Prognostic role of BRAF in Human Cutaneous Melanoma: Gene Versus Protein Expression

by

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Abstract

Melanoma is the deadliest type of skin cancer with an increasing incidence for past two decades. Once melanoma is metastasized (cancer cells are spread out through the body) there is no effective treatment available and 84% of the patients die within 5 years. However, the discovery of *braf* mutation in melanoma increased the hope for developing new treatments.

We first evaluated the effect of *braf* V600E mutation on melanoma patient survival. In a systematic review we revealed that patients with *braf* V600E mutation have almost two times more risk of death compared with patients with wild type *braf.* Next we evaluated the correlation of *braf*V600E mutation with protein expression. We found that compared with nevi samples, BRAF protein expression was remarkably increased in primary melanomas and further increased in metastatic melanoma patients. Higher BRAF protein expression was significantly correlated with other poor prognosis factors and lead to a significant worse five-year survival. However, we did not find a significant correlation between BRAF protein expression and *braf* V600E mutation. In our attempt to investigate the cause of induced BRAF protein, we found novel expression of BRAF splice variants (BRAFsv) in both melanoma patients and cell lines. We identified new kinase-dead BRAFsv, which have a dominant negative effect on full-length BRAF and are able to suppress downstream signaling and reduce melanoma cell proliferation. These variants were highly expressed in primary melanoma compared to normal samples, while the expression was decreased in metastatic and more aggressive types of melanoma. In addition, kinase-dead BRAFsv showed a protective effect on patient survival, which remained significant at the presence of full-length BRAF. Thus, patients who expressed the kinase-dead variant and had lower levels of full-length BRAF expression showed the best survival rate in 5 years. Our in-

vitro analysis also indicates that over expression of kinase-dead BRAFsv in melanoma cell lines enhances the effect of BRAF inhibitor treatment.

All in all, the data presented in this thesis elucidated a new era in the evaluation of melanoma patient prognosis and revealed new possibilities for more effective melanoma treatments.

Preface

Contributions

1. A version of chapter 3 has been published in PLoS One [Ardekani GS] Jafarnejad M, Tan L, Saeedi A, Li G. The Prognostic Value of BRAF Mutation in Colorectal Cancer and Melanoma: A Systematic Review and Meta-analysis. Dr. Li supplied all the facilities and contributed to the overall outline of the manuscript and review of the content. Dr. Jafarnejad M, and Tan L, participated in the literature review and writing of the manuscript. Mr. Saeedi A, helped with the research outline and statistical analysis. I performed the literature review, study design, statistical analysis, and prepared the manuscript.

2. A version of chapter 4 has been published in Br J Dermatology [Ardekani GS], Jafarnejad M, Martinka M, Ho V, Li G. Disease Progression and Patient Survival Are Significantly Influenced by BRAF Protein Expression in Primary Melanoma. Dr. G. Li provided all the required facilities and materials and contributed to the design of experiments. Dr. Jafarnejad M, helped with the design of the experiments, PCR and primer design and the outline of the study. Dr. M. Martinka provided the patients tissue samples and assisted with immunohistochemistry scoring of TMA slides. I contributed to the study design, performed all presented experiments and prepared the final manuscript.

3. A version of chapter 5 has been submitted for publication. [Ardekani GS], Jafarnejad M, Mousavizadeh SR. Khosravi Sh, Martinka M, Zhou Y. McElwee KJ, Duronio V. New Kinase Dead Splice Variant of BRAF Identified in Melanoma that is Correlated with Improved Patient Prognosis and Survival. Dr. V. Duronio provided all the required facilities and materials and contributed to the design of experiments. Dr. Y.

Zhou have contributed to the overall research design and provided some patients samples, and the materials needed for the experiments. Dr. K McElwee has contributed to the research design and helped with the experimental troubleshooting. Dr. M. Jafarnejad helped with the experimental design, PCR and primer design and the outline of the study. Dr. M. Martinka provided the patients tissue samples and assisted with immunohistochemistry scoring of TMA slides. Mr. R. Mousavizadeh and P. Hojabrpor helped with western blot, functional studies. I contributed to the study design, performed all presented experiments and prepared the final manuscript.

List of publications

Below is the list of my publications directly related to the data presented in this thesis. For a complete list of my publications please refer to the appendix.

1. **Ardekani GS**, Jafarnejad M, Mousavizadeh SR. Khosravi Sh, Martinka M, Zhou Y. McElwee KJ, Duronio V. New Kinase Dead Splice Variant of BRAF Identified in Melanoma that is Correlated with Improved Patient Prognosis and Survival (Manuscript submitted for publication)

2. **Ardekani GS**, Jafarnejad M, Martinka M, Ho V, Li G. Disease Progression and Patient Survival Are Significantly Influenced by BRAF Protein Expression in Primary Melanoma. Br J Dermatol. 2013 Apr 1. doi: 10.1111/bjd.12351.

3. **Ardekani GS**, Jafarnejad M, Tan L, Saeedi A, Li G. The Prognostic Value of *BRAF* Mutation in Colorectal Cancer and Melanoma: A Systematic Review and Meta-analysis. PLoS One. 2012;7(10):e47054. doi: 10.1371/journal.pone.0047054.

Ethics certificate

The use of human skin tissues in this study was approved by the Clinical Research Ethics Board of University of British Columbia (certificate number is H09-01321).

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List of abbreviations

Abbreviation	Definition
AA	Amino Acid
ACC	Adenoid Cystic Carcinoma
AJCC	American Joint Committee on Cancer
AKT	Protein Kinase B-α
ALM	Acral Lentiginous Melanoma
Ap-1	Activator protein-1
Apaf-1	Apoptotic protease activating factor-1
ARF	Alternative Reading Frame
Bax	Bcl2- associateed X protein
Bcl2	B-cell lymphoma 2
BRAF	Ras Associated Factor B/ V-raf murine sarcoma homologue B
BRCA1	BReast CAncer 1
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
CDK	Cyclin Dependent Kinase
CDKN1B	Cyclin-Dependent Kinase Inhibitor 1B
cDNA	complementary DNA
c-Myc	cellular Myelocytomatosis viral oncogene
CRD	Cysteine Rich Domain

CREB	cAMP Responsive Element Binding protein
CtBP2	C-terminal-Binding Protein 2
DGCR8	DiGeorge Syndrome Critical Region 8
DMEM	Dulbecco's Modified Eagle Medium
DN	Dysplastic Nevi
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
ECM	Extra-cellular Matrix
EDTA	EthyleneDiamineTetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EMT	Endothelial-Mesenchymal Transition
ERK	Extracellular Regulated Kinase
FDA	Food and Drug Administration for USA
GPCR	G Protein–Coupled Receptor
GTP	Guanosine Triphosphate
H&E	Hematoxylin and Eosin
H_2O_2	Hydrogen Peroxide
HER-2	Human Epidermal Growth Factor Receptor-2
HIF-1	Hypoxia Inducible Factor-1
HMG	High Mobility Group
hMSCs	human Mesenchymal Stem Cells
IFN	Interferon
kb	kilobases

kDa	kilo Dalton
MAPK	Mitogen Activated Protein Kinase
MC1R	Melanocortin 1 Receptor
MDM2	Mouse Double Minute 2
MEK	Mitogen-activated protein kinase Kinase
MITF	Microphthalmia Transcription Factor
MM	Metastatic Melanoma
MMP	Matrix Metalloproteinase
mRNA	messenger Ribonucleic Acid
MSH	Melanocytes-Stimulating Hormone
mTOR	Mammalian Target of Rapamycin
NN	Normal Nevi
NRG1	Neuregulin 1
NSCLC	Non-Small Cell Lung Cancer
nt	nucleotide
ORF	Open Reading Frame
p21	protein 21
PACT	Protein Activator of PKR
PBS	Phosphate Buffered Saline
PBST	PBS containing 0.05% Tween-20
PCR	Polymerase Chain Reaction
PFS	Progression Free Survival
PG	Prostaglandin

PI3K	Phosphoinositide 3-Kinase
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PM	Primary Melanoma
Pol II	Polymerase II
PTEN	Phosphatase and Tensin Homolog
PTH/PTHrP	Parathyroid Hormone /Parathyroid Hormone-related Protein
PUMA	p53 Upregulated Modulator of Apoptosis
PVDF	Polyvinylidene Difluoride
qPCR	quantitative PCR
Rac	GTPase-activating protein
RB	Retinoblastoma
RBD	RAS Binding Domain
RelA	Reticuloendotheliosis viral oncogene homolog A
RGP	Radial Growth Phase
Rho	Ras homolog
RNAse	Ribonuclease
ROCK	RHO-associated serine/threonine kinase
RR	Response Rate
rRNA	ribosomal RNA
SDS-PAGE	Sodium Dodecyl Sulfate -Polyacrylamide Gels Electrophoresis
siRNA	small interfering-RNA
SSM	Superficial Spreading Melanoma
Sox	SRY-related HMG box

SRB	Sulforhodamine B
TGF-β	Transforming Growth Factor Beta
TMA	Tissue Microarray
TOPO2A	Topoisomerase IIA
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VGP	Vertical Growth Phase
Wint	Wingless and int-1

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Dedication

First and foremost, this thesis is dedicated to my beloved angel mother and my father whose unconditional love and support have always been with me throughout my life. Words can never express my gratitude and appreciation for their endless support and huge sacrifices they have made for me. I also express my heartfelt gratitude to my sisters and brother who have always been on my side even when I was far away from home. I am exceedingly grateful for their gracious support, light-heartedness, and unrivaled belief in me.

Last but not least, I owe my deepest gratitude to the love of my life, my wife and dearest friend who has created the most beautiful moments of my life.

Chapter 1. Introduction

1.1. Tumorigenesis and cancer development

Cancer development is a very complex process influenced by genetic or epigenetic alterations that causes sustained proliferative signaling, evading cell death, growth suppressor signal resistance, replicative immortality, sustained angiogenesis, genomic instability, reprogramming of energy metabolism, evading immune destruction and activating invasion and metastasis mechanisms, which all characterize a malignant growth (Hanahan et al. 2011). Normally the functions of single cells are tightly controlled by means of surveillance and repair mechanisms directed by their genetic programming and environmental constraints, leading to tissue homeostasis (Chow et al. 2012). Failure of these mechanisms due to endogenous errors or exogenous stimuli, results in tumorigenesis or inactivation of the restraining measures. The output of these changes is a progressive genetic alteration that transforms the normal healthy cell into one that begins to grow when it should not and eventually may develop into a highly malignant derivative. Several genetic and epigenetic changes are involved in the progression of cancer. Each change will confer one or more new features to the cancer cell, enabling it to defy the internal and external constraints and continue in gradual increases in size, number, disorganization and malignancy (Vogelstein et al. 1993). One of the main characteristics of cancer cells is the ability of indefinite proliferation, which usually happens through alteration of cell proliferation and apoptosis signaling pathways.

1.2. Cutaneous melanoma

1.2.1. Molecular biology of normal melanocytes

Melanoma is the most fatal type of skin cancer, originating from melanocytes, the pigment producing cells in human skin (Gray-Schopfer et al. 2007). Melanocytes are also present in oro-nasal mucosa, esophagus, rectum, penis, vagina, conjunctiva, and meninges (Pandey et al. 1998, Larsson et al. 1999). Embryonic neural crest progenitors, melanoblasts, are the origin of melanocytes. Through a complex of signaling pathways melanoblasts migrate, proliferate and differentiate into melanocytes at their final destinations (Ernfors 2010). Briefly, melanoblasts first lose their adhesions to neighboring cells and undergo epithelial-mesenchymal transition (EMT), which is followed by cell migration and niche localization (Cano et al. 2000). Expression of β-catenin through wingless and int-1 (Wnt) family signaling, and FoxD3 and Notch expression play a critical role in melanocyte lineage development from glial-melanocyte progenitors (Dorsky et al. 1998, Ernfors 2010). However, more recently the role of several other critical genes in melanocyte development, including microphthalmia transcription factor (MITF), c-kit, snail/slug, sox10 and endothelins, has been verified (Uong et al. 2010). The basal keratinocyte layers of epidermis in skin and hair follicles are the final destination of melanocytes in human skin, where keratinocytes regulate their homeostasis (Tanimura et al. 2011). In mature melanocytes, melanin is produced as a consequence of tyrosinase activity on tyrosine within lysosome-liked organelles called melanosomes. Through a guide of protease-activated receptor 2 (PAR-2), melanin is then transferred via melanocytic dendrites to an average of 36 adjacent keratinocytes and hair follicles (Miyamura et al. 2007). In addition to skin color and tone, melanin also protects against UV induced DNA damage. Genetic and epigenetic changes in important growth regulatory genes contribute to disrupted intra-cellular signaling in

melanocytes, allowing them escape the tight regulation by keratinocytes (Haass et al. 2004). Consequently, melanocytes can proliferate and spread in limited amount, leading to formation of a nevus (common mole). The control of growth in nevus cells is disrupted but in general, nevi are benign and their growth potential is limited. Restriction of nevus melanocyte proliferation to the epidermis or the dermis would cause junctional or dermal nevus respectively while the overlap of both components leads to a compound nevus. Dysplastic nevi are more advanced melanocytic lesions with morphologically atypical melanocytes. They are usually characterized by random proliferation of atypical nevi melanocytes, lentiginous or epithelioid-cell pattern, neovascularization, and enhanced inflammatory response (Clemente et al. 1991). Occasionally neval cells can progress to the radial growth-phase (RGP) melanoma, in which the cells acquire the ability to proliferate intra-epidermally. Melanocytes tend to grow horizontally within the epidermis while some small local micro-invasions to the dermis also might happen at this stage (Piris et al. 2009). Once the RGP melanoma cells acquire a more aggressive invasive phenotype, they are considered to have entered the vertical growth phase (VGP). VGP represents a more advanced stage in melanoma development, characterized by the emergence of cells with metastatic potential and nodules or nests of cells invading the dermis (Piris et al. 2009). Usually, RGPs have good prognosis and are curable by excision. However, the cells in VGP are considered to have metastatic potential with poor prognosis (Clark et al. 1984). Notably, not all cases of primary melanoma pass through each and all discussed phases. Indeed, some melanoma cases progress directly to malignant melanoma without any visible earlier stages (Clark et al. 1984). The ultimate stage of melanoma progression (metastatic melanoma) is characterized by dislocation of cells into other areas of the skin and/or other body organs in which they will form secondary colonies (Miller et al. 2006).

1.2.2. Molecular biology of melanoma

The association of several endogenous and environmental risk factors with melanoma development has been discussed over the years. Among all known factors, previous personal or family history of melanoma, multiple dysplastic and/or benign nevi, long-term sun exposure, history of sun burn in early childhood, fair skin type, blond or red hair, and tendency to burn easily and tan poorly, have shown the strongest associations (Bliss et al. 1995, Miller et al. 2006).

Although the prevalence of sporadic melanoma is higher than familial types (Skolnick et al. 1994), studies investigating the genetic basis of familial melanoma have provided much critical information regarding melanoma pathogenesis. Accordingly, mutations in several components of major signaling pathways and regulatory mechanisms have been discovered, which predispose the family members to this malignancy. So far several predisposing genes for melanoma with varying degree of penetrance have been identified, based on the discovery of germline mutations in multiple-case families.

Cyclin-dependent kinase inhibitor (CDKN2A) is the best-characterized gene with high level of penetrance, which is lost or inactivated in 25% to 40% of familial melanoma cases (Nobori et al. 1994, Borg et al. 2000). CDKN2A encodes two distinct melanoma predisposing genes, p16 (INK4a) and p14 (ARF)(Quelle et al. 1995). The INK4a protein binds to Cyclin Dependent Kinase 4/6 (CDK4/6) and inhibits its interaction with cyclin D, preventing the subsequent phosphorylation of the retinoblastoma protein (pRB). Suppression of G1 phase of the cell cycle is the consequence of pRB inactivation (Alcorta et al. 1996). On the other hand, ARF was initially found to function in a cyclin D-independent but p53-dependent manner (Quelle et al. 1995) to induce cell cycle arrest and inhibit cell transformation. It was later demonstrated that

ARF inhibits the ubiquitination of p53 by binding to the mouse double minute 2 (MDM2) and ARF-BP1/Mule ubiquitin ligases (Chen et al. 2005), thereby stabilizing this critical tumor suppressor protein and causing cell cycle suppression and tumorigenesis. Interestingly, genetic change in other component of these pathways, such as CDK4 and MDM2, were described in human melanomas without concurrent loss of INK4a and ARF. This indicates that overexpression of CDK4 and MDM2 may substitute for loss of INK4A and ARF function at least in a subset of melanomas (Muthusamy et al. 2006).

