as the stage in which melanoma has metastasized to secondary organs in the body or distant lymph nodes. A summary of the stages is shown in Table 1.

The Stages of Melanoma

Stage	Characteristics
IA	Tumor ≤ 1.0 mm *without* ulceration; no lymph node involvement; no distant metastases
IB	Tumor ≤ 1.0 mm *with* ulceration or Clark level IV or V; tumor 1.01-2.0 mm without ulceration; no lymph node involvement; no distant metastases
IIA	Tumor 1.01-2.0 mm *with* ulceration; tumor 2.01-4.0 mm without ulceration; no lymph node involvement; no distant metastases
IIB	Tumor 2.01-4.0 mm *with* ulceration
IIB	Tumor > 4.0 mm *without* ulceration; no lymph node involvement; no distant metastases
IIC	Tumor > 4.0 mm *with* ulceration; no nodal involvement; no distant metastases
IIIA	Tumor of any thickness *without* ulceration with 1 positive lymph node
IIIB	Tumor of any thickness *without* ulceration with 2-3 positive lymph nodes
IIIC	Tumor of any thickness and 4 more metastatic lymph nodes *OR* matted nodes *OR* in-transit met(s)/satellite(s) *without* metastatic lymph nodes, or combinations of in-transit met(s)/satellite(s), *OR* ulcerated melanoma *and metastatic* lymph node(s)
IV	Tumor of any thickness with any nodes and any distant metastases

Table 1. Summary of the characteristics of each stage of Melanoma. Table taken from Moncrieff, 2011 at http://skincancersurgery.co.uk/Melanoma/Melanoma_staging.html

Melanoma Treatment

Current therapy for melanoma is based on the stage of the disease. The standard treatment is surgery to remove the lesion (Garbe et al., 2011). If the tumor has spread to the lymph nodes, a lymphadenectomy is advised (Garbe et al, 2010). In addition to surgery, patients with stage II and III melanoma may undergo chemotherapy and/or radiation therapy, and are possibly placed in clinical trials (Garbe et al, 2011). Inferfeuron-α is sometimes used as an adjuvant therapy and has shown a modest increase in survival in some studies. Combination chemotherapy proves to be more promising than single-agent chemotherapy however there is a greater risk for toxicity (Bhatia et al., 2009). Dacarbazine and high does IL-2 are chemotherapeutic agents approved in the United States for metastatic melanoma treatment. However, there is no significant increase in survival for these agents.

The scientific community continues to find improved targeted therapies for advanced staged melanoma. In particular, BRAF inhibitors, such as vemurafenib, have shown promise in regards to treatment for melanoma with BRAF mutations (Livingstone et al., 2012). BRAF is an intermediary kinase in the mitogen-activated protein kinase (MAPK) signaling pathway that regulates cell proliferation. Mutations in BRAF promote activation of the pathway and thus increase cell proliferation. Inhibitors that target BRAF mutations can prevent the lesion from increasing in size. Studies have demonstrated that vemurafenib has increased the length of survival of melanoma patients, however resistance to the

drug and long-term term survival need to be further examined, seeing that patients have quickly developed resistance to the BRAF inhibitor (Flaherty et al., 2010). Other kinase inhibitors of signaling pathways such as MEK inhibitors, and c-KIT inhibitors are also being studied for target therapy (Livingstone et al., 2012). In addition to these pathway inhibitors, Ipilimumab is a monoclonal antibody that has shown to improve overall survival for patients with metastatic melanoma. Ipilimumab acts by blocking cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (Robert and Ghiringhelli, 2009). CTLA-4 is a negative T-cell regulator and blocking this would allow for increased T-cell mediated tumor response. However, autoimmune adverse effects are associated with ipilimumab treatment. Still, ipilimumab displays promise as a targeted therapy for advanced staged melanoma.

Several models of melanoma progression have been proposed in the scientific community. The theory that metastatic melanoma is derived from pre-existing benign nevi was initially suggested by Virchow (Ferreira de Souza et al., 2012). Clark further hypothesized that melanocytes proliferate and transform into nevi and acquire dysplasia, hyperplasia, invasion, and finally metastasis (Miller and Mihm 2006). A Nevus is characterized as a benign lesion that contains melanocytes. Nevi, however, do not typically progress to be melanoma because they have restricted growth. In order for melanoma to develop, there needs to be mutations on a genetic level that leads to an increase in growth as well as diminished DNA repair and cell death (Miller and Mihm, 2006). With these

genetic mutations, the cells have the ability to further multiply without being turned off. Eventually the lesion not only grows laterally, but also grows vertically, deeper into the skin where the cells can come in contact with lymphatics or blood vessels. Here, alterations in cell motility, cell extension, and cell adhesion can allow the malignant cells to metastasize and form secondary tumors in other parts of the body (Ferreira de Souza et al., 2012). Another theory of melanoma progression involves tumor stem cells with the ability of self-renewal, and a high tumorogenicity (Zabierowski and Herlyn, 2008). Metastasis is aided by the microenvironment that contains fibroblasts, endothelial cells, and inflammatory cells (Ferreira de Souza et al., 2012). Other models hypothesize that melanoma progression is a result of epigenetic changes, such as DNA methylation, within tissue specific stem cells (Feinberg et al., 2006). This malignant transformation is caused by tumor suppressor gene inactivation and oncogene activation, which allows the cells to multiply uncontrollably. It is likely that all of these processes are involved in the development and progression of melanoma to a varying degree in individual lesions.

Angiogenesis and Melanoma

The process of angiogenesis plays a significant role in the expansion of a primary melanoma and ultimately metastasis (Mahabeleshwar and Byzova, 2007). As the tumor progresses and grows, it requires oxygen and nutrients that cannot simply diffuse through the initial surrounding environment due to

increased tumor volume. Thus, the malignant lesion secretes growth factors that ultimately allow for an increase in vascular channels to the tumor, a process known as tumor-associated angiogenesis (Ria et al., 2010). In particular, the advancement from lateral growth to vertical growth is associated with the secretion of vascular endothelial growth factor (VEGF) by melanoma cells (Erhard et al., 1997). Other stimulators for angiogenesis in melanoma include fibroblast growth factor-2 (FGF-2), as well as the placental growth factor (PGF) (Ordorisio et al., 2006, Ribatti et al., 2003). Tumor cell secretion of matrix metalloproteinases (MMPs) leads to breakdown of the extracellular matrix leading to subsequent release of the angiogenic growth factor, FGF-2. Melanoma cells express PGF, which is a member of the VEGF family that binds neuropilin-1 (NRP1) and neuropilin-2 (NRP2) leading to enhanced tumor-associated angiogenesis (Ordorisio et al., 2006).

Another key component in tumor metastasis is the interaction of the tumor with its surrounding environment, particularly endothelial cells (Stine et al., 2011). Again, for the melanoma to metastasize, cancer cells must migrate towards, adhere to, and enter the blood or lymphatic system. For this to occur, molecular pathways pertaining to cell adhesion, cell differentiation, cell migration, and extracellular matrix remodeling are upregulated (Stine et al., 2011). In addition to changes in cell communication and angiogenesis, lymphangiogenesis plays a role in melanoma metastasis (Rushing et al., 2011). The interaction between the cancer cells and the endothelium of the lymphatic and vascular vessels is thus

critical for tumor expansion. VEGF modulates the restructuring and proliferation of endothelial cells with the help of neuropilins. Therefore, neuropilins are believed to play a key role in metastasis.

Neuropilins

Neuropilins are upregulated in several tumor types and their expression is shown to correlate with tumor development (Staton et al., 2007). There are currently two known neuropilins: neuropilin-1 (NRP1), and neuropilin-2 (NRP2). They are both transmembrane glycoproteins with large extracellular domains and a cytoplasmic domain that lacks enzymatic activity (Favier et al., 2005, Staton et al, 2007). The extracellular domain has subdomains including an *a1a2 domain* that binds class-3 semaphorins, a *b1b2 domain* that binds VEGF, and a *c domain* that is involved in dimerization (Mamluk, 2002). The basic structure of neuropilins can be viewed in Figure 1.

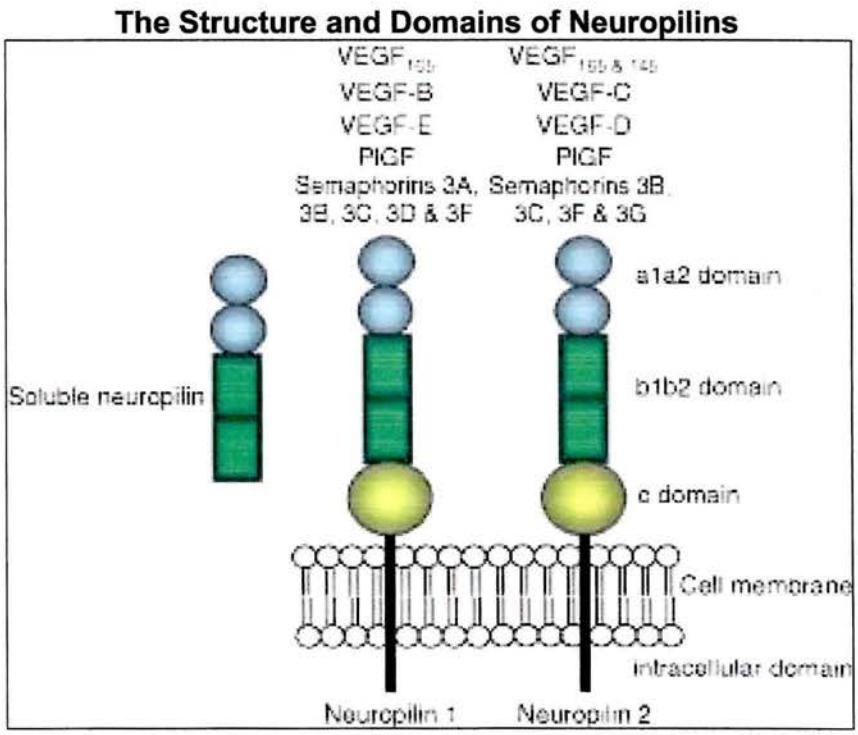

Figure 1. A depiction of the neuropilin structure and its domains as well as its association with common receptors. Figure taken from Staton et al, 2007.

Neuropilins were first discovered to be co-receptors for class-3 semaphorins and were found to be involved in axonal growth guidance during embryologic development (Cai and Reed, 1999). Subsequently, neuropilins were found to serve as co-receptors for VEGF and contribute to angiogenesis (Mamluk et al., 2002). It is notable that neuropilins have been found to be present in multiple tissue types (Bielenberg et al., 2006). NRP1 and NRP2 have similar structures but differ in their expression throughout the body as well as in their

regulation and binding properties. For example, in embryonic endothelium, NRP1 is expressed in arterial endothelium while NRP2 is expressed in venous and lymphatic endothelium. Studies show that knocking out NRP1 is lethal in mice while knocking out NRP2 diminishes lymphatics and capillaries (Bielenberg et al., 2006, Ellis, 2006).

Both neuropilins are associated with tumor cells; however, the expression of each neuropilin varies depending on the type of cancer. For example, NRP1 is seen in epithelial carcinomas like prostate, colorectal, lung, and breast cancers whereas melanoma cells, human glioblastoma cells, and human neuroblastoma cells all express NRP2 suggesting that expression of NRP2 is restricted to tumors derived from the neural crest (Bielenberg et al., 2006, Ellis, 2006). In addition, the expression of neuropilins may be associated with the progression of a particular tumor and correlate with a worse prognosis. For example, NRP2 expression is associated with more advanced stages of bladder cancer (Ellis, 2006).

Since it is critical to identify and treat melanoma before it advances to metastasis, the relationship between NRP2 and tumor progression may prove to be extremely valuable as a prognostic marker for primary tumors and may serve to identify subsets of early stage tumors that would benefit from adjuvant therapy. Currently, the most useful tumor characteristics for predicting the prognosis of primary melanomas are the phenotypic aspects of the disease such as the thickness of the lesion, proliferative index, and whether there is ulceration seen in

the primary lesion. Whether a particular primary melanoma lesion will remain limited to skin or metastasize to distant sites remains extremely difficult to determine at the time of biopsy. A reliable biomarker, such as NRP2, may provide insight as to whether metastasis is predicted to occur in a particular lesion and whether adjuvant therapies might be considered for a particular tumor.

Melanoma Biomarkers

The National Cancer Institute defines a biomarker as a biological molecule found in blood, body fluids, or tissues that is a sign of a normal or abnormal process, a condition, or a disease (National Cancer Institute, 2011). A melanoma biomarker would not only be an efficient way of diagnosing the disease, but also aid in prognosis and further show how the tumor is responding to treatment. The National Cancer Institute's Surveillance, Epidemiology, and End Results Program (SEER) Cancer Statistics Review states that from 2001 to 2007, the 5-year relative survival at the time of diagnosis for localized lesions that remain at the primary site was 98.2%, for regional tumors that spread to the lymph nodes was 61.7%, and for distant melanomas that have metastasized was 15.2% (Howlander et al., 2010). A melanoma biomarker could substantially increase survival rates among patients with early stage melanoma either to improve diagnosis of primary malignant lesions or predict the outcome for localized disease and whether such disease would warrant additional therapies. In addition to increased survival, being able to screen for the biomarker and then provide a

targeted therapy specific to the biomarker would allow for a more cost effective approach for identifying and treating melanoma (Atherly and Camidge, 2012).

The scientific community has identified several melanoma biomarkers. Some only state the presence of melanocytic/melanoma cells such as MART-1/Melan-A, Tyosinase, Mitf, and gp100 (Medic et al., 2006). Other proposed biomarkers have a connection with the progression of melanoma such as Nuclear Factor Kappa B (NFkB), which is associated with the transition from radial growth to vertical growth and from pro-apoptotic to anti-apoptotic. In addition, factors that are associated with angiogenesis such as VEGF-C and VEGF-D as well as those associated with lymphangiogenesis such as lymphatic endothelial hyaluronal receptor-1 (LYVE-1) aid in the analysis of the tumor (Medic et al., 2006).

Objectives

NRP2 has emerged as a promising biomarker because it is associated with angiogenesis and lymphangiogenesis in melanoma. NRP2 may show utilization when differentiating between primary melanomas of low metastatic potential and primary melanomas of high metastatic potential. The goals of the current study is to demonstrate if there is a relationship between the level of expression of NRP2 in primary melanocytic lesions and the prognosis of melanoma, and determine the potential utility of NRP2 as a biomarker for melanoma.