CDK4 itself is also a key regulator of cell cycle progression, which encodes the cyclin-dependent kinase 4 protein (Zuo et al. 1996). Activation of CDK4 allows the cell to bypass the G1/S cell-cycle checkpoint through phosphorylation and inhibition of pRB that result in release and activation of the E2F transcription factor, leading to initiation of DNA replication and progression of cell cycle (Kato et al. 1993). The majority of germ line CDK4 mutations (repeatedly seen in familial melanoma) affect the critical Arg-24 amino acid that is essential for INK4a–CDK4 interaction. When this amino acid is mutated INK4a can no longer bind to and inactivate the CDK4 protein allowing uncontrolled cell proliferation (Clemente et al. 1991).

Melanocortin 1 receptor (MC1R) is a melanoma susceptibility gene that encodes a seven trans membrane G protein–coupled receptor (GPCR) expressed on epidermal melanocytes. In normal melanocytes, binding of the melanocytes-stimulating hormone (α-MSH) to MC1R stimulates adenylyl-cyclase and leads to generation of cAMP, which in turn enhances transcription of microphthalmia transcription factor (MITF), tyrosinase, and other enzymes necessary for production of melanin through cAMP responsive element binding protein (CREB) (Schaffer et al. 2001). Therefore MC1R is a key regulator in production of eumelanin (brown/black) and pheomelanin (red) in human skin, ratio of which controls the skin and hair

color. Some variants of MC1R gene have been shown to control skin response to UV radiation and to be associated with melanoma development in humans while they are also associated with the phenotypic risk factors for melanoma like red hair and fair skin types (Valverde et al. 1996, Flanagan et al. 2000, Healy et al. 2000, Palmer et al. 2000). Dutch families with familial atypical multiple mole melanoma show an increased risk of melanoma when they carry MC1R in addition to p16-Leiden variant alleles. Although some parts of increased melanoma risk in patients with MC1R variants might be attributable to its effect on skin type, van der Velden and colleagues (van der Velden et al. 2001) showed that the R151C variant contributes to an increased melanoma risk even after statistical correction for its effect on skin type. These findings suggest a dual involvement of MC1R variant in melanoma tumorigenesis including a determinant of fair skin type (as a melanoma risk factor) and as an independent component in melanoma genesis pathways.

The Extracellular Regulated Kinase/Mitogen Activated Protein Kinase (ERK/MAPK) pathway is the most studied signaling pathway in melanoma. Activation of receptor tyrosine kinases triggers the pathway through recruitment of the rapidly accelerated fibroblast (RAF) family of serine/ threonine kinases (RAF kinase) members (RAF-1, BRAF and ARAF) to the plasma membrane (Chang et al. 2001). RAF kinases phosphorylate and activate the MAPK/extracellular-signal-regulated kinase (MEK1 and MEK2) that in turn phosphorylate extracellular signal-regulated kinase ERK (ERK1 and ERK2) (Chang et al. 2001). Activation of this pathway plays an important role in melanoma progression and will be discussed in more detail in the upcoming chapters.

The phosphoinositide 3-Kinase (PI3K) pathway, and more importantly PTEN protein, are also involved in melanoma genesis and progression. PI3K phosphorylates the 3-O-hydroxyl

group of phosphoinositides producing phosphatidylinositol-3,4,5-trisphosphate or PIP3, a critical second messenger which recruits multiple downstream mediators such as PDK1 and AKT for activation of growth, proliferation and survival signaling. This signal is mostly transduced through activation of several prominent downstream cascades such as mTOR and MAPK pathways (Cantley 2002). Mutation, overexpression or alteration in activity of the PI3K pathway has been commonly observed in melanoma (Meier et al. 2005). However, PTEN phosphatase mostly plays the important role of negatively regulating this pathway by dephosphorylation of PIP3 at the 3 position (Cantley 2002). PTEN was initially identified as a candidate tumor suppressor after its positional cloning from a region of small arm of chromosome 10, known to be lost in a wide range of tumors (Steck et al. 1997). Loss of PTEN expression due to chromosomal deletion has also been observed in melanoma while its mutation has been detected in a variety of human cancers as well as melanoma (Guldberg et al. 1997, Teng et al. 1997). Consistent with a tumor suppressor role for PTEN, induced overexpression of PTEN in melanoma cells lacking PTEN protein suppressed tumor development in mice, evidently through suppression of AKT activity, with consequent up-regulation of the apoptotic pathway of melanoma cells (Stahl et al. 2003).

The Nuclear Factor κB (NF-κB) is another molecular pathway whose deregulation has been frequently observed in melanoma. In mammals, there are five NF-κB subunits (RelA/p65, RelB, p105/p50, c-Rel, and p100/p52) all of which contain a Rel homology domain (RHD) in their N-termini mediating DNA-binding and dimerization (Perkins 2000). Typically, in non-stimulated normal cells, NF-κB subunits are held in an inactive cytoplasmic form bound to a member of the IκB protein family, IκB α, β or ε. Exposure to inflammatory cytokines like TNFα stimulates the signaling pathways that result in the activation of the IκB kinase (IKK) complex.

The IKK complex phosphorylates members of the IκB family (α, β or ε), resulting in their ubiquitination and proteasomal degradation. Upon degradation of IKB, NF-κB translocates to the nucleus where the regulation of target gene expression is controlled (Ghosh et al. 1998). The NF-κB pathway is known to induce several oncogenic mechanisms and is believed to be constitutively active in multiple types of cancers, including melanoma (Davis et al. 2001, Yang et al. 2001, Gilmore 2003). A variety of mechanisms have been found by which the NF-κB pathway is uncoupled from its normal modes of regulation, thereby promoting cancer development. Previous studies have shown that the expression and DNA binding ability of NF-κB is elevated in melanoma cells relative to normal melanocytes (Dhawan et al. 2002) and the level of its inhibitor IκB is significantly lower in metastatic melanoma biopsies (McNulty et al. 2004). Moreover, recently our lab also reported that NF-κB p50 expression is increased with melanoma progression and overexpression of NF-κB p50 enhanced melanoma cell migration (Gao et al. 2006) and angiogenesis (Karst et al. 2009, Wani et al. 2011).

1.2.3. Epidemiology of melanoma

Melanoma incidence is almost ten fold less than epithelial skin cancers, but it has the highest disease burden because of its capacity for early metastasis and young age involvement. The incidence of melanoma has been reported to increase over the past 10 years among Caucasians (Linos et al. 2009). Studies have shown that the lifetime risk of melanoma development has risen from 1:1,500 in 1935 to 1:120 in 1987 and lately up to 1:55 (Erickson et al. 2010). This increase in risk is actually at a faster rate than any other cancer and raises concerns for a melanoma epidemic. The increase of annual melanoma incidence was estimated between 2 to 7%, which means a doubled incidence rate every 10 years (Garbe et al. 2009, Linos et al. 2009, Nikolaou et al. 2014).

According to the Canadian Cancer Society, melanoma is the seventh most common cancer in women in Canada. In 2014, an estimated 6500 (14.45 per 100,000 capita) new melanoma cases are expected to be diagnosed in Canada (Canadian Cancer (2014). The same report also predicted an estimated 970 cases of melanoma diagnosis in British Columbia in 2014.

1.2.4. Melanoma diagnosis and classifications

It is very important to remember that the dermatologic examination for melanoma diagnosis should include evaluation of the whole body not a single lesion. A major part of melanoma diagnosis is made based on examination of the general appearance, morphology and the distribution pattern of pigmented lesions. For example, the "Ugly duckling sign" which refers to a distinct appearance of a pigmented lesion compared to others, is one of the diagnostic criteria that is even used by patients themselves for self-evaluation. Other main criteria for initial melanoma evaluations are categorized as A (asymmetry), B (border), C (color), D (diameter), and E (evolution), which are very helpful but not always reliable, especially in early melanoma cases (Tronnier et al. 2013). However, the gold standard for diagnosis of melanoma is histopathology evaluation. The signs for melanoma in a routine Hematoxilin and Eosin staining are larger epithelioid melanocytes arranged in pagetoid pattern with hyper chromatic nuclei, usually accompanied with a few abnormal mitoses and altered nuclear-cytoplasmic ratio. Based on the stage of melanoma lesion, invasion of melanocytes into the connective tissue in the form of sheets, cords and islands might be detectable as well. Other types of specific staining like Fontana could be used to detect melanin content, usually 50-70% (Batsakis et al. 1982). Immunohistochemistry, as a more specific method has been utilized to detect S100 and HMB45 in melanoma. S100 is a calcium binding protein involved in cell surface transport and in RNA polymerase activity, while HMB45 is an antibody against gp-100 glycoprotein, as part of a pre-

melanosome complex. S100 has a low specificity as it is expressed in many other cells as well, but it has a very high sensitivity and is positive in almost all melanoma cases (Argenyi et al. 1994, Piris et al. 2009). On the other hand, HMB45 is more specific for melanoma than S100 but it has low sensitivity (Marincola et al. 1996). Anti MART-1 and anti Melan-A are two other antibodies against the same melanocyte differentiation antigen (Fetsch et al. 1999). This protein represents the trans-membrane part of the pigment-producing apparatus and is recognized by T cells (Fetsch et al. 1999). Because the genes coding MART-1/Melan-A is only expressed in melanocytes, these two antibodies have more specificity than S100 and more sensitivity than HMB45 (Piris et al. 2009).

There are four main clinical subtypes of primary melanoma. Superficial spreading melanoma (SSM) is usually flat with an intra-epidermal component, particularly at the edges and is associated with severe sunburn, especially at an early age. SSM is the most common form of melanomas (about 70%) and is the third most common cancer in young people in the UK and USA (Ishihara et al. 2001, Gray-Schopfer et al. 2007). Nodular melanoma consists of raised nodules without a significant flat portion, and accounts for 15% to 30% of all melanomas. Acral lentiginous melanoma (ALM) that is mainly found on the palms and soles (hand & feet) and in the nail bed, accounts for only 5% of melanomas. Interestingly, ALM is not associated with UV exposure and accounts for about 50% of melanoma in non-Caucasian populations (Gray-Schopfer et al. 2007). The last main subtype of melanoma is lentigo maligna, which comprises roughly 5% of all melanomas and is generally flat in appearance. It is associated with chronic sun exposure; therefore mainly occurs on sun-exposed regions in elderly individuals.

As for melanoma classification, the standard method adopted by the American Joint Committee on Cancer (AJCC) is currently the most commonly used method (Balch et al. 2009).

The current AJCC staging system is based on TNM cancer staging, which was substantially revised in 2001 on the basis of analysis for 17,600 patients and then revised in 2009 with a much higher pool of patients (Balch et al. 2009). In fact, tumor thickness and ulceration status was first added to the conventional TNM staging (T; extent of tumor, N; lymph node involvement, and M; distant metastasis) for primary melanoma staging in the AJCC system. In the most recent version of the AJCC staging system, mitotic rate was added to the criteria for primary melanoma and the two most important components for metastatic melanoma evaluation remained as site of distant metastasis and the level of serum lactate dehydrogenase (LDH) (Balch et al. 2009).

Briefly, in AJCC staging, primary melanoma tumor thickness <1.0 mm or between 1.01-2.0 mm without ulceration are considered stage I. Primary melanoma patients with tumors thicker than 2.0 mm or between 1.01-2.0 mm with ulceration are categorized as stage II. Stage III consists of patients with lymph node involvement (even microscopic nodal metastasis) with no distant metastasis, and patients with distant metastasis are classified as stage IV (Balch et al. 2001). The significant prognostic value of AJCC staging has been shown over the time while higher stages confer a poorer prognosis for the patients (Baitei et al. 2009).

1.3. MAPK pathway

Three mitogen activated protein kinase families have been clearly characterized in mammalian cells; namely classical MAPK (known as ERK), C-Jun N-terminal kinse/ stress activated protein kinase (JNK/SAPK) and p38 kinase. Each of these cascades at least contains three typical kinases that act sequentially; a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAP kinase (MAPK). So far, at least 14 different MAPKKKs, 7 MAPKKs, and 12 MAPKs have been identified in mammalian cells (Widmann et al. 1999, Zhang et al. 2002). MAPK pathways receive the signals from a diverse range of stimuli and conduct a critical

role in cell proliferation, differentiation, development, inflammatory response and apoptosis (Zhang et al. 2002). Over activation of the MAPK pathway is a very common event in many cancers that causes a continuous proliferation signaling (Hanahan et al. 2011). The MAPK pathway is comprised of a series of critically conserved enzymes (mostly kinases) connecting events at the cell surface to the important regulatory transcription factors in the nucleus. In normal situations, MAPK stimulation starts with binding of an external ligand (e.g. epidermal growth factor family) to receptor tyrosine kinases (RTKs) (e.g. epidermal growth factor receptors (EGFRs)). Upon stimulation, RTKs interact to form receptor dimers, which changes the conformation of their cytoplasmic domain and reveals its latent tyrosine kinase activity. The phosphorylated tyrosines act as docking sites for Src-Homology 2 (SH-2) domain containing proteins such as Grb-2 adaptor proteins, which in turn recruit guanine nucleotide exchange factors (like SOS) to the site. Interaction of Grb-2 and SOS recruits inactive RAS-GDP complex to the site and induces its activation by the exchange of GTP in place of GDP (Downward 2003). Activation of RAS then induces a cascade of kinases and subsequent downstream activity both through MAPK and PI3K/Akt/mTOR pathways. In normal cells with no stimulation, RAF is stabilized in an inactive dimer conformation by a dimer of 14-3-3 proteins. Each 14-3-3 monomer binds to a phosphoserine residue in RAF (Ser-259 and Ser-621). Binding of activated RAS to RAF dimers induces a conformational change in RAF that disrupts their association with 14-3-3 proteins. Release of dephosphorylated Ser-259 then induces RAF kinase activity, which in turn recruits MEK, ERK, and scaffolding proteins to the cell membrane, thus leading to the phosphorylation of MEK and ERK (Salomon et al. 1995, Lemmon et al. 2010). Active ERK also would exert its functions through phosphorylation of downstream effectors such as fos, MITF, myc and Ap-1 (Lemmon et al. 2010).

1.3.1. MAPK pathway alterations in melanoma

Three isoforms of RAS (HRAS, KRAS, and NRAS) are the most frequently mutated oncogenes in human cancers (Bos 1989). KRAS mutations are widely found in colorectal cancer (CRC), Non-Small Cell Lung Cancer (NSCLC), and 90% of pancreatic adenocarcinoma. NRAS is the second most commonly mutated gene in melanoma patients with wild type *braf* (40%), and 56% of congenital nevi (Papp et al. 1999, Dereure 2014). NRAS mutations are also present in 14% of non-cutaneous melanomas including brain, uveal and mucosal primaries and are absent in melanoma of soft parts (Wong et al. 2005). Interestingly, NRAS mutations were also reported in a majority of congenital nevi samples while they did not harbor *braf* mutations (Bauer et al. 2007). Activating NRAS point mutations were also reported in primary melanoma (33%) (Papp et al. 1999) and metastatic melanoma samples (26%) (Demunter et al. 2001). Because *braf* mutations induce a RAS-independent activation of MAPK pathway, it is not surprising to find *nras* and *braf* mutations mutually exclusive (Tsao et al. 2004, Goel et al. 2006). Spitzoid melanoma is a distinguished type of melanoma lesion like a papule or nodule that could be amelnotic and sometimes ulcerated. Although Spitzoid melanoma resembles a benign lesion called Spitz nevi which has a high rate of HRAS mutation, the malignant lesion has been reported to be very low in HRAS mutation rate (van Dijk et al. 2005, Da Forno et al. 2009). In contrast to *braf* and *nras*, the mutations in downstream factors of this pathway (*mek1* and *mek2*) are not prevalent in cancers or other human diseases (Schubbert et al. 2007). However, MEK mutations have been reported in patients undergoing BRAF inhibitor treatments as a mechanism for treatment resistance (Emery et al. 2009).