METHODS

Sample Selection

Patient samples were randomly selected and provided by the Department of Dermatology at Boston University School of Medicine, Boston, MA. Samples were in the form of formalin-fixed, paraffin-embedded tissues and consisted of nevi (n = 12), primary melanomas (n = 11), and metastatic melanomas (n = 12) in order to assess the level of expression of NRP2 throughout different phases of the disease. Two to four tissue slides were used from each patient sample for processing and analysis. All of the primary melanomas had a depth equal to or less than 1.65 mm. Four of these primary melanomas possessed ulcerations; however, none experienced vascular invasion or microsatellites.

RNA Extraction and Isolation

Tissue samples were processed with Arcturus Paradise PLUS Whole Transcript Reverse Transcription Reagent System from Applied Biosystems by Life Technologies. RNA extraction and isolation was performed according to manufacturer's instructions. In summary, tissue slides underwent a series of Xylene washes for a total of 15 minutes to remove the paraffin. The tissue samples were then scraped and incubated with a Proteinase K solution for 16 to 20 hours. Following the incubation, 10ng of luciferase RNA was added to each sample as an internal control for processing and analysis. Sample RNA was

isolated using a conditioned purification column along with a binding buffer and ethanol solution. The samples then underwent a series of washes and finally an elution buffer was used to isolate approximately 11μL of RNA. DNase treatment was also performed according to manufacturer's instructions to ensure the RNA sample was clean and free of contaminating DNA.

Reverse Transcription

The isolated RNA was converted to cDNA using SuperScript III First-Strand Synthesis System for RT-PCR by Invitrogen. Reverse transcription was carried out according to manufacturer's instructions. One third, about 4.5μL of each isolated RNA was used for cDNA synthesis. The RNA was primed with random hexamers, combined with 10mM dNTP mix, and denatured at 65˚C for 5 minutes then 4˚C for at least 1 minute. A previously prepared cDNA synthesis mix containing 10X RT Buffer, 25mM $MgCl_2$, 0.1M DTT, RNaseOUT, and SuperScript III RT was then added and the samples were heated to 25˚C for 10 minutes, 50˚C for 50 minutes, and 8˚C for 5 minutes. RNase H was then added and the samples were heated to 37˚C for 20 minutes. The entire procedure yielded approximately 20μL of cDNA for each sample.

Primer Design

Primers for qRT-PCR were designed for the genes NRP2, Actin, beta (ACTB), Melan-A, and Luciferase, using the program Primer Blast provided by

the PubMed website and then ordered through Invitrogen. Each nucleotide was searched for the Homo sapiens sequence and selected primers were between 80 and 100 base pairs. Once received, the primers underwent optimization and appropriate annealing temperatures were determined. The primer sequence for each gene is shown in Table 2:

Primer Sequences
NRP2 Forward: 5'-GAGTACCAGATTGTGTTC-3' Reverse: 5'-CTTATCCGAATGTCATCAA-3'
ACTB Forward: 5'-GCATTGTTACAGGAAGTCC-3' Reverse: 5'-CATTACATAATTTACACGAAGC-3'
Melan-A Forward: 5'-AGGAAGGTGTCCTGTGCCCTGA-3' Reverse: 5'-GAGTGGCCGTGCCCCTTCTTG-3'
Luciferase Forward: 5'-TCAGGTGGCCCCCGCTGAAT-3' Reverse: 5'-GTCGGGAAGACCTGCCACGC-3'

Table 2. Forward and reverse nucleotide sequences for primers used to amplify sample cDNA in qRT-PCR. Size ranged from 80 to 100 base pairs.

qRT-PCR

MyiQ Single-Color Real-Time PCR Detection System by Bio-Rad was used to amplify cDNA samples for analysis. Protocol was designed based on

manufacturer's recommendations. All samples were run at once in duplicates with both positive and negative controls, using only one primer per run to minimize bias, for a total of four runs. The positive control was cDNA derived from melanoma cell line WM35 and the two negative controls consisted of a mock from the cDNA synthesis and a mock of only nuclease free distilled water. A 96-well plate was used in each procedure and each well contained 0.5μL cDNA, 12.5μL Sybr Green Master Mix provided by Bio-Rad, 0.25μL of the forward primer, 0.25μL of the reverse primer, and 11.5μL of nuclease free distilled water. All samples were heated to 95˚C for 5 minutes then ran for 60 cycles of 95˚C for 0.5 minutes, 55˚C for 0.5 minutes, and 72˚C for 0.5 minutes, and then finally heated to 95˚C for 1 minute and 65˚C for 1 minute. The MyiQ software provided with the detection system determined the cycle threshold (Ct) values for the samples and generated melting curves to make sure the correct fragment was amplified. However, the Ct baseline for all samples was manually set at 50 to ensure standardization throughout each run for each primer.

Standard Curves

For sample analysis, standard curves for each gene were generated to determine gene copy numbers in each tissue sample. First, qRT-PCR was performed to amplify cDNA from melanoma cell line WM35 with 500 base pair primers for the genes NRP2, ACTB, and Melan-A. The 500 bp primers were

designed using the same Primer Blast tool as the short primers. The sequences are shown in Table 3.

Primer Sequences
NRP2 Forward: 5'-CGGCTTTTGCAGTGGACATCCCA-3' Reverse: 5'-CCCTGTGCGGCTGCATCTCC-3'
ACTB Forward: 5'-CACAGAGCCTCGCCTTTGCCG-3' Reverse: 5'-ACGGCCAGAGGCGTACAGGG-3'
Melan-A Forward: 5'-TGCAGAACAGTCACCACCACCT-3' Reverse: 5'-GGCCAGTCAACCCTTTGTCTTAACC-3'

Table 3. Forward and reverse nucleotide sequences for 500 base pair primers used to amplify DNA in qRT-PCR.

Gel electrophoresis was then run with the amplified qRT-PCR samples in 2% agarose gel. The band was excised and DNA was extracted using Qiagen QIA Quick Gel Extraction Kit. The DNA concentration was determined with Bio-Rad Smart-Spec Plus Spectrometer and 10 fold dilutions were made starting with 1ng. The dilutions were then run in duplicate for qRT-PCR with the 500 bp primers and Ct values were determined. The copy numbers were generated from the following formula: m = [n][1.096e-21 g/bp], where n = 500 and m = mass of one gene copy. A ratio was then set up to determine how many copies were in each dilution. The copy numbers were then plotted against Ct values and the equation of the linear slope was used in analysis of tissue samples.

RESULTS

To evaluate the possible utility of NRP2 as a biomarker for melanoma, the level of expression of NRP2 was assessed in formalin-fixed, paraffin-embedded tissues of nevi, primary melanomas, and metastatic melanomas. Quantitative RT-PCR was performed and sample cDNA was amplified with NRP2, ACTB, Melan-A, and Luciferase primers. To minimize statistical error from procedural variation between samples, 10ng Luciferase RNA was added as an exogenous control to each sample RNA before processing. The Ct values obtained from amplification with Luciferase primers were then used to normalize the Ct values obtained from amplification with NRP2, ACTB, and Melan-A primers, allowing for a more accurate statistical analysis. Once this normalization was performed, the copy numbers of NRP2, ACTB, and Melan-A were determined using the equations obtained from the standard curves. The standard curves for NRP2, ACTB, and Melan-A are shown in Figures 2a, 2b, and 2c respectively, along with the corresponding equation to calculate gene copy numbers.

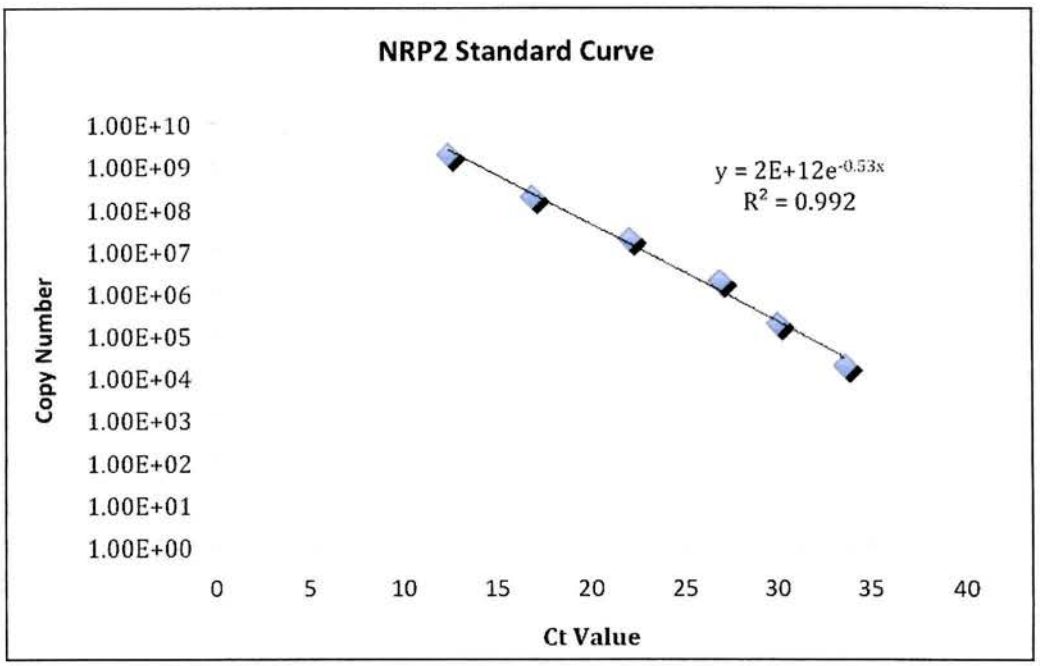

Figure 2a. The cycle threshold (Ct) value (x) of each NRP2 dilution was plotted against the corresponding copy number (y). As the NRP2 DNA solution becomes more diluted, the Ct value increases and the number of NRP2 gene copies decreases.

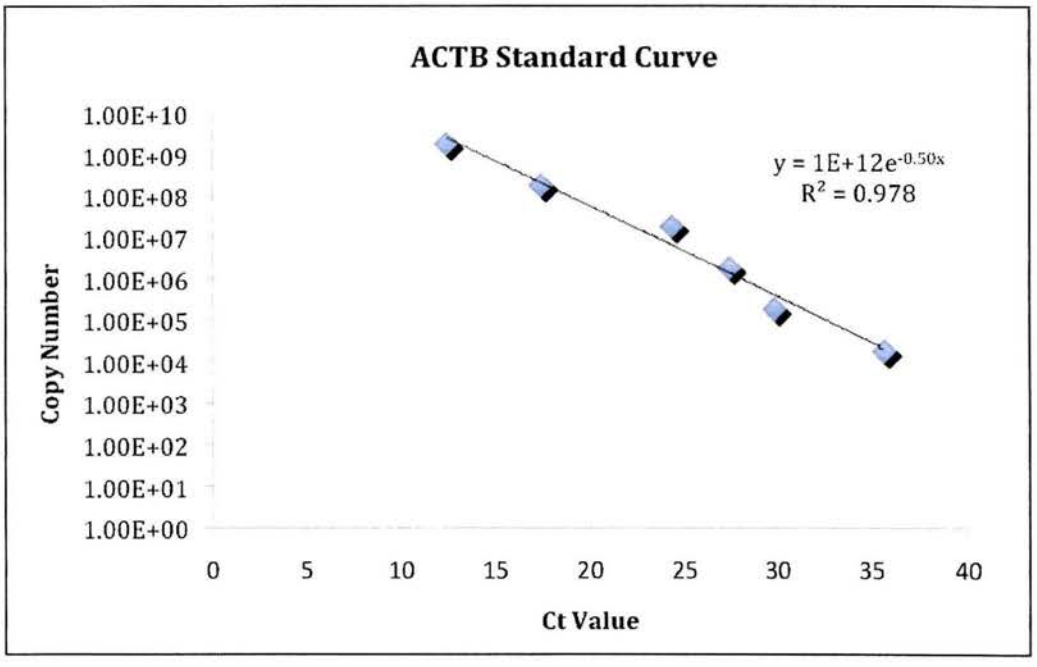

Figure 2b. The cycle threshold (Ct) value (x) of each ACTB dilution was plotted against the corresponding copy number (y). As the ACTB DNA solution becomes more diluted, the Ct value increases and the number of ACTB gene copies decreases.

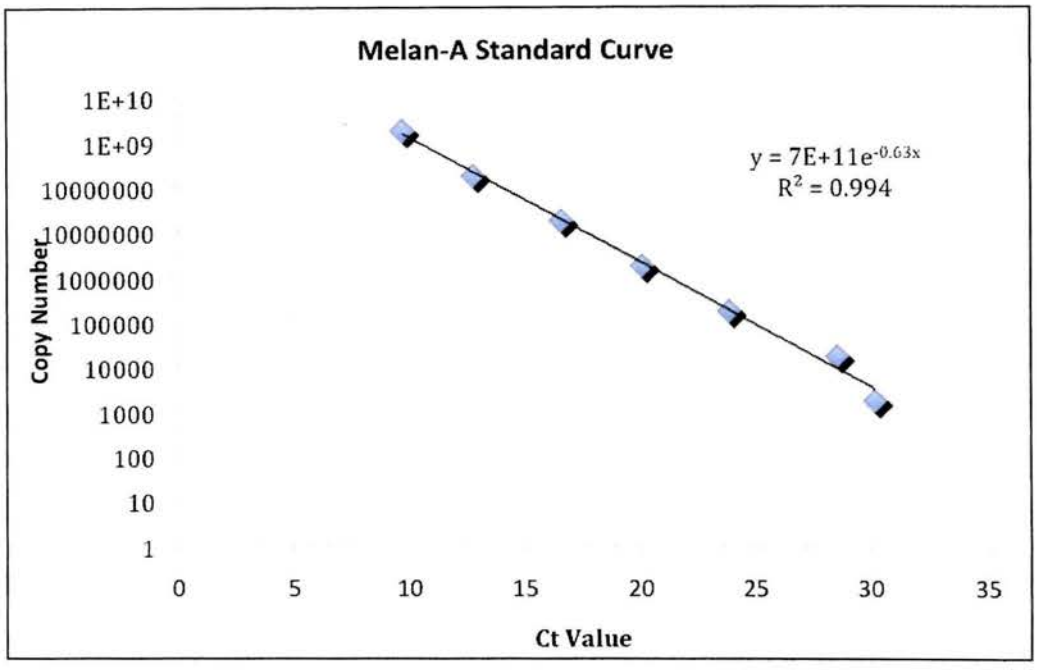

Figure 2c. The cycle threshold (Ct) value (x) of each Melan-A dilution was plotted against the corresponding copy number (y). As the Melan-A DNA solution becomes more diluted, the Ct value increases and the number of Melan-A gene copies decreases.

The copy numbers for NRP2, ACTB, and Melan-A can be seen in Tables 4a, 4b, and 4c respectively.