1.4. RAF gene family

The first description of BRAF was in 1983, when a murine retroviral oncogene with a mammalian cell homologue, termed CRAF (also known as RAF1) was introduced (Papp et al. 1999). Another avian retroviral oncogene that is orthologous to v-Raf, v-mil, was also characterized at the same time (Jansen et al. 1983). In 1984, both v-RAF and v-MIL were identified as the first oncoproteins that show serine/threonine kinase activity (Meier et al. 2005). Two other related genes to CRAF were subsequently found in mice and humans: ARAF and BRAF (Bonner et al. 1985). However, identification of MEK1 as the physiological substrate of CRAF happened almost ten years later (Kyriakis et al. 1992).

In the human genome, *braf* is located on the long arm of chromosome 7 at position 34 (7q34). The *braf* gene is comprised of 18 exons with a total length of 2478 bp at the mRNA level, and 766 amino acids at the protein level. It shares three conserved regions with ARAF and CRAF. Conserved region 1 (CR1) with 131 amino acids (aa) contains the cysteine rich domain (CRD) and most of the RAS binding domain (RBD), both of which bind to RAS-GTP complex. Conserved region 2 (CR2) (16aa) is rich in serine and threonine residues including S365 as an inhibitory phosphorylation site. The last conserved region (CR3) comprises of 293 aa within the kinase domain that also contains the G-loop motif (a highly conserved motif in most human kinases), the activation segment and the regulatory phosphorylation sites S446, S447, D449, T599 and S602 (Schubbert et al. 2007).

1.4.2. BRAF in human developmental disorders

Cardio-Facio- Cutaneous (CFC) syndrome is caused by increased MAPK activity mostly caused by *braf* mutations. About 36 *braf* mutations have been verified in patients with CFC

syndrome, all of which increase the MAPK activity. This skewed signaling interferes with the normal development of many organs and tissues, resulting in the characteristic features of CFC syndrome including facial abnormalities, heart defects, impaired growth and development and predisposition to cancers (Krajewska-Walasek et al. 1996, Tartaglia et al. 2005).

braf mutations are also associated with multiple lentiginous syndrome (known as LEOPARD syndrome) which is characterized by multiple brown skin spots (lentigines), heart defects, short stature, genital abnormalities, hearing loss, and distinctive facial features. Two well-known *braf* mutations associated with this syndrome are substitution of threonine with proline at position 241 (T241P) and leucine with phenylalanine at position 245 (L245F) (Tartaglia et al. 2005, Bentires-Alj et al. 2006). Noonan syndrome, is another developmental disorder associated with *braf* mutations. This syndrome is characterized with heart defects, growth problems, and skeletal abnormalities along with susceptibility to cancer (Tartaglia et al. 2005, Schubbert et al. 2007).

1.4.3. *braf* gene mutation in cancer

Oncogenic *braf* mutations were reported by Sanger institute for the first time in 2002 (Davies et al. 2002). Almost 8% of all cancers and on average 50% of melanoma cases harbor a point mutation in codon 1799 (T1799A) that results in valine to glutamic acid substitution (V600E) (Holderfield et al. 2014). Since the original report, close to 300 distinct missense mutations have been identified in cancer cell lines and tumor samples (Forbes et al. 2011). While the missense mutations encompass 115 of the total 766 BRAF aa, most of them are located within the kinase domain. Activation loop (A-loop) that is located very close to V600, and the GSGSFG phosphate-binding loop (P-loop) at residues 464-469 are the most prevalent sites of

mutations (Garnett et al. 2004, Forbes et al. 2011, Holderfield et al. 2014). Researchers showed that interactions of A-loop and P-loop, especially V600 with F468 is the main cause of BRAF stabilization in an inactive conformation (Wan et al. 2004). Under normal circumstances, interaction of A-loop and P-loop is regulated by reversible phosphorylation of T599 and S602 in the A-loop, which allows a flexible switch between kinase active and inactive states of BRAF. Consequently, all the mutations in either A-loop or P-loop disrupt the normal interaction and mostly lead to a constitutive activation of BRAF. *braf* -T1799 point mutations leading to V600 substitution is thus by far the most common site for oncogenic mutations in melanoma. Accordingly, V600 lysine (V600K), aspartic acid (V600D), and arginine (V600R) mutations have also been characterized as activating mutations in *braf*. Most *braf* mutations would lead to an increase in kinase activity of BRAF, few of which (e.g. G469A) even to the same extent as V600E mutation (Davies et al. 2002, Wan et al. 2004). It is noteworthy to mention that even the *braf* mutations which do not increase its kinase activity were also found to demonstrate an oncogenic role through CRAF activation (Wan et al. 2004, Smalley et al. 2009).

Interestingly, *braf* V600E mutation was reported to have a significant reverse correlation with patient's age in melanoma while a direct relation between age and likelihood of braf mutation was found in thyroid cancer (Adeniran et al. 2006, Viros et al. 2008).

In one report on melanoma patients, *braf* mutations are found to be restricted to lesions that arise from intermittently sun-exposed skin on the trunk and extremities (Curtin et al. 2005). Conversely, melanomas that arise from highly sun-exposed skin or sun protected areas (mucosal and acral-lentiginous) expressed a low frequency of *braf* mutations (Curtin et al. 2005).

1.4.4. *braf* gene mutation in melanoma

The prevalence of *braf* point mutations is reported to be approximately 60% in thyroid cancers, 10% in colorectal carcinomas and 6% in lung cancers (Holderfield et al. 2014). However, this mutation was reported in nearly all cases of papillary cranio-pharyngioma (Brastianos et al. 2014) classical hairy-cell leukemia (HCL-C) (Tiacci et al. 2011, Cerami et al. 2012) and metanephric kidney adenoma (Choueiri et al. 2012).

Unlike most cancers where V600 site mutations predominate, *braf* mutations in lung cancer mostly occur in the P-loop, at G466 and G469 sites (Holderfield et al. 2014). Compared to other cancers in which *braf* mutations are reported, colon cancer and lung cancer have lower frequencies of *braf* mutation (10% and 6% respectively) (Holderfield et al. 2014). However, the higher morbidity of colon cancer in United States and Canada (50,830 and 9,200 deaths per year respectively) and lung cancer (159,480 and 20,200 deaths per year respectively)(American Cancer Society 2013, Canadian-Cancer-Society 2014) , dictates a huge upsurge in the affected cancer population and annual death rate to braf–mutated cancers.

1.5. Melanoma treatment and BRAF

Melanoma at the early stages is usually curable through a simple surgical resection procedure (5-year survival rate 98.1%). However, almost 15% of new melanoma cases diagnosed each year will have unresectable stage III or IV disease with a 5-year survival of 15% (National Cancer Institute (U.S.) et al. 2002). Treatment options for metastatic melanoma have remained nearly unchanged for the past 40 years. In fact, available treatments for metastatic melanoma before late 2011 were limited to dacarbazine (1975), interferon-alpha (INF- α) (1995) and interleukin 2 (IL-2) (1998) (Luke et al. 2013). Dacarbazine has remained the only

chemotherapy reagent approved by the US Food and Drug Administration (FDA) for advanced melanoma. Dacarbazine and the analog drug temozolomide are alkylating agents that cause cell death via DNA damage (adding alkyl groups to guanine bases) and eventually apoptosis and other cell death mechanisms (Bajetta et al. 2002). Although dacarbazine was considered the standard of treatment for melanoma chemotherapy, it was only associated with a response rate (RR) of approximately 10-20% and a progression free survival (PFS) of 3-6 month (Patel et al. 2011). The only advantage of temozolomide over dacarbazine was its oral formulation and it was never approved by the FDA for melanoma treatment (Luke et al. 2013). Immunological treatment for melanoma has gained interest, especially with some primary results of INF-α and IL-2 efficacy. These two agents were combined with various chemotherapy regimens in search for a better response. Although some studies reported an impressive RR of up to 64% for chemo-immunotherapy, the OS still remained unchanged as compared with dacarbazine alone (Luke et al. 2013). However, there is an important dose dependency to the efficacy of IL-2 as some researchers reported a response to high dose IL-2 in patients who had failed previous chemo-immunotherapy (Tarhini et al. 2007). An analysis of 30 clinical trials (phase III) comparing chemotherapy with chemo-immunotherapy revealed an increase in RR for combination therapy while the toxicity was also increased in those patients and no difference was found in OS compared with dacarbazine alone (Eggermont et al. 2004, Eggermont et al. 2009). The most promising improvement in melanoma treatment has happened in the past 2 years when Anti-cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) monoclonal antibody (Ipilimumab) and ATP competitive BRAF inhibitor (Vemurafenib) have improved patients' survival and have been approved by the FDA for melanoma treatment (Hodi et al. 2010, Chapman et al. 2011, Robert et al. 2011). Sorafenib is the first multi-kinase inhibitor, which was tested for BRAF inhibition in

melanoma. Sorafenib is an oral small-molecule targeting BRAF, CRAF, vascular endothelial growth factor receptor (VEGF-R), platelet-derived growth factor receptor (PDGFR), c-KIT, Flt-3 and RET. It has been tested in phase II/III studies as monotherapy or in combination with chemotherapy (carboplatin/paclitaxel and dacarbazine) but failed to show an improvement in OS (McDermott et al. 2008, Hauschild et al. 2009, Eisen et al. 2011) RAF-265 is another small-molecule multi-kinase inhibitor of mutant/wild-type BRAF and VEGFR. In a phase I study, RAF-265 showed a RR of 16% in *braf*-mutant melanoma and 13% in *braf* wild-type melanoma (Sharfman et al. 2011). However, selective BRAF inhibitors have shown the best clinical efficacy thus far. Vemurafenib (PLX4032, Zelboraf, Roche Pharma AG, Germany) is an oral, highly selective and competitive inhibitor of mutant *braf*. Comparison of Vemurafenib with dacarbazine as first line treatment for *braf* V600E mutant metastatic melanoma showed an obvious superiority for Vemurafenib (RR of 48% compared to 5% for Vemurafenib and dacarbazine respectively) (Chapman et al. 2011). Vemurafenib also showed a higher over all RR and longer OS (13.6 months) compared with dacarbazine (9.7 months) (HR 0.70, 95% CI 0.57–0.87, $p < 0.001$) (Chapman et al. 2011).

Dabrafenib (GSK2118436) is another selective BRAF mutant inhibitor. On a phase III clinical trial (BREAK-3) dabrafenib resulted in a significant improvement in PFS and RR over dacarbazine (PFS of 5.1 months for dabrafenib versus 2.7 months for dacarbazine (HR 0.30 95%, CI 0.18–0.53, $p < 0.0001$) (Hauschild et al. 2012). Despite being a drug of the same class as vemurafenib, the incidence of some adverse effects like SCC/KA (6%) and photosensitivity (3%) seems to be less with dabrafenib compared with vemurafenib. However, dabrafenib caused pyrexia in 28% (all grade) of patients, a side effect that is not commonly observed with vemurafenib treatment (Hauschild et al. 2012).

MEK inhibitors are another new family of drug for melanoma treatment. Trametinib (GSK1120212) is a selective inhibitor for MEK1 and MEK2 that showed a median PFS of 4.8 months compared with 1.5 months for chemotherapy (HR 0.45, 95% CI 0.33–0.63, $p < 0.001$) (Flaherty et al. 2012). Also trametinib showed a better OS rate over the period of 6 months compared with chemotherapy (81% versus 67% respectively; HR 0.54, 95% CI 0.32–0.92, $p = 0.01$) (Flaherty et al. 2012).

1.5.1. RAF inhibitors for melanoma treatment

Sorafenib was the first RAF inhibitor that entered clinical trials even before the identification of RAF mutations in cancer. Initially, Sorafenib was discovered as a result of screening for recombinant, activated CRAF and was introduced to treat renal cell carcinoma (Lyons et al. 2001). It is a chemical competitor for ATP that directly binds to and inactivates the kinase domain in CRAF. However, considering the common structural homology of the ATP-binding site in other kinases (FMS-like tyrosine kinase 3 (FLT3), platelet-derived growth factor receptor-β (PDGFRβ), KIT and vascular endothelial growth factor receptor 2 (VEGFR2)(Wilhelm et al. 2004), the exact predominant efficacy of Sorafenib in cancer treatment is unclear. As a case in point, even though the treatment response in hepatocellular carcinoma is correlated with ERK phosphorylation (Abou-Alfa et al. 2006), there is no association between treatment response and RAS mutation status. Also the efficacy of Sorafenib in renal cell carcinoma is mostly linked to VEGFR2 inhibition rather than RAS inhibition.

Sorafenib was also the first multi-kinase inhibitor tested for *braf* V600E inhibition in melanoma patients. However, sorafenib did not show any clinical efficacy in melanoma treatment and lead to the development of ATP-competitive RAF inhibitors particularly designed

to target BRAF. As a result of these attempts, the primary analogue Vemurafenib (PLX-4720) was the first drug, which was introduced as a specific ATP-competitive RAF inhibitor. Interestingly, PLX-4720 showed almost 50-fold more potency on cell lines with *braf* V600E mutation compared with wild type *braf* (Tsai et al. 2008). The selectivity of the Vemurafenib drug family for mutant BRAF protein is explained by the type I binding mode (a tendency to bind to the active conformation of BRAF) in contrast to sorafenib with type II binding tendency (favors the inactive conformation of BRAF and thus has lower efficacy on BRAF V600E mutants). This oncoprotein-binding selectivity has translated to a high therapeutic index for BRAF V600E inhibitors, which allows a high dose treatment without experiencing the acute toxicity associated with general BRAF inhibition (Flaherty et al. 2010, Joseph et al. 2010). As a matter of fact, the high therapeutic index plays a big role in favor of the BRAF V600E inhibitors facilitating more than 80% target inhibition, which is required for a clinical response (Bollag et al. 2012). Interestingly, studies have shown that all ATP-competitive BRAF inhibitors (vemurafenib, dabrafenib, and even sorafenib) are not only poor inhibitors of BRAF wild type but also trans-activate RAF dimers and induce MAPK signaling in BRAF wild type cells (Hatzivassiliou et al. 2010, Heidorn et al. 2010, Poulikakos et al. 2010). The high therapeutic index for ATP- competitive BRAF inhibitors and lack of potency in wild type BRAF are attributed to their ability to induce CRAF homo- and hetero- dimerization with BRAF and RAS-mediated activity of the MAPK pathway. It is shown that BRAF suppresses CRAF activity in RAS mutant cells, therefore inhibition of BRAF activity (e.g. through drug treatment) also alleviates the catalytic CRAF suppression and activates it in a RAS dependent manner (Hatzivassiliou et al. 2010, Poulikakos et al. 2010). In line with these findings, Holderfield and colleagues (Holderfield et al. 2013) also reported a new RAF auto-inhibitory phosphorylation

site in wild type BRAF. They showed that a sub-saturating concentration of BRAF inhibitor in fact alleviates this BRAF wild type auto-inhibitory effect and induces a RAF dimerization and RAS activation. However, high concentrations of BRAF inhibitor were able to block the catalytic effect and disrupted the downstream signaling through the MAPK pathway.

1.5.2. Primary resistance to BRAF inhibitor treatment

Although BRAF mutation has been detected in multiple malignancies, so far melanoma patients are the only ones who benefit from BRAF inhibitors. The reason why other malignancies with BRAF V600E mutation do not respond to BRAF inhibitor treatment is attributed to the parallel activity of other signaling pathways. As an example, low success rate in the treatment of metastatic colorectal cancer patients harboring braf V600E mutation with vemurafenib was associated with increased activity of Epidermal Growth Factor Receptor (EGFR) signaling pathway (Prahallad et al. 2012). It is shown that in CRC patients the base line MAPK pathway has a negative feedback effect on RTK-EGFR signaling which then would be removed upon treatment with BRAF inhibitors and induces a primary resistance to BRAF inhibitor treatment (Corcoran et al. 2012, Prahallad et al. 2012). Similarly, Fagin and colleagues, showed that treatment of papillary thyroid cancer patients with BRAF inhibitor alleviates a negative feedback and increases the activity of Human Epidermal Growth Factor Receptor 2 (HER2) and HER3 through the section of their ligand Neuregulin 1 (NRG1) (Montero-Conde et al. 2013).