Copy Number of NRP2 in Each Sample		
Nevi (n = 11)	**Primary Melanomas (n =11)**	**Metastatic Melanomas (n = 12)**
24498.65	85184.29	7270.40
31047.35	35926.72	1463534.66
22018.00	2244.32	9980.16
28177.76	18623.17	87667.21
22946.47	28729.20	51887.08
21573.15	24188.65	5503117.84
10538.80	23609.43	220911.56
22841.52	358.97	131480.94
9512.18	3322.77	28964.14
45609.44	2938708.64	474692.14
341810.29	19860.88	659355.96
		64385.22

Table 4a. NRP2 copy numbers were calculated using the equation $y = 2E+12^{-0.53x}$, where x = cycle threshold value and y = number of NRP2 gene copies in the sample. The equation was obtained from the corresponding standard curve seen in figure 1a. n = sample size of each group. The mean copy number of NRP2 in nevi was 52,779.42, in primary melanomas was 289,159.7, and in metastatic melanomas was 725,270.61. Any sample that did not amplify in qRT-PCR or showed a poor melting curve was negated.

Copy Number of ACTB in Each Sample		
Nevi (n = 12)	**Primary Melanomas (n =11)**	**Metastatic Melanomas (n = 12)**
262821.37	749462.66	36150.78
798751.97	409322.75	1540994.64
279819.32	34516.19	9601.35
327142.58	267363.99	92305.02
69651.71	44166.29	88180.49
445615.19	16644.40	25120972.46
38968.88	63572.18	579622.05
440912.44	72334.08	818224.23
206628.38	56062.61	300142.39
5372.22	16943299.22	1051441.51
474047.35	143928.22	2450935.17
2106183.04		344534.03

Table 4b. ACTB copy numbers were calculated using the equation $y = 1E+12^{-0.50x}$, where x = cycle threshold value and y = number of ACTB gene copies in the sample. The equation was obtained from the corresponding standard curve seen in figure 1b. n = sample size of each group. The mean copy number of ACTB in nevi was 454,659.54, in primary melanomas was 1,709,152.06, and in metastatic melanomas was 2,702,758.68. Any sample that did not amplify in qRT-PCR or showed a poor melting curve was negated.

Copy Number of Melan-A in Each Sample		
Nevi (n = 12)	**Primary Melanomas (n =11)**	**Metastatic Melanomas (n = 12)**
1686.57	2593.12	329.00
7517.80	581.30	10242.34
3969.10	173.73	108.83
1194.74	94.87	1460.63
181.61	0.02	230.89
3431.33	77.25	173933.67
203.19	448.92	784.99
11678.39	68.13	698.75
866.92	191.27	325.30
197.14	159864.31	14440.67
6385.64	1265.58	36302.30
57030.36		7446.72

Table 4c. Melan-A copy numbers were calculated using the equation $y = 7E+11^{-0.63x}$, where x = cycle threshold value and y = number of Melan-A gene copies in the sample. The equation was obtained from the corresponding standard curve seen in figure 1c. n = sample size of each group. . The mean copy number of Melan-A in nevi was 7,861.90, in primary melanomas was 15,032.59, and in metastatic melanomas was 20,525.34. Any sample that did not amplify in qRT-PCR or showed a poor melting curve was negated.

Once the copy numbers for each gene were computed, further normalizations were performed to provide more reliable results. ACTB is found throughout skin cells and was used as a reference gene in qRT-PCR to correct for the size of the entire tissue sample. Melan-A is found in melanocytes and was used as a reference gene to correct for the size of just the lesion. Furthermore, any sample that did not amplify in qRT-PCR or showed an incorrect melting curve was negated from the analysis.

When normalized by only Luciferase, the mean level of expression of NRP2 in nevi was 52,779.42 copies, in primary melanomas was 289,159.73 copies, and in metastatic melanomas was 725,270.61 copies. Then, when normalized by ACTB, the mean level of expression of NRP2 in nevi was 635.65 copies, in primary melanomas was 1,556.43 copies, and in metastatic melanomas was 2,459.23 copies. The relative levels of expression of NRP2 when normalized by Luciferase and ACTB are depicted in figures 3a and 3b.

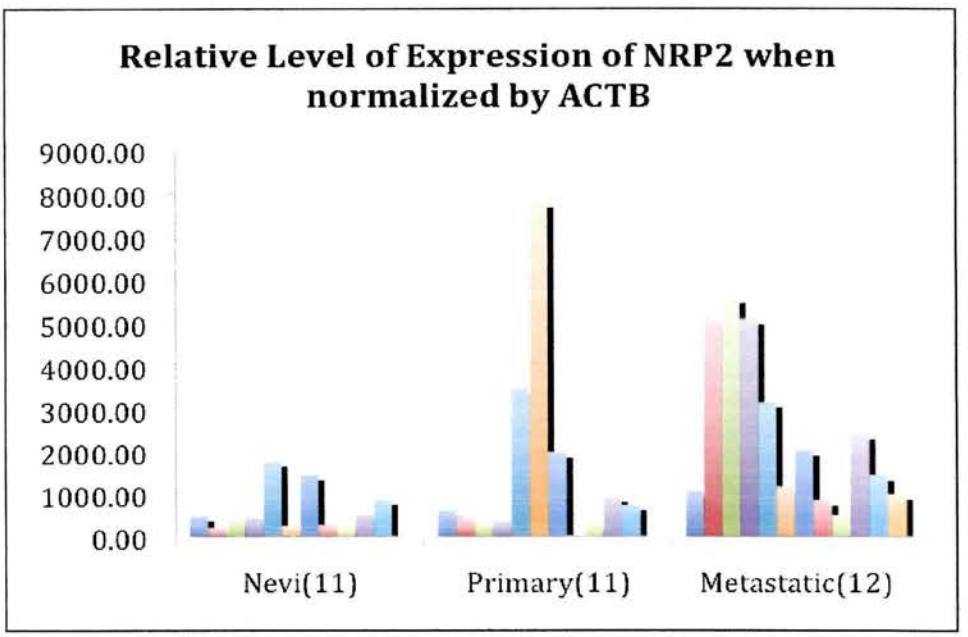

Figure 3a. A bar graph depicts the copy numbers of NRP2 when normalized by ACTB among nevi, primary melanomas, and metastatic melanomas. The numbers next to each group correspond to the sample size. Any sample that did not amplify in qRT-PCR or showed a poor melting curve was negated.

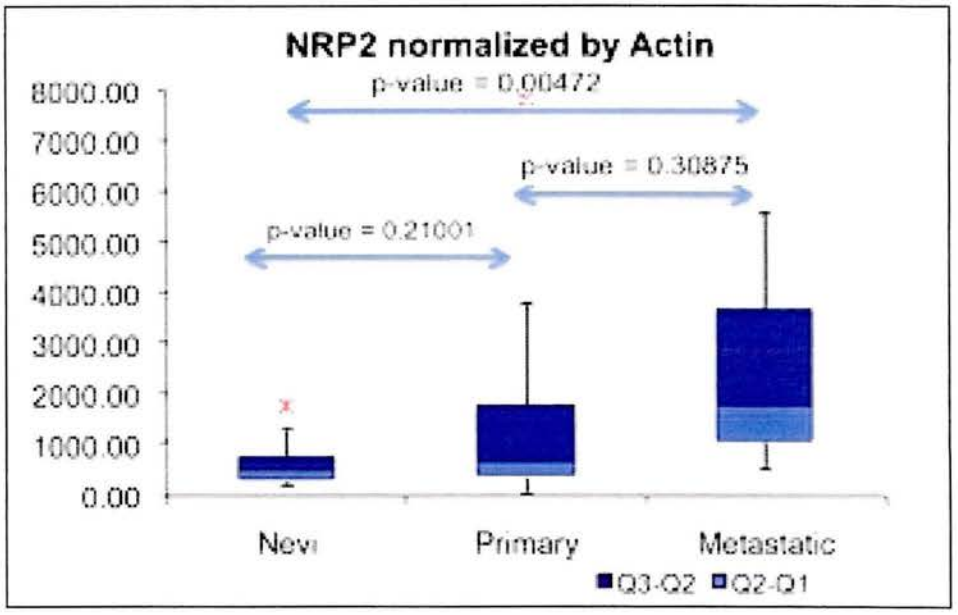

Figure 3b. A box and whisker plot showing the p-values and relative expression of NRP2 among nevi (n=11), primary melanomas (n=11), and metastic melanomas (n=12) when normalized by ACTB. Arrows correspond to the calculated p-value between those two groups. P-values were caluclated using two-tailed hypothesis testing assuming equal variances. Any sample that did not amplify in qRT-PCR or showed a poor melting curve was negated.

When just normalized by Luciferase and Melan-A the mean level of expression of NRP2 in nevi was 4630.28 copies, in primary melanomas was 9,050.67 copies, and in metastatic melanomas was 19,572.76 copies. The relative levels of expression of NRP2 when normalized by Luciferase and Melan-A are depicted in Figures 4a and 4b.

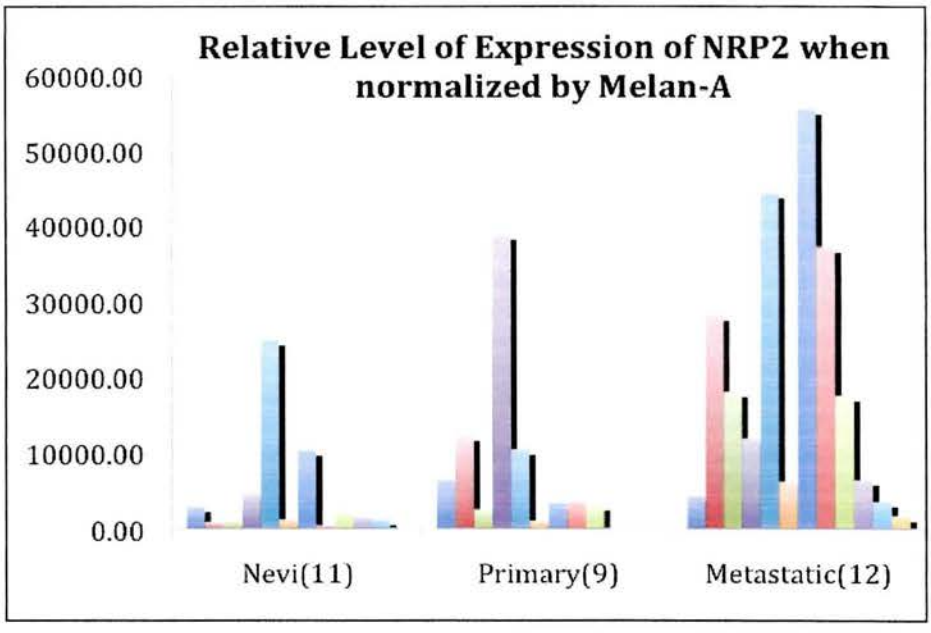

Figure 4a. A bar graph depicts the copy numbers of NRP2 when normalized by Melan-A among nevi, primary melanomas, and metastatic melanomas. The numbers next to each group correspond to the sample size. Any sample that did not amplify in qRT-PCR or showed a poor melting curve was negated.

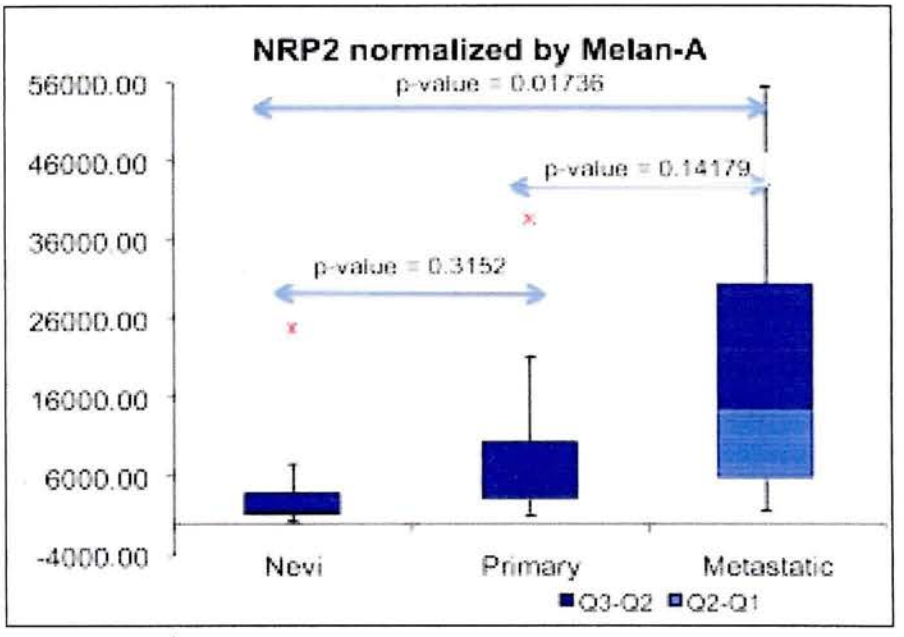

Figure 4b. A box and whisker plot showing the p-values and relative expression of NRP2 among nevi (n=11), primary melanomas (n=9), and metastic melanomas (n=12) when normalized by Melan-A. Arrows correspond to the calculated p-value between those two groups. P-values were caluclated using two-tailed hypothesis testing assuming equal variances Any sample that did not amplify in qRT-PCR or showed a poor melting curve was negated.

Finally the NRP2 copy numbers were normalized by both ACTB and Melan-A. Using all three genes for normalization of data, the mean level of expression of NRP2 in nevi was 326.10, in primary melanomas was 308.71, and in metastatic melanomas was 1282.51. The relative levels of expression of NRP2 when normalized by luciferase, ACTB, and Melan-A are depicted in Figures 5a and 5b.