Primary resistance is also reported in *braf* mutant melanoma patients as well. In the phase II of clinical efficacy of dabrafenib in melanoma, 16% of patients with braf V600E and 31% of patients with braf V600K experienced disease progression in spite of the treatment with dabrafenib (Ascierto et al. 2013). This resistance to BRAF inhibitor treatment is attributed to

wild type RAF activation and the loss of feedback mechanisms, which in turn leads to elevated levels of RAS-GTP (Lito et al. 2012).

1.5.3. Acquired resistance to BRAF inhibitor treatment

After an initial dramatic response to vemurafenib in *braf* V600E mutant melanoma, unfortunately almost all patients developed resistance to the drug treatment (Chapman et al. 2011, Sosman et al. 2012, Ascierto et al. 2013). The resistance to braf inhibitor treatment in melanoma patients is mostly explained through reactivation of the MAPK pathway. Paradoxical activation of MAPK pathway after initial BRAF inhibitor treatment has been one of the main observations to justify the resistance. Accordingly, mutational activation of NRAS, MEK1 or MEK2 or increased levels of CRAF protein all have been reported to contribute to MAPK reactivation after BRAF inhibitor treatment and induce an acquired resistance (Montagut et al. 2008, Emery et al. 2009, Nazarian et al. 2010, Su et al. 2012, Van Allen et al. 2014). One of the mechanisms described for this observation is inhibition of the negative regulatory signals from MEK for the MAPK pathway. In another word, blocking the activity of MAPK pathway (through BRAF inhibition) removes the inhibitory signal, which normally restricts the pathway activity. While the mutations in downstream effectors of the pathway bellow the BRAF level, like MEK mutation, basically oppose the inhibitory effect that BRAF inhibition was supposed to induce on the MAPK pathway. Another discussed mechanism for BRAF inhibitor resistance is dimerization of the inhibited BRAF molecule with other molecules such as CRAF or wild type BRAF. However, the efficacy of high CRAF protein levels in inducing BRAF inhibitor resistance is only shown at the cellular level and its clinical implication needs further investigation, especially after recent reports showing a negative regulatory effect for CRAF on

BRAF-V600E (Karreth et al. 2009). Another way to acquire resistance to BRAF inhibitor treatment is activation of other genes or more broadly the signaling pathways, which are parallel to MAPK pathway and induce the same end effect. For example Johannessen and colleagues found the over-activation of serine/threonine MAPK kinase kinase (MAP3K8 also known as COT) induced MEK activation and vemurafenib resistance. They reported an increased mRNA copy number for COT in metastatic melanoma biopsies after BRAF inhibitor treatment (Johannessen et al. 2010). In addition, receptor tyrosine kinase has been reported to induce the signaling independent of the MAPK pathway. Likewise, platelet-derived growth factor receptor β, insulin like growth factor 1 receptor, and hepatocyte growth factor receptor have been shown to induce the BRAF inhibitor resistance (Nazarian et al. 2010, Villanueva et al. 2010, Straussman et al. 2012).

On the other hand, changes in the *braf* gene itself like increased BRAF V600E copy number (as the drug target) (Schimke et al. 1978, Shi et al. 2012) or the emergence of BRAF V600E splice mutants also have been reported in resistant patients. Particularly, Poulikakos and colleague reported a novel splice variant of BRAF in resistant patients, which is missing the RBD from the N-terminal region. Therefore the splice variant becomes independent of upstream RAS signaling for dimerization and works in an independent manner (Poulikakos et al. 2010).

Reactivation of parallel pro-survival signaling pathways like PI3K is also reported as an alternative mechanism for MAPK reactivation in vemurafenib-resistant patients. Increased expression of PDGFRβ or Insulin-Like Growth Factor 1 receptor (IGF1R) was identified both at the cell culture level and tissue specimens of vemurafenib resistant melanoma cases (Nazarian et al. 2010, Villanueva et al. 2010). Abel and colleagues reported up regulation of transcription factor Forkhead Box protein D3 (FOXD3) in response to MAPK inhibition, which in turn

increases the expression of HER3 and its signaling pathway as an alternative mechanism for BRAF inhibitor treatment (Abel et al. 2013).

Furthermore, over expression of MITF or its downstream factor (BCL2 related protein A1 (BCL2A) (Johannessen et al. 2010, Haq et al. 2013, Smith et al. 2013) and growth factor secretion (e.g. Hepatocyte Growth Factor (HGF) by tumor stromal cells (Straussman et al. 2012, Wilson et al. 2012) have been reported to confer BRAF inhibition resistance.

1.6. Objective and hypothesis

Over the past few years, since the introduction of *braf* mutations in multiple cancers it has received a lot of attention and these have become the focus of multiple research projects. The importance of *braf* mutation and its role in the progression of cancer has been discussed in detail frequently, however the effect of *braf* mutation on cancer patient survival has been a matter of controversy (Chang et al. 2004, Akslen et al. 2005, Barault et al. 2008, French et al. 2008, Zlobec et al. 2010, Stanojevic et al. 2011, Van Cutsem et al. 2011). In a genuine effort to generate level-one medical evidence to address this question, **we hypothesized that braf V600E mutation is a risk factor by itself for cancer patient survival and utilized the powerful method of systematic review and meta-analysis to evaluate our hypothesis.** We reviewed and analyzed the pool of studies published in the medical literature between 2002 and 2011 to investigate the effect of *braf* V600E mutation on patient survival in melanoma, colorectal cancer and thyroid carcinoma (malignancies with the highest burden among the *braf* associated cancers). These results are reported in Chapter 3.

Despite all the advancements in defining characteristics of *braf* and its mutations in cancer, the status of BRAF protein expression and its role in melanoma prognosis was not well explored.

We hypothesized that BRAF protein expression is involved in melanoma disease progression and patient survival. We used our database consisting of a large cohort of melanoma patients at different stages of disease progression to evaluate the prognostic role of BRAF protein expression in melanoma and its correlation with patient survival. Also we investigated the correlation of BRAF protein expression and *braf* V600E mutation in our melanoma patient samples. These results are presented in Chapter 4.

In the last section of my research in Chapter 5, **we hypothesized that new splice variants (SV) of BRAF are affecting the variable level of BRAF protein expression at different stages of melanoma progression.** We first identified the new variants of BRAF in melanoma cell lines and their effect on cancer cell proliferation and then used tissue microarray (TMA) and immunohistochemistry to study the expression pattern of BRAFsv and its correlation with clinical-pathological features of melanoma.

Chapter 2. Materials and methods

2.1. Literature search strategy and selection criteria

We conducted a comprehensive search of medical literature on studies evaluating the effect of *braf*-V600E mutation on cancer patient survival. We searched MEDLINE and EMBASE using the terms "*braf*", "*braf* mutation", "*braf* V600E", "cancer", "patient survival", "colorectal cancer", "melanoma", and "papillary thyroid carcinoma" in different combinations from June 2002 to December 2011. We initially narrowed our search based on research title followed by abstract and finally full texts were reviewed if they were categorized as relevant reports. We did not restrict the language in our research. All of the references from review papers and original reports were checked for further relevant studies in the systematic review.

Studies were excluded if they contained no clinicopathologic data, survival analysis, or no comparison between wild type and mutant *braf*. In addition, studies, which only reported a progression free survival as well as those with only *in vitro* and animal reports, were also excluded.

2.2. Data extraction and study assessment

Two independent reviewers (GSA and LT) reviewed each full text report for eligibility and extracted required data. For each study the data on the number of patients in each group, mean survival time, hazard ratio and mean progression free survival time for randomized controlled trials (RCT), cancer type and study design were obtained and a consensus was achieved on all items. In the cases of incomplete required information, authors were contacted for additional information, which was added as best as possible. Duplication of data was avoided by matching the author's name and the name of the research centers.

2.3. Meta-analysis

We started summarizing the effect of *braf*V600E mutation on patient survival separately based on study design - RCT versus cohort and cancer type. We evaluated the publication bias using funnel plot analysis. We also assessed the heterogeneity of the studies using chi-square test of heterogeneity and I^2 measure of inconsistency. Significant heterogeneity was defined as a Chi-square test *P* value of <0.10 or as an I^2 measure >50% (Greenland 1998). Estimated hazard ratio (HR) was calculated using odds ratio and confidence interval in studies where HR was not available. In the absence of heterogeneity HRs and CIs were calculated according to a fixed model (Greenland 1998) which assumes that results across studies differ only by sampling error. In those studies where only the survival curve was available with no other detailed information, survival rates were extracted over multiple time periods in order to reconstruct HR and its variance with the assumption that patient censor rate was constant during study follow-up. This method has been described previously by Parmar *et al.* (Parmar et al. 1998) to extract summary statistics for meta-analysis. A HR>1 was considered as a risk factor for worse survival in patients with positive *braf* mutation. In the end we used a log hazard ratio in the pooled data for the final analysis using R software (2011, The R Foundation for Statistical Computing). The impact of *braf* mutation on patient survival was considered statistically significant if 95% confidence interval for individual or overall log HR did not overlap zero.

2.4. Cell lines and cell culture, antibodies, reagents and expression plasmids

The MMRU, MMAN, SK110 (metastatic melanoma cell line), RPEP (derived from a recurrent melanoma) and PMWK (a primary melanoma cell line) cells were kind gifts from Dr. H.R. Byers (Boston University School of Medicine, Boston, MA, U.S.A.). These cells were

selected among a number of cell lines with similar features representing different stage of melanoma progression. All melanoma cell lines were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Fisher Scientific, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (Fisher Scientific) in the presence of 100 units/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin B (Invitrogen, Burlington, ON, Canada) in 5% CO2 humidified atmosphere at 37^{o}C.

Anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA); rabbit anti-BRAF (BRAF 431-445) from Sigma-Aldrich (St. Louis, MO, USA); mouse anti-BRAF (BRAF12-156) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti Pan-phospho-Mek 1/2 from Cell Signaling Technology (Danvers, MA, USA) mouse anti-Flag from Sigma-Aldrich (St. Louis, MO, USA).

2.5. Cohort study patient population and construction of TMAs

We recruited 564 melanoma samples from the tissue bank of the Pathology Department at Vancouver General Hospital, Vancouver, Canada and constructed tissue-microarrays (TMAs) from the paraffin-embedded melanoma tissue samples. The tissues were collected from patients who had been diagnosed with melanoma both clinically and pathologically between February 1992 and July 2009. Tissues from 34 normal nevi, 38 dysplastic nevi, 369 primary melanomas, and 195 metastatic melanomas were used in assembly of the tissue microarray construct. The use of human skin biopsies was approved by the Clinical Research Ethics Board of the University of British Columbia and the study was performed in accordance with Declaration of Helsinki guidelines.

Patients, who were recruited for our cohort study, were prospectively followed to the last date of follow-up time or death (median follow-up time 60.0 months). For each tissue block, the most representative site of the tumor was carefully chosen and marked on hematoxylin and eosin (H&E) stained slides by a dermatopathologist. TMAs were assembled using a tissue-array instrument (Beecher Instruments, Silver Spring, MD, USA). After taking duplicate 0.6-mm tissue cores from each biopsy specimen, a Leica microtome (Leica Microsystems, Bannockburn, IL, USA) was used to cut multiple 4-µm sections, which were transferred to adhesive-coated slides. The location of tumors in each category is summarized in table 2.1, and 2.2.

Table 2. 1. Tissue locations for 369 primary melanoma cases in our TMA construct

Location	No. of cases
Arm	56
Back	53
Foot	14
Head	97
Leg	58
Neck	11
Trunk	70
Unspecified	3
Vulva	7

Table 2.2. Tissue locations of 195 metastatic melanoma cases in our TMA construct

Location	No. of cases
Arm	7
Axillary	38
Bowel	1
Brain	17
Breast	1
Duodenum and Esophagus	1
Eye	1
Femoral Lymph Nodes	1
Foot	1
Frontal Sinus	1
Gastric	1
Groin	6
Hard Palate	1
Head	15
Hematoma	1
Iliac	1
Ilio-obturator	1
Inguinal	34
Leg	8
Liver	1
Medistinal	1
Neck	23
Parotidectomy	2
Peritoneal	1
Periumbilical	1
Pleural	2
Postauricular Node	1
Spleen	3
Subumilical	1
Temporal Hematoma	1
Trunk	14
Unspecified	6
Vulva	1

2.6. Immunohistochemistry of TMAs

Tissue sections were deparaffinized by heating at 55^{o}C for 30 minutes followed by three xylene washes, 5 minutes each. Specimens were rehydrated by consecutive washing in 100%, 95%, and 80% ethanol and distilled water, 5 minutes each. Antigen retrieval was done by

incubating samples in 0.01M sodium citrate buffer (pH 6.0) for 30 minutes at 95^{o}C. Endogenous peroxidase blocking in 3% hydrogen peroxide and blocking with the universal blocking serum (Dako Diagnostics, Glostrup, Denmark) were performed for 30 minutes each. Sections were incubated with a polyclonal anti-BRAF antibody against the kinase domain of BRAF (BRAF 431-445) (1:100 dilution; Sigma, St. Louis, MO, USA) at 4^{o}C overnight. The specificity of this antibody has been shown by Sigma-Aldrich;

(http://www.sigmaaldrich.com/catalog/product/SIGMA/B1687?lang=en&region=CA).

The sections were incubated with a biotin-labeled secondary antibody for 30 minutes followed by streptavidin-peroxidase (DAKO Diagnostics) for 30 minutes. The staining was developed by diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlington, ON, Canada) and counterstained with hematoxylin. The slides were then dehydrated and sealed with coverslips. Negative controls were included by omitting the BRAF primary antibody.

2.7. Immunohistochemistry of TMAs for BRAFsv

The protein expression of BRAFsv was evaluated in our patient population with the help of two separate antibodies against BRAF N-terminal (BRAF 12-156, SantaCruz Biotechnology) and BRAF C-terminal (BRAF 431-445, Sigma) regions (the numbers indicate the amino acid sequence in BRAF protein structure). Because the epitope against which BRAF 12-156 antibody is developed is located at the N-terminal side of BRAF (which is conserved in both kinase-deleted BRAFsv and full length BRAF) this antibody could detect both targets. On the other hand, BRAF 431-445 antibody is able to detect the full length BRAF but not the kinase-deleted BRAFsv as its epitope is part of the missing section in this variant. Therefore, BRAF 431-445

antibody was used in this study to exclude full-length BRAF staining and distinguish the patients with kinase-deleted BRAFsv expression (Fig. 2.1.).

2.8. Evaluation of immunohistochemical staining

Full-length BRAF protein staining in TMAs was examined blindly by two independent observers (including one dermatopathologist and one physician scientist) simultaneously, and a consensus score was reached for each core. The expression of BRAF was scored in two quantitative aspects: intensity of the staining (0, negative; 1+, weak; 2+, moderate; and 3+,

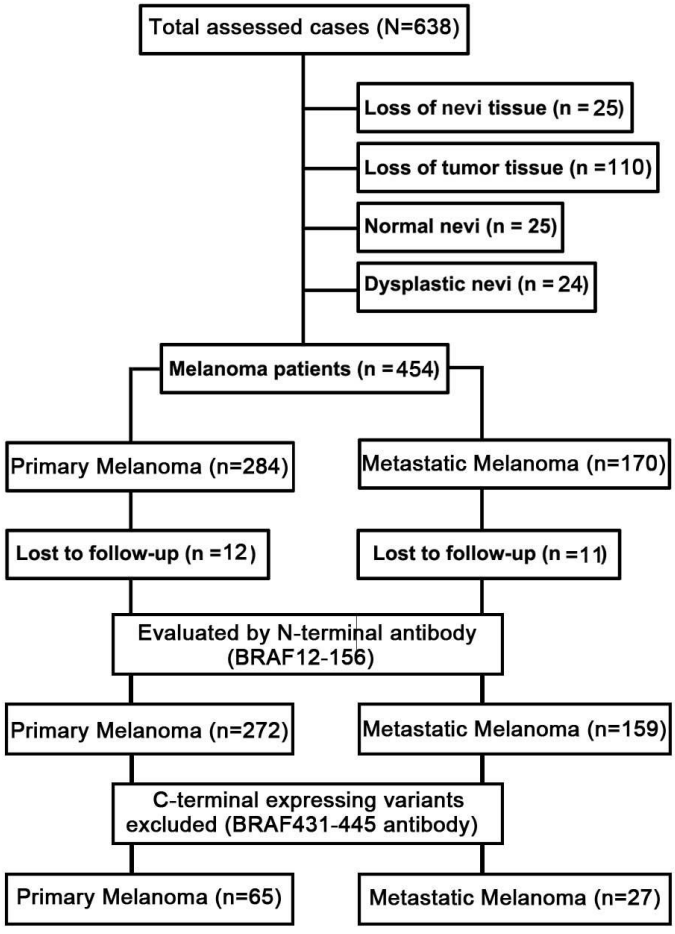

Figure 2.1. Diagram shows patient inclusion and exclusion into our cohort study.

strong) and the percentage of positive cells (0 [0%], 1 [1-25%], 2 [26-50%], 3 [51-75%], and 4 [76-100%]). The higher score was used in case of discrepancy between the duplicate tissue cores. Multiplying the intensity score by the percentage of positive cells, a BRAF immunoreactive score (IRS) was calculated. Utilizing frequency histogram plots and X-Tile software (Yale University, New Haven, CT), the best cut-off point for IRS categorization was chosen: low BRAF expression (IRS 0-4), and high BRAF expression (IRS 6-12) (Fig. 2.2.). Similar unbiased analysis for BRAFsv staining (BRAF 12-156 antibody) using frequency histogram plots and X-Tile software indicated negative versus positive BRAF expression (IRS 0 and IRS1-12 respectively) as the best cut-off point for further statistical analysis (Figure. 2.3.).

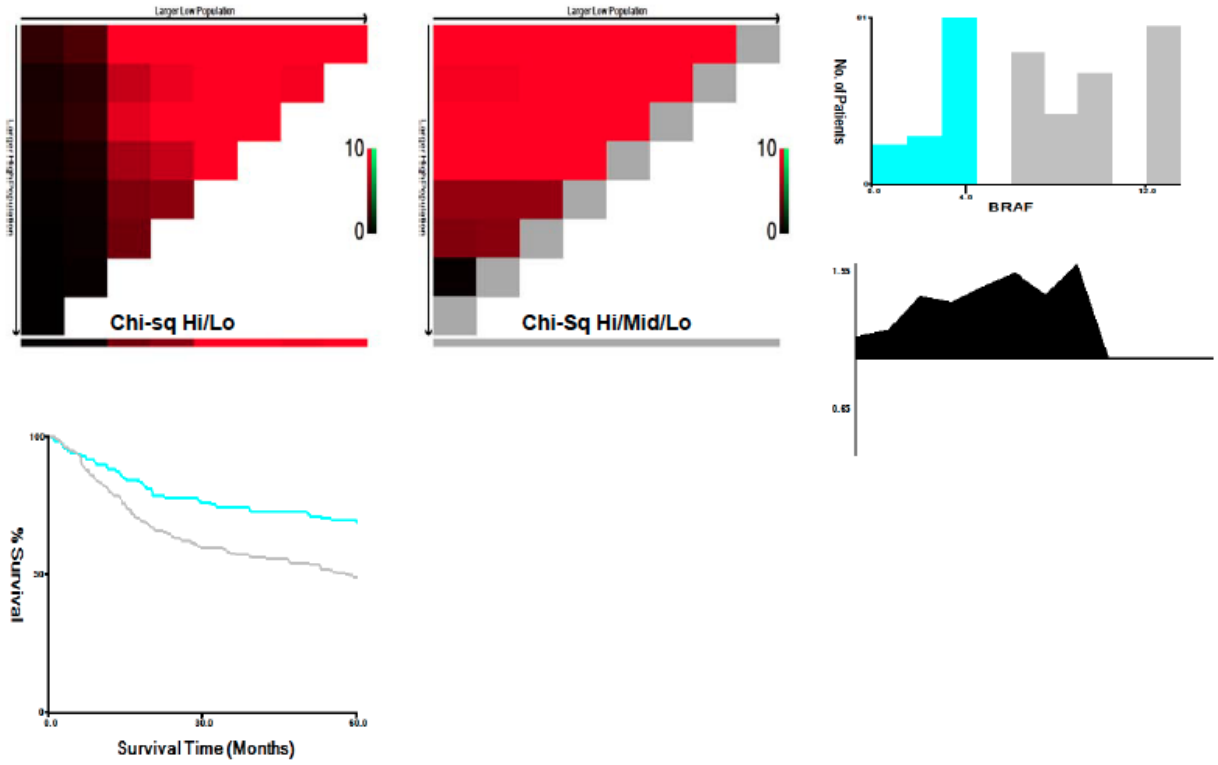

Figure 2.2. Unbiased assessment of immuno-reactive scores (IRS) for full-length BRAF protein staining. Protein expression of full length BRAF was evaluated using BRAF 431-445 antibody. Unbiased evaluation of IRS with X-tile software indicates an IRS cut of point of 0-4 as low (blue color throughout the graphs) versus 6-12 (Gray color throuout the graphs) as high for further statistical analysis.

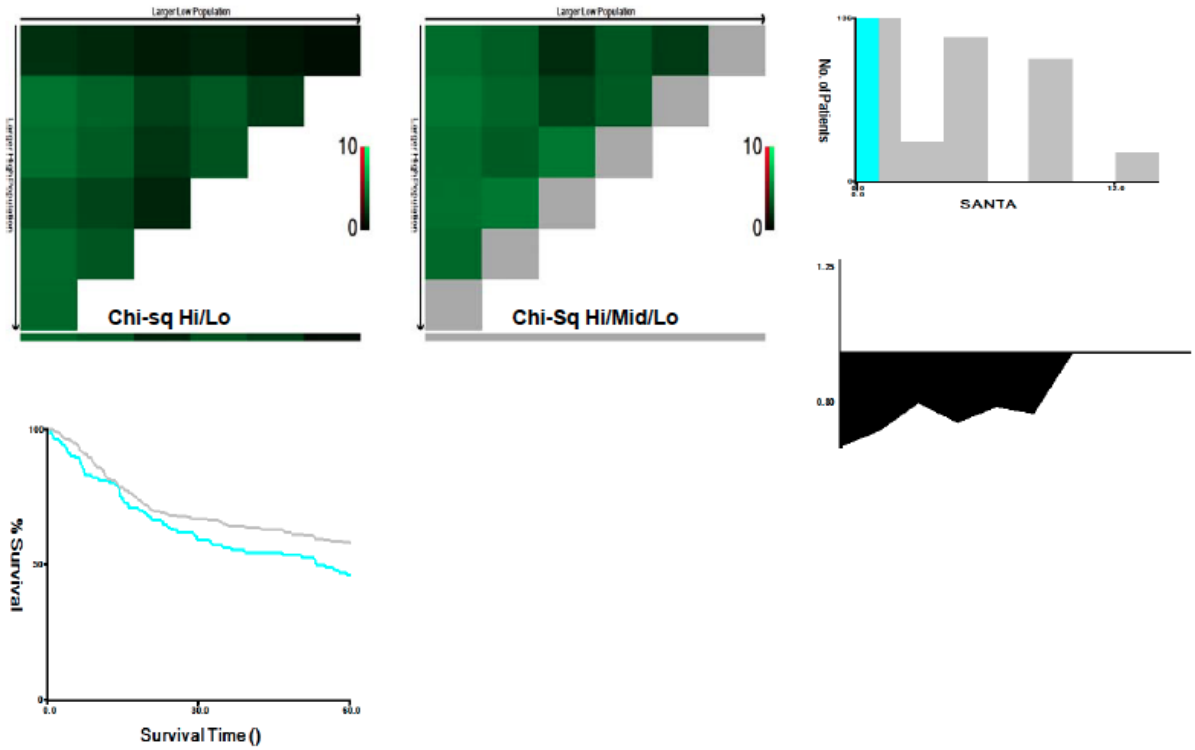

Figure 2.3. Unbiased assessment of immuno-reactive scores (IRS) for BRAF N-terminal epitope protein staining. Protein expression of BRAF N-terminal epitope was evaluated using BRAF 12-156 antibody (detecting both full length and BRAFsv). Unbiased evaluation of IRS with X-tile software indicates an IRS cut of point of negative (blue color throuout the graphs) versus positive (gray color throughout the graphs) for further statistical analysis.

2.9. DNA extraction and polymerase chain reaction direct sequence for *braf* V600E mutation

Out of all primary and metastatic melanoma patients, a group of 80 were randomly selected for DNA extraction based on full length BRAF protein expression level (40 with low and 40 with high BRAF expression). DNA was extracted from formalin-fixed and paraffin-embedded tissue samples using QIAamp DNA FFPE Tissue kit (QIAGEN, Hilden, Germany). Briefly, two 10 μm sections of each sample were deparaffinized mechanically, using sterile scalpel, and chemically, using Xylene incubation for two minutes, and washed with 70% ethanol. The dried samples were incubated at 56°C for one hour with 200 μl lysis buffer (180 μl ATL buffer and 20 μl proteinase K). After proteinase K inactivation by heating samples at 90°C for one hour, genomic DNA was extracted in ATE buffer based on the standard protocol provided by QIAGEN. The extracted DNA was used as a template for polymerase chain reaction (PCR). We analyzed the T1799A transverse mutation in exon 15 of BRAF gene by direct PCR sequencing. Specific primers for BRAF exon 15 were designed to amplify a 227-base-pair (bp) product using the following primers: forward 5′-TGCTTGCTCTGATAGGAAAATG-3′ and reverse 5′-GCATCTCAGGGCCAAAAAT-3′. Amplification was performed in a reaction volume of 50 μl containing DNA template, 1× PCR buffer, 1 mM MgSO4, 0.3 mM of each dNTP, 0.3 μM each primer, and 1U PfX DNA polymerase (Invitrogen, Carlsbad, CA). PCR conditions were as follows: (i) 94°C for 5 minutes; (ii) 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds; (iii) 72°C for 10 minutes; and (iv) held at 4°C. PCR products were visualized by electrophoresis on 1.5% agarose gels with SafeView (Applied Biological Material Inc. Richmond, BC, Canada) staining. Amplified PCR products with sharp bands on the gel were prepared for direct sequencing using an ABI 3730 DNA Analyzer system (Applied Biosystems,

Foster City, CA). In order to make sure about the quality of the sequencing analysis we designed the primer in a way that the target sequence would be at the middle of the PCR product and double checked the reverse sequencing results for randomly selected samples. Considering the possibility of contaminating DNA signals from non-melanoma cells, we manually set a lower peak amplitude threshold setting for heterozygous mutation signals, and used the same size standard setting for all samples. In addition, we re-analyzed the sequencing results with mixed-base calling setting (for 25%) turned on, if the second (smaller) peak was 25% the height of the main peak.

2.10. DNA amplification and sequence analysis for *braf*

We recruited seven patients at different stages of melanoma who had not received any BRAF inhibitor treatment before. Total RNA was extracted from formalin-fixed and paraffin-embedded tissue samples using RNeasy FFPE Kit (QIAGEN, Hilden, Germany). Reverse transcription was performed using EasyScript™ cDNA Synthesis Kit (Applied Biological Materials Inc., Richmond, BC, Canada). *braf* cDNA molecular cloning was done through polymerase chain reaction (PCR) amplification with primers BRAF42-FW-NheI (GGCTG-GCTAGC-CCCGGCTCTCGGTTAT) and BRAF-2388-RV-XbaI (GGC-TCTAGA-CTCCTGAACTCTCTCACTCATTTG) as described before (Hirschi et al. 2013). In order to verify the accuracy of the results, the same series of experiments were performed on six melanoma cell lines (PMWK, MMRU, MMLH, MMAN, A375, and SK110). Utilizing a PCR-based screening method and eight primer sets targeting smaller segments of the *braf* gene (table 2.3.), alternative splicing of *braf* was assessed in melanoma cell lines. Each successfully amplified PCR product (of full length *braf* amplification in each cell line) was separately

extracted from 1.5% agarose gel electrophoresis and subsequently inserted into the Escherichia-coli TOP10 cells plasmid vector using TOPO® TA Cloning Kit for Sequencing (Invitrogen, Life Technologies, Carlsbad, CA, USA). Plasmids containing *braf* cDNA amplicons from MMRU, MMAN and SK110 cell lines were prepared (QIAprep Spin Miniprep Kit, Qiagen) and sequenced using BRAF-1727-FW sequencing primer (ATTGCACGACAGACTGCACA) as explained previously (Hirschi et al. 2013). In case of any inconclusive results, a second sequencing was performed with BRAF-2077-RV sequencing primer (TCCTCGTCCCACCATAAAAA) and the results were aligned with NCBI Reference Sequence NM_004333.4.

Table 2.3. Primer sequences for BRAF gene segmental amplification

BRAF segment	Product size	Forward	Reverse
Exon 1-2	191	GCTCTGTTCAACGGGGACAT	TCCAGATATATTGATGGTGGATTATG
Exon 1-3	421	GCTCTGTTCAACGGGGACAT	CGATAGGTTTTTGTGGTGACTTG
Exon 3-5	198	AGAGTCTTCCTGCCCAACAA	TCTCCAACACTTCCACATGC
Exon 6-8	429	AAGCTGCTTTTCCAGGGTTT	CATTGGGAGCTGATGAGGAT
Exon 8-11	366	AACGAGACCGATCCTCATCA	CCCACTGTAATCTGCCCATC
Exon 11-13	380	GACGGGACTCGAGTGATGAT	TGCAGTCTGTCGTGCAATATC
Exon 14-17	388	GCCAAGTCAATCATCCACAG	TCCTCGTCCCACCATAAAAA
Exon 17-18	405	TTTTTATGGTGGGACGAGGA	GTTTCAGTGGACAGGAAACG

2.11. Evaluation of MAP kinase activity of BRAFsv in melanoma cell lines

MMRU, a human melanoma cell lines derived from metastatic melanoma patient (Byers et al. 1991), was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 5% CO2 at 37°C. Using EcoRV and XbaI restriction sites, BRAFsv was sub-cloned into 3×Flag-CMV-10 vector (Sigma-Aldrich, St. Louis, MO, USA). Cells were grown to 60% confluence before transfection with either empty vector plasmid or 3×Flag-BRAFsv using Effectene transfection reagent (QIAGEN, Hilden, Germany). The cells were harvested for Western blot assay at 48 hours after transfection. Proteins were extracted as described previously(Hirschi et al. 2013) and concentration was determined by protein assay (Bio-Rad) and Western blot analysis was performed. The following antibodies were used for Western blot: mouse anti BRAF 12-156 (1:250; SantaCruz, Santa Cruz, CA, USA), rabbit anti BRAF 431-445 (1:1000; Sigma-Aldrich), rabbit anti Pan-/phospho Mek 1/2 (1:1000; Cell Signaling Technology, Danvers, MA, USA), mouse anti flag (1:500; Sigma-Aldrich), and rabbit anti Vinculin (1:1000; Cell Signaling Technology, Danvers, MA, USA). Infrared-dye−labeled secondary antibody was applied to the blot for 1 hour at room temperature and then signals were detected and quantified using Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA).

2.12. Cell viability assay

MMRU cell lines were cultured in 6 well plates (10.0×10^4 cell/well) and transfected with BRAFsv or control vector in triplicates as described in previous section. Standard MTS assay was used to study the viability of the cells after 48 hours from the transfection time. Briefly, cells were treated with a prepared MTS/PMS solution (20/1 ratio), incubated at 37^oC and 5% CO2 for one hour and the absorbance of the samples were measured in 490nm. Absorbance of the

samples was normalized to the control sample. The result of MMRU cell line over expressing BRAF ΔEx 4-17 was compared with its control expressing empty vector using 2-tailed student *t*-test. Displayed images are representative and the associated graphs are normalized composites of three independent experiments.