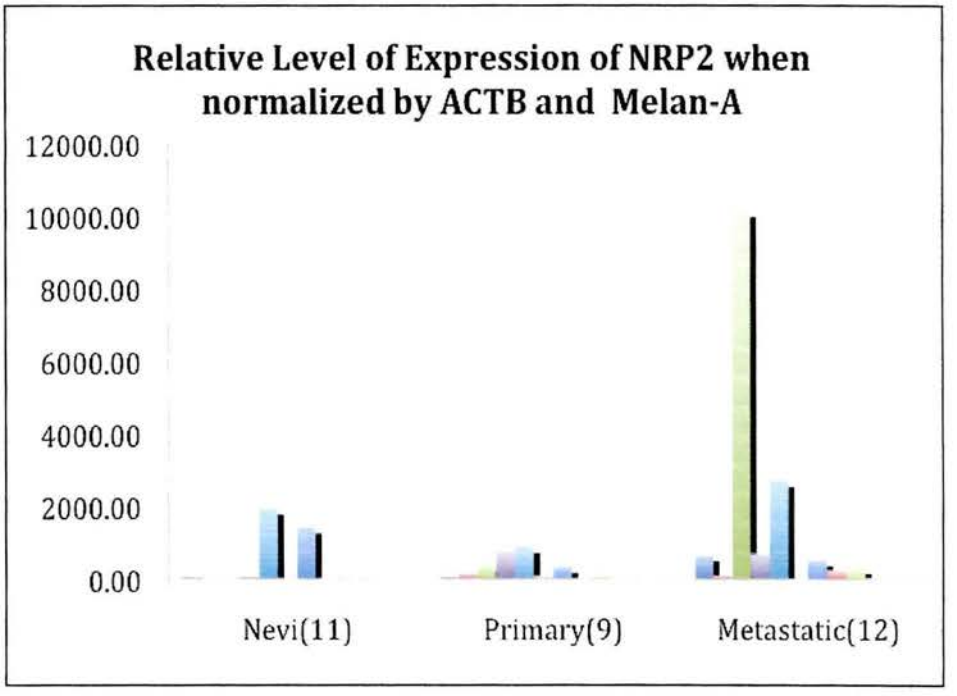

Figure 5a. A bar graph depicts the copy numbers of NRP2 when normalized by ACTB and Melan-A among nevi, primary melanomas, and metastatic melanomas. The numbers next to each group correspond to the sample size. Any sample that did not amplify in qRT-PCR or showed a poor melting curve was negated.

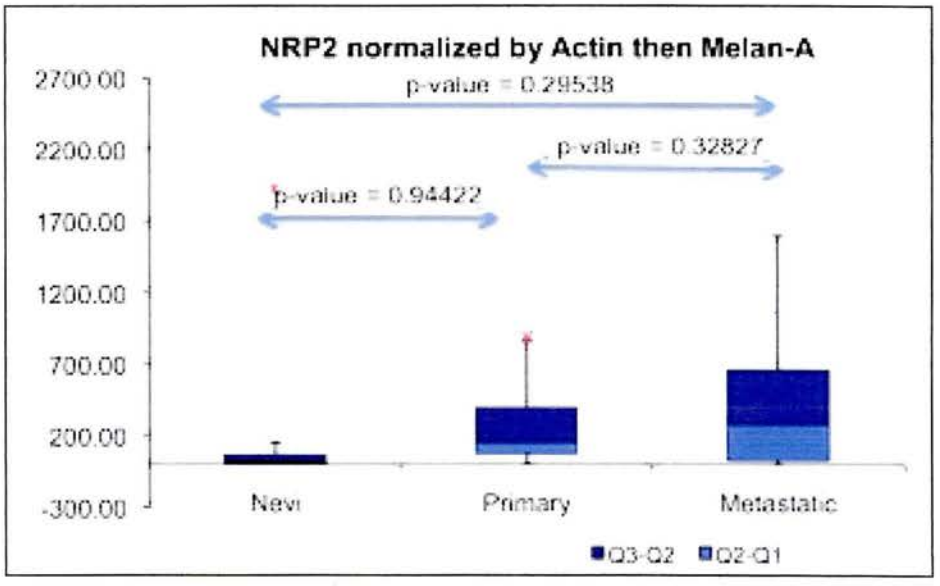

Figure 5b. A box and whisker plot showing the p-values and relative expression of NRP2 among nevi (n=11), primary melanomas (n=9), and metastic melanomas (n=12) when normalized by ACTB and Melan-A. Arrows correspond to the calculated p-value between those two groups. P-values were caluclated using two-tailed hypothesis testing assuming equal variances. Any sample that did not amplify in qRT-PCR or showed a poor melting curve was negated.

DISCUSSION

It is observed that there are varying levels of expression of NRP2 among nevi, primary melanomas, and metastatic melanomas, thus demonstrating that NRP2 shows promise as a biomarker for melanoma. To further assess the relationship between the level of expression of NRP2 in primary melanocytic lesions and the prognosis of melanoma, p-value analysis was performed. P-values were calculated using two-tailed hypothesis testing and assuming equal variances between nevi and primary melanomas, nevi and metastatic melanomas, and primary melanomas and metastatic melanomas among all of the performed normalizations.

In theory, using both ACTB and Melan-A to correct for differences between tissue samples should provide the most statistically accurate analysis. Unfortunately for this normalization, the differences in the level of expression of NRP2 between nevi and primary melanomas ($p=0.94422$), nevi and metastatic melanomas ($p=0.29538$), and primary melanomas and metastatic melanomas ($p=0.32827$) all failed to show statistical significance. In addition when normalized by only ACTB, there is no statistical significant difference in the level of expression of NRP2 between nevi and primary melanomas ($p=0.21001$), or primary and metatastic melanomas ($p=0.30874$). However, there is a difference between nevi and metastatic melanomas ($p=0.00472$) that is statistically significant when just normalized by ACTB. Furthermore, when just normalized by Melan-A, there is no significant difference in the level of expression of NRP2

between nevi and primary melanomas (p=0.3152) or primary melanomas and metastatic melanomas (p=0.14179). However, there is a statistically significant difference in the level of expression of NRP2 between nevi and metastatic melanomas (p=0.01736).

The fact that there is a statistically significant difference in the level of expression of NRP2 between nevi and primary melanomas demonstrates its ability to be used as a biomarker. Again, NRP2 is a protein expressed during angiogenesis and lymphangiogensis. In order for metastasis to occur and for the tumor to be able to spread to other parts of the body, these processes are crucial. Thus it is logical that the levels of NRP2 would continue to increase as both angiogenesis and lymphangiogenesis become more and more evident, or as the cancer progresses to become metastatic. Since both nevi and primary melanomas show no sign of metastasis it is acceptable that their levels of expression of NRP2 are not significantly different. A reason as to why there is no significant difference in the level of expression between primary melanomas and metastatic melanomas may be because even though the primary tumor has no signs of vascular or lymph invasion, there still may be something developing in the lesion to prepare for such processes. This is where establishment of a threshold for the level of expression of NRP2 may serve as prognosis factor for melanoma. It would be able to determine primary melanomas of low metastatic potential versus primary melanomas of high metastatic potential. For instance, if this sort of analysis was performed on a tissue biopsy, one could use this

threshold to determine if the lesion is going to progress to be metastatic and therefore give the patient a more accurate report on the current and future state of the disease.

Small sample size may also be a contributing factor as to why there is no difference among selected groups. The inadequate sample size may lead to invalid inferences because it has a decreased probability of providing a result that is statistically significant (Kalla, 2009). Unfortunately, resources were limited and the experiments could only be performed on the formalin-fixed paraffin-embedded tissue sections that were available. Assessing the NRP2 expression level in a larger sample size may be the next step in this study. Increasing the size would offer a more accurate result and thus a more legitimate conclusion.

In addition to increasing sample size, one may consider analyzing other potential biomarkers in order to develop somewhat of a multi-biomarker panel for evaluating melanoma. This is because tumor-related gene expression tends to be heterogeneous in melanoma (Medic et al., 2006). The levels of expression vary among lesions as seen in the NRP2 data from this experiment. Having more than one biomarker to analyze in the cancer would improve the methods for both diagnosis and prognosis and give the patient a more accurate assessment of their condition. In addition one could test the levels of expression to determine the effects of treatment on the patient.