Chapter 3. The prognostic value of *braf* V600E mutation in melanoma, colorectal cancer and papillary thyroid carcinoma

3.1. Background and rationale

Constitutive activation of the MAPK pathway in cancers has been frequently observed in various malignancies which is usually due to activating mutations in upstream factors such as RAS and RAF (Malumbres et al. 2003). Accordingly, mutations of *braf* are reported in up to 70% of cancer cell lines (Davies et al. 2002) and they are highly prevalent in most common cancers with poor prognosis such as malignant melanoma, colorectal cancer and papillary thyroid carcinoma (Brose et al. 2002, Davies et al. 2002). Mutations in *braf* have been reported in up to 60% of melanoma cases, between 40 to 70% of thyroid carcinomas, and up to 18% of colorectal cancers (Davies et al. 2002, Cohen et al. 2003).

So far, over 300 distinct mutations have been identified in the *braf* gene, which are present either in the glycine-rich P-loop of the N lobe or the activating segment in the exon 15 region (Wan et al. 2004, Holderfield et al. 2014). Most of these mutations increase BRAF activity by 1.5 to 700-folds depending on the type of the mutation (Wan et al. 2004). Of all *braf*-activating mutations, a transitional mutation in nucleotide 1799 (T-A), also known as BRAF V600E, is the most common change. In fact, this single mutation dramatically increases BRAF activity and accounts for more than 80% of all reported *braf* mutations in tumors (Davies et al. 2002, Wan et al. 2004). As a result, malignant cells with V600E mutation proliferate in a growth factor-independent manner in culture as well as in tumors in animal models (Wellbrock et al. 2004). In addition, it has been demonstrated that *braf* mutations are highly involved in the main steps of cancer development and progression (Hoeflich et al. 2006).

Although there are multiple reports on the correlation of *braf* mutation with a variety of cancer progression steps, the correlation between *braf* mutation and cancer patient survival is still a matter of controversy in different reports (Chang et al. 2004, Akslen et al. 2005, Barault et al. 2008, French et al. 2008, Zlobec et al. 2010, Stanojevic et al. 2011, Van Cutsem et al. 2011). In this part of the study, we used systematic review and meta-analysis, to investigate whether *braf* V600E mutation is associated with patient outcome.

3.2. Results

3.2.1. Number of studies

A total of 565 studies were retrieved from our electronic search. Of these, 120 abstracts were considered relevant and full texts were reviewed in detail. By the end of the review 26 studies on colorectal cancer (5 RCTs and 21 cohorts; 11,773 patients) met our inclusion criteria for meta-analysis. In addition, four studies on melanoma (1 RCT and 3 cohorts; 674 patients) including one study that was published at the time of our statistical analysis (Si et al. 2012) (which is outside of the mentioned time period for our medical database search) were incorporated in our final meta-analysis (Figure 3.1.). We were able to extract the overall survival information from two studies on papillary thyroid carcinoma (Elisei et al. 2008, Musholt et al. 2010). However, we did not perform meta-analysis on papillary thyroid carcinoma subject due to the small number of studies (Table 3.1.). The funnel plot for colorectal cancer but not for melanoma studies showed a publication bias in our collected data.

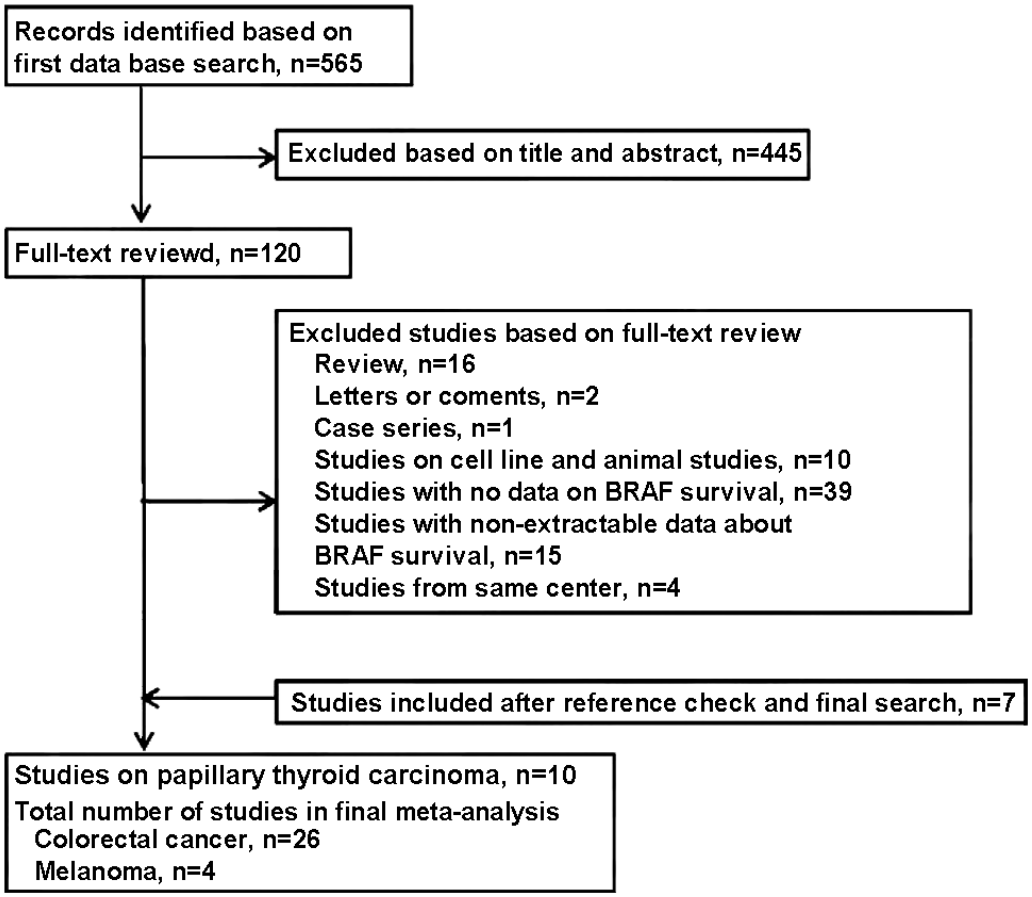

Figure 3.1. Flow diagram demonstrates the study selection process.

Table 3.1. Summary of studies that evaluated the impact of BRAF mutation on overall patient survival in colorectal cancer and melanoma.

	Country	Study design	Number of study patients				Overall Survival		Hazard ratio
			Overall	*braf* subgroup	*braf* WT	*braf* mutant	*braf* mutant	*braf* WT	
Colorectal Cancer									
Barault L (Barault et al. 2008)	France	Cohort	582	582	506	76 (13.1%)			1.2 (0.55-2.61)
De Roock W (De Roock et al. 2010)	Belgium	Cohort	886	761	725	36 (4.7%)	26	54	2.93 (1.85-4.65)
Farina-Sarasqueta A (Farina-Sarasqueta et al. 2010)	Netherland	Cohort	258	203	165	38 (18.7)			2.22 (0.87-3.57)
Ferracin M (Ferracin et al. 2008)	Italy	Cohort	93	79	72	7 (8.9%)			3.37
French AJ (French et al. 2008)	USA	Cohort	533	490	413	77 (15.7%)	71	68	1.2 (0.8-1.8)
Laurent-Puig P (Laurent-Puig et al. 2009)	France	Cohort	173	115	110	5 (4.3%)	14.4	17.9	
Liao W (Liao et al. 2010)	China	Cohort	61	61	58	3 (4.9%)	9	11	2.016 (0.61-6.58)
Liou JM (Liou et al. 2011)	Taiwan	Cohort	314	314	302	12 (3.8%)			3.91 (1.31-11.66)
Loupakis F (Loupakis et al. 2009)	Italy	Cohort	138	87	74	13 (14.9%)	4.1	13.1	1.96 (0.48-3.44)
Maestro ML (Maestro et al. 2007)	Spain	Cohort	351	324	312	12 (3.7%)	41	68	1.62 (0.50-5.21)
Maughan TS (Maughan et al. 2011)	UK	RCT	1630	1291	1189	102 (7.9%)	8.8	14.4	
Ogino S (Ogino et al. 2009)	USA	Cohort	649	631	526	105 (16.6%)			1.97 (1.13-3.42)

	Country	Study design	Number of study patients		Overall Survival	Hazard ratio			
			Overall	*braf* subgroup	*braf* WT	*braf* mutant	*braf* mutant	*braf* WT	
Price TJ (Price et al. 2011)	Australia	Cohort	471	315	282	33 (10.5%)	8.6	20.8	2.04 (1.20-2.87)
Richman SD (Richman et al. 2009)	UK	RCT	2135	692	638	54 (7.8%)			1.82 (1.36-2.43)
Roth AD (Roth et al. 2010)	Switzerland	RCT	1404	1307	1204	103 (7.9%)			1.59 (0.65-3.91)
Samowitz WS (Samowitz et al. 2005)	USA	Cohort	763	763	723	40 (5.2%)			4.23 (1.65-10.84)
Saridaki Z (Saridaki et al. 2011)	Greece	Cohort	112	112	104	8 (7.1%)	4.3	15.1	3.6 (1.7-7.5)
Shaukat A (Shaukat et al. 2010)	USA	Cohort	194	165	129	36 (21.8%)			1.95 (1.18-3.20)
Souglakos J (Souglakos et al. 2009)	Greece/USA	Cohort	168	168	155	13 (7.7%)	10.9	40.5	4.5 (2.4-8.4)
Tie J (Tie et al. 2011)	Australia	Cohort	525	525	473	52 (9.9%)	2.8	13.5	2.48 (1.31-4.72)
Tol J (Tol et al. 2010)	Netherland	RCT	559	518	473	45 (8.7%)	12.9	24.5	3.2
Tran B (Tran et al. 2011)	Australia/USA	Cohort	524	524	467	57 (10.9%)	10.4	34.7	11.11 (6.27-19.17)
Van Cutsem E (Van Cutsem et al. 2011)	Belgium	RCT	999	625	566	59 (9.4%)	14.1	25.1	1.1 (0.42-1.78)
Yokota T (Yokota et al. 2011)	Japan	Cohort	319	229	214	15 (6.5%)	11	40.6	4.23 (1.76-10.2)
Zlobec I (Zlobec et al. 2010) (Left side)	Switzerland	Cohort	404	242	223	19 (7.9%)			0.53 (0.3-1.2)
Zlobec I (Zlobec et al. 2010) (Right side)	Switzerland	Cohort	404	127	102	25 (19.7%)			2.82 (1.5-5.5)

	Country	Study design	Number of study patients		Overall Survival	Hazard ratio			
			Overall	*braf* subgroup	*braf* WT	*braf* mutant	*braf* mutant	*braf* WT	
Melanoma									
Kumar R (Kumar et al. 2003)	Finland	Cohort	38	38	12	26 (68.4%)			2.16 (1.02-4.59)
Long GV (Long et al. 2011)	Australia	Cohort	197	197	102	95 (48.2%)	11.1	46.1	
Si L (Si et al. 2012)	China	Cohort	432	395	297	98 (24.8%)	33	53	1.54 (1.11-2.12)
von Moos R (von Moos et al. 2011)	Switzerland	RCT	62	44	22	22 (50.0%)	9.2	12	

3.2.2. Impact of *braf* V600E mutation on colorectal cancer patient survival

In our pooled data for colorectal cancer only one paper reported a protective HR for *braf* mutation. Accordingly, Zlobec et al (Zlobec et al. 2010) observed a protective HR 0.53 (0.3-1.3) for left side colon cancer. However, they reported a higher HR of 2.82 (1.5-5.5) for *braf* mutation as a risk factor for right side colon cancer in the same report. We considered these two analyses as separate reports in our final analysis. The heterogeneity of data on colorectal cancer was significant ($P < 0.0001$) and I^2 estimate of variation between analyzed studies was 74.3% (Figure 3.2). The pooled log HR of *braf* mutation effect on patient survival in colorectal cancer for cohort and RCT studies were 0.88 (0.60-1.16) and 0.61 (0.28-0.94), respectively. The final log HR for all studies on colorectal cancer was 0.81 (0.60-1.03), which corresponds to a HR of 2.24 (1.82-2.83, 95% CI) (Figure 3.3).

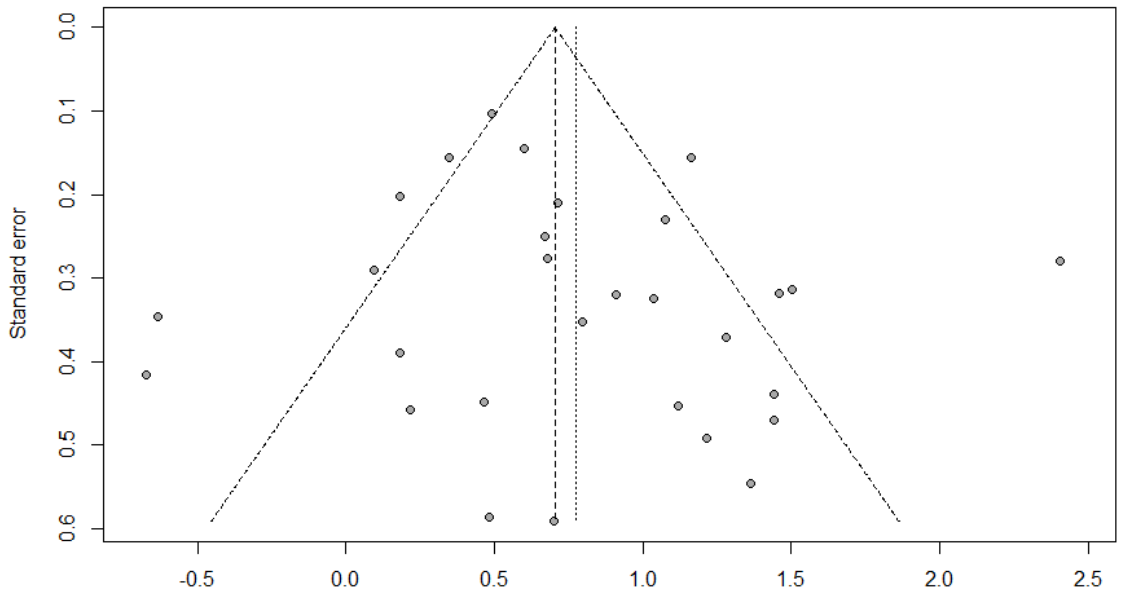

Figure 3.2 estimate of variation between analyzed studies on CRC. Heterogeneity of CRC reports is significant (I2 estimate 74.3%; P < 0.0001).

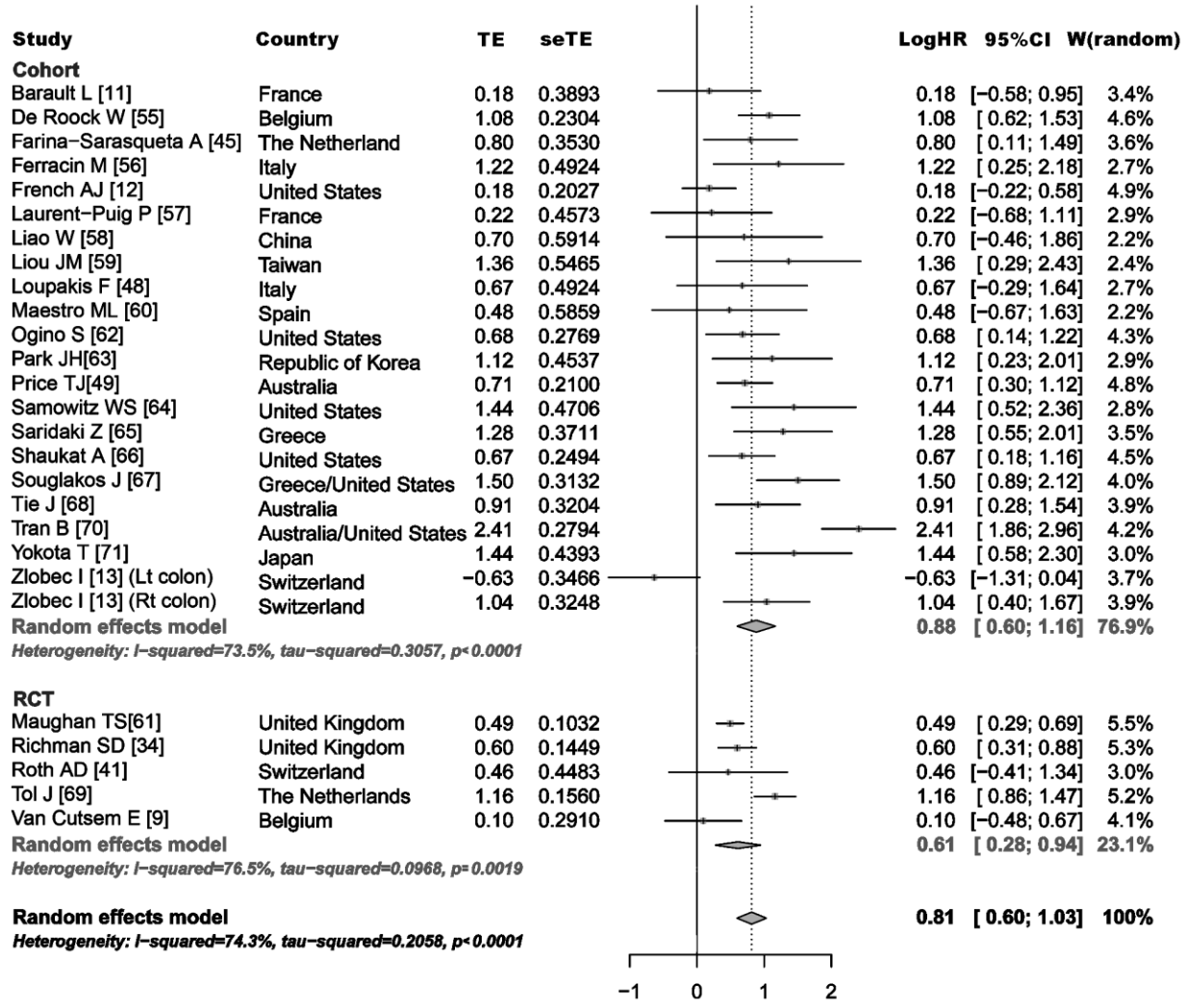

Figure 3.3. Random effect model of Log hazard ratio (LogHR) with 95% confidence interval for studies comparing the effect of braf V600E mutation on overall survival of colorectal cancer patients. A LogHR <0 implies a survival benefit for patients with BRAF mutation. The square size indicates the power of each study in meta-analysis based on the number of patients in that study. The center of diamond shape at the lowest part indicates the combined LogHR for meta-analysis and its extremities the 95% confidence interval.

3.2.3. Impact of *braf* V600E mutation on melanoma patient survival

One RCT study (Board et al. 2009) compared *braf* mutation in patients' serum level with tumor samples but had no data on wild type BRAF status. Two other RCTs evaluated progression-free survival (PFS) with either no overall survival information (Amaravadi et al. 2009) and non-significant PFS or no overall survival data on wild type *braf* group (Flaherty et al. 2010). One cohort study used age <55 years as a surrogate marker for *braf* mutation while others either reported PFS or non-significant difference with no detailed information or survival curve graphs (Table 3.1). The heterogeneity of the data was not significant (P = 0.467) and I^2 estimate of variation between analyzed studies was 0.0% (Figure 3.4). Pooled log HR for *braf* mutation effect on patient survival in melanoma for cohort studies was 0.57 (0.35-0.80) and the final pooled log HR including one RCT was 0.53 (0.32-0.75) corresponding to a HR of 1.70 (1.37-2.12, 95% CI) (Figure 3.5).

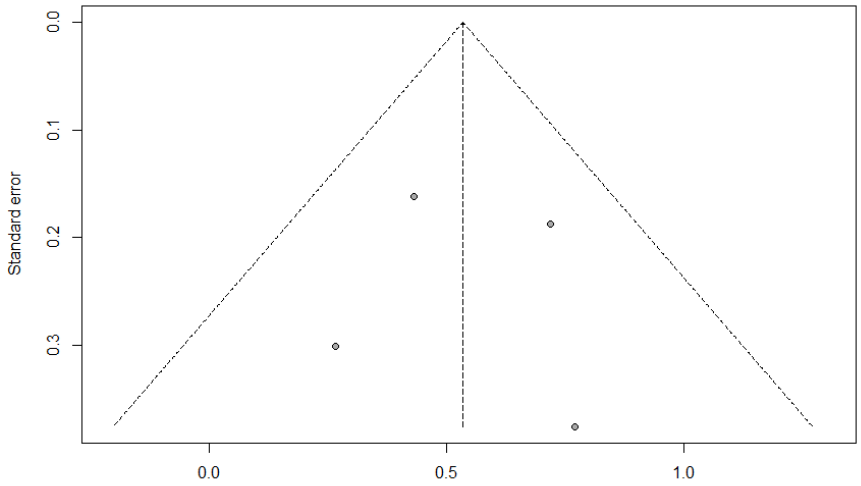

Figure 3.4. I2 estimate of variation between analyzed studies on melanoma. The heterogeneity of the data was not significant (I2 estimate 0.0%; P = 0.467).

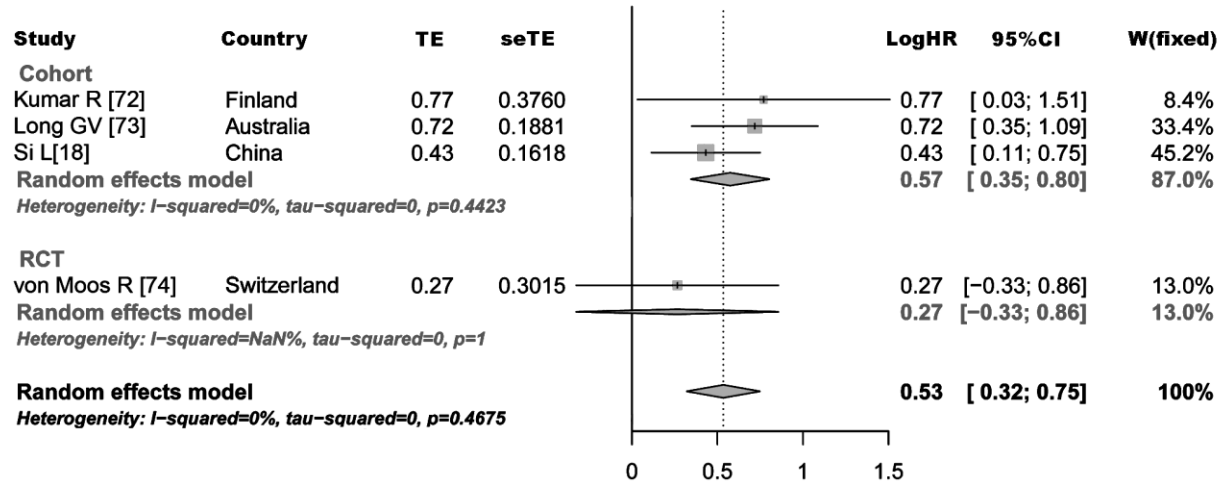

Figure 3.5. Random effect model of Log hazard ratio (LogHR) with 95% confidence interval for studies comparing the effect of *braf* V600E mutation on overall survival in melanoma patients. A LogHR <0 implies a survival benefit for patients with BRAF mutation. The square size indicates the power of each study in meta-analysis based on the number of patients in that study. The center of diamond shape at the lowest part indicates the combined LogHR for meta-analysis and its extremities the 95% confidence interval.

3.2.4. Impact of *braf* V600E mutation on papillary thyroid carcinoma patient survival

One study (O'Neill et al. 2010) reported no deaths in the wild type *braf* group after almost 221 months of follow up. Another study (Elisei et al. 2008) reported just one death in the wild-type *braf* group after 20 years of follow up with odds ratio of 14.63 (1.28-167.29) for mutant BRAF. The study by Musholt et al (Musholt et al. 2010) reported no difference in overall survival (HR = 1.04), while two other reports (Ito et al. 2009, Stanojevic et al. 2011) showed no difference in disease-free survival between mutant and wild-type *braf* patients. However, another study by Abubaker et al (Abubaker et al. 2008) found *braf* mutation as a risk factor for disease free survival and Costa et al (Costa et al. 2008) reported that *braf* mutation would affect patient survival if it is considered in combination with other mutations but not when considered alone. In addition, Wang et al (Wang et al. 2012) reported that patients with synchronous bilateral papillary thyroid carcinoma, which harbor more *braf* mutation, have worse survival compared with those who have unilateral papillary thyroid carcinoma (Table 3.2).

Table 3.2. Summary of studies that reported the status of BRAF mutation in papillary thyroid carcinoma with information on patient survival.

	Number of study patients				Overall Survival		Hazard ratio	Progression free Survival		Hazard ratio
	Overall	*Braf* subgroup	*braf* WT	*braf* mutant	*braf* mutant	*braf* WT		*braf* mutant	*braf* WT	
Abubaker J (Abubaker et al. 2008)	536	296	143	153 (51.7%)				Poor		
Costa AM (Costa et al. 2008)	49	49	22	27 (55%)	No diff. Poor, when combined with other markers					
Elisei R (Elisei et al. 2008)	102	102	64	38 (37.3%)	Sig. Lower		OR 14.63 (1.28-167.29)			
Ito Y (Ito et al. 2009)	631	631	389	242 (38.4%)				DFS No diff.		
Musholt TJ (Musholt et al. 2010)	290	290	168	122 (42%)	No diff.		1.04			
Oler G (Oler et al. 2009)		120	62	57 (48%)				No data on survival		
O'Neill CJ (O'Neill et al. 2010)	104	101	41	60 (59%)				80%	75%	
Stanojevic B (Stanojevic et al. 2011)		266	182	84 (31.6%)				DFS. No diff.		1.15 (0.42-3.19)

	Number of study patients		Overall Survival	Hazard ratio	Progression free Survival	Hazard ratio	Number of study patients		Overall Survival	Hazard ratio
	Overall	***Braf* subgroup**	***braf* WT**	***braf* mutant**		***braf* mutant**	**Overall**	***Braf* subgroup**	***braf* WT**	***braf* mutant**
Wang W (Wang et al. 2012)	891 177 714	208 67 (SBiPTC) 141 (UiPTC)	93 23 70	115 (55.3%) 44 (65.7%) 71 (50.4%)		SBiPTC with more *BRAF* mutation had lower survival compared to UiPTC (*P* = 0.091)				
Xing M (Xing et al. 2005)		219	112	107 (48.9%)		Recurrence free probability				

DFS, Disease free survival; OR, Odds Ratio; SBiPTC, Synchronous bilateral papillary thyroid carcinoma; UiPTC, Unilateral papillary thyroid carcinoma.

3.3. Discussion

Studies investigating *braf* mutation have become an important research topic in cancer biology since the original observation by Davies et al (Davies et al. 2002) reporting a high prevalence of *braf* mutations in multiple human cancers in 2002. Since then, numerous studies investigated the role of *braf* mutation in cancer development and progression. From a mechanistic point of view, BRAF V600E mutation, as the most prevalent mutation in BRAF protein, changes the inactive conformation of BRAF kinase to a very active state (Wan et al. 2004). This simple point mutation leads to a constitutive activation of whole MAPK pathway, which mediates the cell surface growth signals to transcriptional activity of cell cycle regulatory genes. The key regulatory role of *braf* mutation in MAPK activation especially in melanoma generated a tremendous research effort to block this signaling pathway for cancer treatment. Among most available multi-kinase inhibitors at that time, sorafenib was the first one chosen to potentially target BRAF inhibition. Despite early promising results in cell culture and animal studies, sorafenib was found to be unsuccessful in melanoma patients' treatment even among those harboring mutant *braf* (Sharma et al. 2005, Hauschild et al. 2009). A number of other small molecule inhibitors have been tested for targeted BRAF inhibition; however, so far only PLX4032 and GSK2118436 have successfully been used in clinical trials (Bollag et al. 2010, Hauschild et al. 2012). Taking everything into account, the main goal in cancer treatment is to increase patient survival, yet the possibility of whether *braf* mutation per se actually affects patient survival has been a matter of debate. In this study, by conducting meta-analysis on data reported in 30 independent studies, we evaluated the effect of *braf* V600E mutation on patient survival in colorectal cancer and melanoma. We also reviewed another 10 independent studies on papillary thyroid carcinoma in which *braf* mutation is prevalent.

In a population of 11,773 patients from 26 independent studies, we found that the risk of mortality in colorectal cancer patients harboring *braf* V600E mutation is more than two times higher than those with wild-type *braf*. We also demonstrated that melanoma patients with *braf* mutation have a 1.7 times higher risk of mortality when compared with their counterparts without *braf* mutation in a population of 674 patients from the pooled result of 4 studies. In fact, this significant hazard ratio for *braf* mutation in our study can indirectly explain the previously reported promising improvement of melanoma patient survival harboring *braf* mutation after selective BRAF inhibitor treatments (Richman et al. 2009, Hauschild et al. 2012, Young et al. 2012). However, short periods of symptom free survival and resistance to drug therapy are new emerging problems with BRAF-specific inhibitor treatments in melanoma patients. Although the preliminary results for BRAF inhibitor treatments were promising, resistance to drug treatment usually appeared in almost all cases (Flaherty et al. 2010, Chapman et al. 2011). Typically a reactivation of the MAPK pathway happens in resistant cases through other mechanisms including RAS or MEK1 mutations, COT overexpression or BRAF truncation (Johannessen et al. 2010, Paraiso et al. 2010, Poulikakos et al. 2011, Wagle et al. 2011). Nevertheless, the response rate of colon cancer patients harboring *braf* V600E mutation to BRAF inhibitor treatment is much lower than melanoma patients (Kopetz et al. 2010, Roth et al. 2010). In fact, over activation and crosstalk of parallel pathways like phosphatidylinositol 3-kinase (PI3 kinase) – AKT with MAPK in colorectal cancer plays a major role in the observed differential response to BRAF inhibitor treatments in colorectal cancer. Likewise, a recent study by Prahallad et al (Prahallad et al. 2012) revealed the important role of epidermal growth factor receptor (EGFR) activation in colon cancer patients as well. They showed that a feedback activation of EGFR occurs very quickly in colon cancer cells after BRAF-V600E inhibition. In fact, this feedback

activation of EGFR in colon cancer cells leads to continuous malignant cell proliferation even in the presence of BRAF-V600E inhibition. However, this mechanism would not be applicable to melanoma cells as they express a very low level of EGFR (Prahallad et al. 2012).

braf mutation in papillary thyroid cancer was reported to be a risk factor for worse survival in two studies (Elisei et al. 2008, O'Neill et al. 2010). Notwithstanding a notably long term follow-up of patients for 18 to 20 years in these studies from Australia and Italy, the authors either did not observe any death (O'Neill et al. 2010) or only one death (Elisei et al. 2008) in the *braf* wild-type group of patients. Authors reported only one death in a population of 64 or no death among 41 wild-type *braf* patients while Standardized Death Rate for the general population in Australia was found to be 6.9 and 4.7 per 1000 standard populations for male and female respectively (http://www.abs.gov.au/ausstats/abs@.nsf/Lookup/by+Subject/4125.0~Jul+2011~Main+Features~Death+rate~3210).

Also, based on the report from the Centers for Disease Control and Prevention, age specific mortality rate for normal population within the same age group as patients in these two studies (45 to 54 years) is 420.9 per 100,000 of population (http://www.cdc.gov/nchs/nvss/mortality_tables.htm). Altogether, it seems that more studies with larger sample size would be required to be more confident in the significance of *braf* V600E mutation effect on papillary thyroid carcinoma patient survival.

The number of studies comparing molecular and clinico-pathological differences between right and left side colon cancers has increased during the past two decades. For instance, a higher frequency of microsatellite instability, which is a poor prognostic factor for colon cancer, has been reported to be more prevalent in right side compared with left side colon cancer (Nash et al.