In conclusion, the results from this study show that there is a potential relationship between the levels of expression of NRP2 and the different stages of

melanoma. In particular there is a statistically significant difference ($p<0.05$) in the level of NRP2 expression between the nevi tissues that were analyzed and the metastatic melanoma tissues that were analyzed, inferring that NRP2 does play a role in metastasis and could be used as both a diagnostic and prognostic biomarker for melanoma. Due to the limitations in this study, future investigations with a larger sample size would provide more accurate results and possibly show a stronger correlation between the levels of expression of NRP2 and the stage melanoma. Even so, NRP2 does show promise as a biomarker for melanoma.

REFERENCES

American Cancer Society. (2012). *Skin cancer facts.* Retrieved February 11, 2012, from http://www.cancer.org

Atherly, A., & Camidge, D. (2012). The cost-effectiveness of screening lung cancer patients for targeted drug sensitivity markers. *The British Journal of Cancer, 106*, 1100-1105.

Bhatia, S., Tykodi, S., & Thompson, J. (2009). Treatment of metastatic melanoma: An overview. *Oncology, 23*(6), 488-96.

Bielenberg, D., Pettaway, C., Takashima, S., & Klagsbrun, M. (2006). Neuropilins in neoplasms: Expression, regulation, and function. *Experimental Cell Research, 312*(5), 584-593.

Cai, H., & Reed, R. (1999). Cloning and characterization of neuropilin-1-interation protein: A PSD-95/Dlg/Zo-a domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1. *The Journal of Neuroscience, 19*(15), 6519-6527.

Ellis, L. (2006). The role of neuropilins in cancer. *Molecular Cancer Therapeutics, 5*(5), 1099-1107.

Erhard, H., Rietveld, F., van Altena, M., Brocker, E., Ruiter, D., & de Waal, R. (1997). Transition of horizontal to vertical growth phase melanoma is accomplished by induction of vascular endothelial growth factor expression and angiogenesis. *Melanoma Research, 2*, 19-26.

Favier, B., Alam, A., Barron, P., Bonnin, J., Laboudie, P., Fons, P., et al. (2005). Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. *Blood Journal, 108*, 1243-1250.

Feinberg, A., Ohlsson, R., & Henikoff, S. (2006). The epigenetic progenitor origin of human cancer. *Nature Reviews Genetics, 7*, 21-33.

Ferreira de Souza, C., Morais, A., & Jasiulionis, M. (2012). Biomarkers as key contributers in treating malignant melanoma metastases. *Dermatology Research and Practice, 2012*, 14.

Flaherty, K., Puzanov, I., Kim, K., Ribas, A., McArthur, G., Sosman, J., O'Dwyer, P., Lee, R., Grippo, J., Nolop, K., & Chapman, P. (2010). Inhibition of mutated, activated BRAF in metastatic melanoma. *The New England Journal of Medicine, 363*(9), 809-819.

Garbe, C., Eigentler, T., Keilholz, U., Hauschild, A., & Kirkwood, J. (2011). Systematic review of medical treatment in melanoma current status and future prospects. *The Oncologist, 16*(1), 5-24.

Garbe, C. et al. (2010). Diagnosis and treatment of melanoma: European consensus-based interdisciplinary guideline. *European Journal of Cancer, 46*(2), 270-283.

Hearing, V., & Leong, S. (2006). Melanocyte distribution and function in the skin. *From melanocytes to melanoma - the progression to malignancy* (pp. 102). New Jersey: Humana Press Inc.

Howlader, N., et al. (2010). *SEER cancer statistics review.* Retrieved February 17, 2012, from http://seer.cancer.gov/statfacts/html/melan.html#incidence-mortality

Kalla, S. (2009). Statistical Significance And Sample Size. Retrieved 17 Mar. 2012 from Experiment Resources: http://www.experiment-resources.com/statistical-significance-sample-size.html

Livingstone, E., Zimmer, L., Vaubel, J., & Schadendorf, D. (2012). Current advances and persepctives in the treatment of advanced staged melanoma. *The Journal of the German Society of Dermatology*, doi:10.1111/j.1610-0387.2012.07895.x

Mahabeleshwar, G., & Byzova, T. (2007). Angiogenesis in melanoma. *Seminars in Oncology, 34*(6), 555-565.

Mamluk, R., Gechtman, Z., Kutcher, M., Gasinuas, N., Gallagher, J., & Klagsbrun, M. (2002). Neuropilin-1 binds vascular endothelial growth factor 165, placenta growth factor-2, and heparin via its b1b2 domain. *The Journal of Biological Chemistry, 277*, 24818-24825.

Medic, S., Pearce, R., Heenan, P., & Ziman, M. (2006). Molecular markers of circulating melanoma cells. *Pigment Cell Research, 20*(2), 80-91.

Merk and Company Incorporated. (2011). *Melanoma.com.* Retrieved February 11, 2012, from http://www.melanoma.com/whatis.html

Miller, A. J., & Mihm, M. C. (2006). Melanoma. *The New England Journal of Medicine, 355*(1), 51-65. doi:10.1056/NEJMra052166

Moncrieff, M., & Garioch, J. (2011). *The Stages of Melanoma.* Retrieved February 11, 2012, from http://skincancersurgery.co.uk/Melanoma/Melanoma_staging.html

National Cancer Institute. (2011). *Dictionary of Cancer Terms*. Retrieved February 11, 2012, from http://www.cancer.gov/dictionary?cdrid=45618

National Cancer Institute. (2011). *Stages of melanoma.* Retrieved February 11, 2012, from http://www.cancer.gov/cancertopics/pdq/treatment/melanoma/Patient/page2#Keypoint10

Odorisio, T., Cianfarani, F., Failla, C., & Zambruno, G. (2006). The placenta growth factor in skin angiogenesis. *Journal of Dermatological Science, 43*(1), 71.

Ria, R., Reale, A., Castrovilli, A., Mangialardi, G., Dammacco, F., Ribatti, D., et al. (2010). Angiogenesis and progression in human melanoma. *Dermatology Research and Practice, 2010*, 185687.

Ribatti, D., Vacca, A., Ria, R., Marzullo, A., Nico, B., Filotico, R., et al. (2003). Neovascularisation, expression of fibroblast growth factor-2, and mast cell with tryptase activite increase simultaneously with pathological progression in human malignant melanoma. *European Journal of Cancer, 39*(5), 666-674.

Robert, C. & Ghiringhelli, F. (2009). What is the role of cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma? *Oncologist, 14*, 848-861.

Rushing, E., Stine, M., Hahn, S., Shea, S., Eller, M., Naif, A., et al. (2011). Neuropilin-2: A novel biomarker for malignant melanoma? *Human Pathology, 43*(3), 381-389.

Soengas, M., & Lowe, S. (2003). Apoptosis and melanoma chemoresistance. *Oncogene, 22*, 3138-3151.

Staton, C., Kumar, I., Reed, M., & Brown, N. (2007). Neuropilins in pathological angiogenesis. *Journal of Pathology, 212*, 237-248.

Stine, M., Wang, C., Moriarty, W., & et al. (2011). Integration of genotypic and phenotypic screening reveals molecular mediators of melanoma - stromal interaction. *Cancer Research, 71*, 2433-2444.

Zabierowski, S., & Herlyn, M. (2008). Melanoma stem cells: The dark seed of melanoma. *Journal of Clinical Oncology, 26*(17), 2890-2894.

VITA

[redacted]