2010, Rampazzo et al. 2010). A number of studies also reported more prevalent *braf* mutations in right side colon cancer (Farina-Sarasqueta et al. 2010, Zlobec et al. 2010). Although different biological and clinico-pathological characteristics have been described for right and left side colon cancer, this issue is still a matter of controversy. Accordingly, investigating a large number of patients (29,568) in a recent study, Benedix et al (Benedix et al. 2011) revealed a remarkable clinic-pathological variation among colonic sub-sites irrespective of the side of tumor (right versus left). They showed that these differences are more related to the anatomical site of the cancer origin rather than a simple right or left categorization (Benedix et al. 2011, Benedix et al. 2012). Despite a number of descriptive reports on the prevalence of *braf* mutation and its correlation with clinico-pathological characteristics, there has been no comprehensive comparison of the effect of *braf* mutation on patient survival in separate groups of right and left side colon cancers. Accordingly, a controversial HR of 0.53 has been reported by Zlobec et al (Zlobec et al. 2010) for *braf* in left side colon cancer, which shows a protective effect for *braf* mutation on patient survival, as it is less than one. On the other hand, in the same study they reported a HR of 2.82 for *braf* mutation in right side colon cancer, which reveals *braf* mutation as a strong risk factor for patent survival. They were not able to reconfirm the same protective effect for *braf* mutation on left side colon cancer considering other risk factors in a multifactorial analysis, although the negative effect of *braf* on right side colon cancer was persistently significant in multifactorial analysis. One main reason for this controversial observation could be the big difference in the number of mutant (19) versus wild-type *braf* (223) group in the left side colon cancer patients. Furthermore, the frequency of *braf* mutation in the left side colon cancer arm of the study was reported to be much less than in the right side (7.9% versus 19.7%; Table 3.1), which might also affect the survival analysis.

A number of other researchers from our pooled studies also observed a considerable decrease in patient survival with *braf* mutation compared with wild-type *braf*; however, the specific HR for wild-type or mutant *braf* was not determined (Loupakis et al. 2009, Farina-Sarasqueta et al. 2010, Price et al. 2011, Van Cutsem et al. 2011). Based on the significantly poor patient survival in the mutant *braf* group in those studies according to survival curves and reported survival time difference, we estimated the HR of mutant *braf* in our meta-analysis.

With respect to reports on melanoma, Ellerhorst et al (Ellerhorst et al. 2011) reported no difference in patient survival in a group of 223 patients. However, they considered the mutation of *braf* in combination with *nras* mutation as a single entity in their survival analysis. There was no data available for the effect of *braf* mutation alone on patient survival in this report. In a very similar study, Houben et al (Houben et al. 2004) evaluated the effect of combined mutation of *braf* and *nras* mutation in 200 patients and reported a poor overall survival prognosis for metastatic samples which harbor either *braf* or *nras* mutation. However, they did not observe the same pattern in primary melanoma patients. As these two reports did not provide any information on the effect of *braf* mutation per se on patient survival we did not include them in our final meta-analysis. The inconsistency of results in these reports could be due to the fact that they combined *braf* and *nras* mutation and classified this group of patients together. In addition, Akslen et al (Akslen et al. 2005) and Chang et al (Chang et al. 2004) reported no difference in patient survival in 69 and 68 cases respectively according to their *braf* mutation status. However, no details on patient survival have been provided in these reports. Akslen et al (Akslen et al. 2005) mostly focused on different *braf* and *nras* mutations and their combinations and possible correlation with clinico-pathological characteristics. They reported that *braf* and *nras* mutations are mutually exclusive except for one case but they did not find any correlation with tumor cell

proliferation, thickness or vascular invasion. Although they reported a median follow-up time of 76 months for the patients, no detailed information on mean survival time in each arm of the study was provided. There was no survival curve available in this report either. In a separate study, Chang et al (Chang et al. 2004) observed a significant trend for liver metastasis and tendency for multiple organ metastasis in *braf* mutant group but they did not detect a significant difference in either clinico-pathological characteristics or in patient survival. Basically in this study the authors chose a descriptive method to explain their observation and just mentioned that they did not find any correlation between patient survival and *braf* mutation. Unfortunately, no more detailed information including mean survival time in each group of study or survival graph were provided by the authors. A need for a conclusive meta-analysis on the effect of *braf* mutation on melanoma patient survival has been emerged due to the controversial reports on this issue. In our meta-analysis, we combined the results of four independent studies and measured the pooled risk of *braf* mutation on melanoma patient survival. So far our report is the first study that used meta-analysis to generate level-one clinical evidence demonstrating the correlation between *braf* mutation and poor melanoma patient survival. The number of reports on *braf* mutation and colorectal cancer were enough to pool the results together and perform a meta-analysis. Therefore, our findings in the pooled data suggest that with successful BRAF inhibition we would be able to increase the survival of colorectal cancer and melanoma patients harboring *braf* mutations.

In summary, we used systematic review and meta-analysis approach to investigate possible association between *braf* V600E mutation and cancer patient survival. We found that BRAF V600E mutation increases the risk of mortality in colorectal cancer patients for more than two-fold. In addition, we revealed that BRAF V600E mutation also significantly increases the risk of

mortality in melanoma patients. This data highlights the important role of mutant *braf* in patient survival and suggest that with successful BRAF inhibition we may be able to increase the survival of colorectal cancer and melanoma patients harboring *braf* mutation.

Chapter 4. Melanoma disease progression and patient survival are significantly influenced by BRAF protein expression

4.1. Background and rationale

Identification of several important oncogenes has influenced our understanding of the molecular basis of melanoma, which has led to new drug development, especially during the past few years. The MAPK signaling pathway is by far the most prominent oncogenic pathway involved in the progression of melanoma (Inamdar et al. 2010). Most predominant changes associated with aberrant MAPK activity in melanoma are mutations in the *ras* and *raf* genes. Mutations in the *nras* gene are reported in almost 20% of melanoma patients, while *braf* mutation has been detected in 50% of melanomas and up to 62% of melanoma cell lines (Davies et al. 2002, Dong et al. 2003, Gorden et al. 2003, Aguissa-Toure et al. 2011). Substitution of valine at location 600 (V600) (usually as a consequence of a point mutation) and more specifically its exchange for glutamic acid (V600E) is the most prevalent *braf* mutation in melanoma (Wan et al. 2004, Rubinstein et al. 2010). This single substitution leads to an excessive elevation in BRAF kinase activity and constitutively stimulates ERK activity independent of upstream RAS signal, both in vitro and in vivo (Davies et al. 2002, Wan et al. 2004).

Despite the abundant studies investigating mutations of *braf* and its significance in initiation and progression of cancers, so far there has been no report on BRAF expression at the protein level in different stages of melanoma and its possible correlation with patient outcome. In this section of the study, we investigated the expression pattern of BRAF protein in various

stages of melanocytic lesions and its correlation with clinico-pathologic characteristics and 5-year patient survival. In addition, we evaluated the possible correlation between BRAF protein expression level and V600E mutation.

4.2. Results

4.2.1. Clinicopathologic and demographic features of the patients

Originally, biopsies from 638 patients were used for construction of the TMAs. Due to absence of cores in the TMA blocks, core loss, and insufficient tumor cells in the cores, 389 melanoma and 49 nevi samples were included in this part of our analysis. After censoring the 19 cases lost to follow-up, 232 primary melanoma cases, 138 metastatic melanoma, 25 nevocytic nevi, and 24 dysplastic nevi were included in our final analysis. Of 232 primary melanoma cases, age ranging from 7 to 95 years (median 66), 104 were female and 128 male. One hundred thirty six cases of primary melanoma were $\leq$2 mm (low-risk), and 96 were thicker than 2 mm (high-risk). Ulceration was detected in 46 cases of primary melanoma patients. For 138 metastatic melanoma patients, 45 were female and 93 male, age ranging from 23 to 94 years (median 59). We also used American Joint Committee on Cancer (AJCC) stage to categorize the patients: 126 cases were at AJCC stage I, 106 at stage II, 59 at stage III, and 79 at stage IV at the time of diagnosis (Table 4.1).

Table 4.1. Full-length BRAF staining and clinicopathologic characteristics of melanoma patients

Variables	Low BRAF No. (%)	High BRAF No. (%)	Total	P value
All melanoma (n= 370)				
Age, y				
≤ 62	71 (37.6)	118 (62.4)	189	0.338
> 62	60 (33.1)	121 (66.9)	181	
Sex				
Male	77 (34.8)	144 (65.2)	221	0.782
Female	54 (36.2)	95 (63.8)	149	
AJCC stage				
I	78 (61.9)	48 (38.1)	126	3.6×10^{-13}
II	23 (21.7)	83 (78.3)	106	
III	9 (15.3)	50 (84.7)	59	
IV	21 (26.6)	58 (73.4)	79	
Primary melanoma (n= 232)				
Age, y				
≤ 66	59 (50.4)	58 (49.6)	117	0.129
> 66	42 (36.5)	73 (63.5)	115	
Sex				
Male	56 (43.8)	72 (56.3)	128	0.941
Female	45 (43.3)	59 (56.7)	104	
Tumor thickness (mm)				
≤ 1.0	54 (66.7)	27 (33.3)	81	1.5×10^{-7}
1.01 -2.00	24 (43.6)	31 (56.4)	55	
2.01 -4.00	14 (31.8)	30 (68.2)	44	
>4.00	9 (17.3)	43 (82.7)	52	
Ulceration				
Absent	94 (50.5)	92 (49.5)	186	1.5×10^{-5}
Present	7 (15.2)	39 (84.4)	46	
Subtype				
Lentigo maligna	34 (59.6)	23 (40.4)	57	8.7×10^{-5}
Superficial spreading	37 (50.7)	36 (49.3)	73	
Nodular	6 (15.0)	34 (85.0)	40	
Unspecified	24 (38.7)	38 (61.3)	62	
Site [a]				
Sun-protected	76 (45.8)	90 (54.2)	166	0.273
Sun-exposed	25 (37.9)	41 (62.1)	66	
Metastatic melanoma (n= 138)				
Age, y				
≤ 59	18 (25.0)	54 (75.0)	72	0.446
> 59	12 (18.2)	54 (81.8)	66	
Sex				
Male	21 (22.6)	72 (77.4)	93	0.730
Female	9 (20.0)	36 (80.0)	45	

4.2.2. Correlation between BRAF expression and clinicopathologic parameters

A significant difference in BRAF staining was observed between dysplastic nevi and primary melanoma as well as between primary and metastatic melanoma (P = 0.032 and 2.2×10-5, respectively; Kruskal-Wallis test, Figure 4.1c). However, no significant difference was detected between nevocytic nevi and dysplastic nevi, P > 0.05; Kruskal-Wallis test, Figure 4.1.c and d). Similarly, $\chi 2$ test confirmed that high BRAF expression was significantly increased from dysplastic nevi (33.3%) to primary melanoma (56.5%) (P = 0.03) and further increased in metastatic melanoma compared with primary melanoma (78.3%) (P = 2.2×10-5; Figure 4.1.d).

In all 370 melanoma patients, we found that high BRAF expression is significantly increased from early to advanced AJCC stages (P = 3.6×10-13, $\chi 2$ test; Table 4.2). Because of the big surge of high BRAF expression from stage I to stage II, we then compared BRAF expression between AJCC stage I (38.1%) and stages II-IV (79.3%) which clearly showed statistical significance (P = 1.0×10-14, $\chi 2$ test). In primary melanoma cases, high BRAF expression was detected in 84.4% of patients with ulceration at the time of diagnosis compared to 49.5% of patients without ulceration (P = 1.5×10-5, $\chi 2$ test, Table 4.1). Also, high BRAF expression was found to be gradually increased along with tumor thickness (P = 1.5×10-7, $\chi 2$ test; Table 4.1 and Figure 4.2). Moreover, high BRAF expression was detected in 49.3% of superficial spreading and 40.4% of lentigo maligna while nodular subtype showed the highest rate of high BRAF expression 85.0% (P = 8.7×10-5, $\chi 2$ test; Table 4.1). However, we did not find any significant correlation between BRAF expression and the location of the lesions or other clinicopathologic variables such as age and gender in primary or metastatic melanoma patients (Table 4.1).

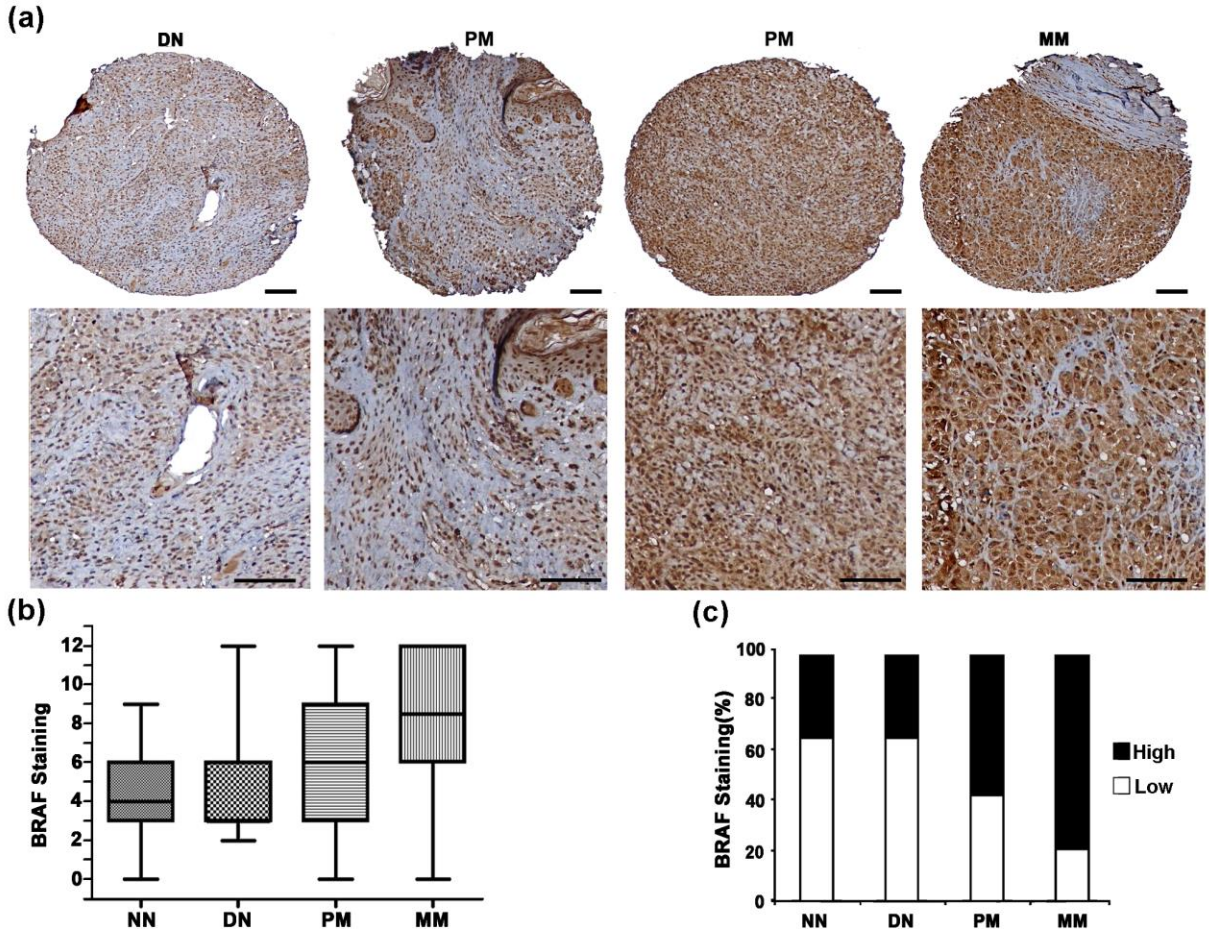

Figure 4.1 Correlation between BRAF expression and melanoma progression. (a) Representative images of cytoplasmic BRAF immunohistochemistry staining which shows a gradual increase in BRAF protein expression from nevi samples (IRS: 2) to thin primary (≤2mm) (IRS: 4), thick primary (>2mm) (IRS: 8) and metastatic melanoma (IRS: 12). Bar = 100 µM in core pictures and 10 µM in high magnifications. (b) Kruskal-Wallis test for differences in BRAF staining among nevocytic nevi (NN), dysplastic nevi (DN), primary melanoma (PM), and metastatic melanoma (MM). (c) High expression of BRAF protein increases from dysplastic nevi to primary melanoma and further to metastatic melanoma.

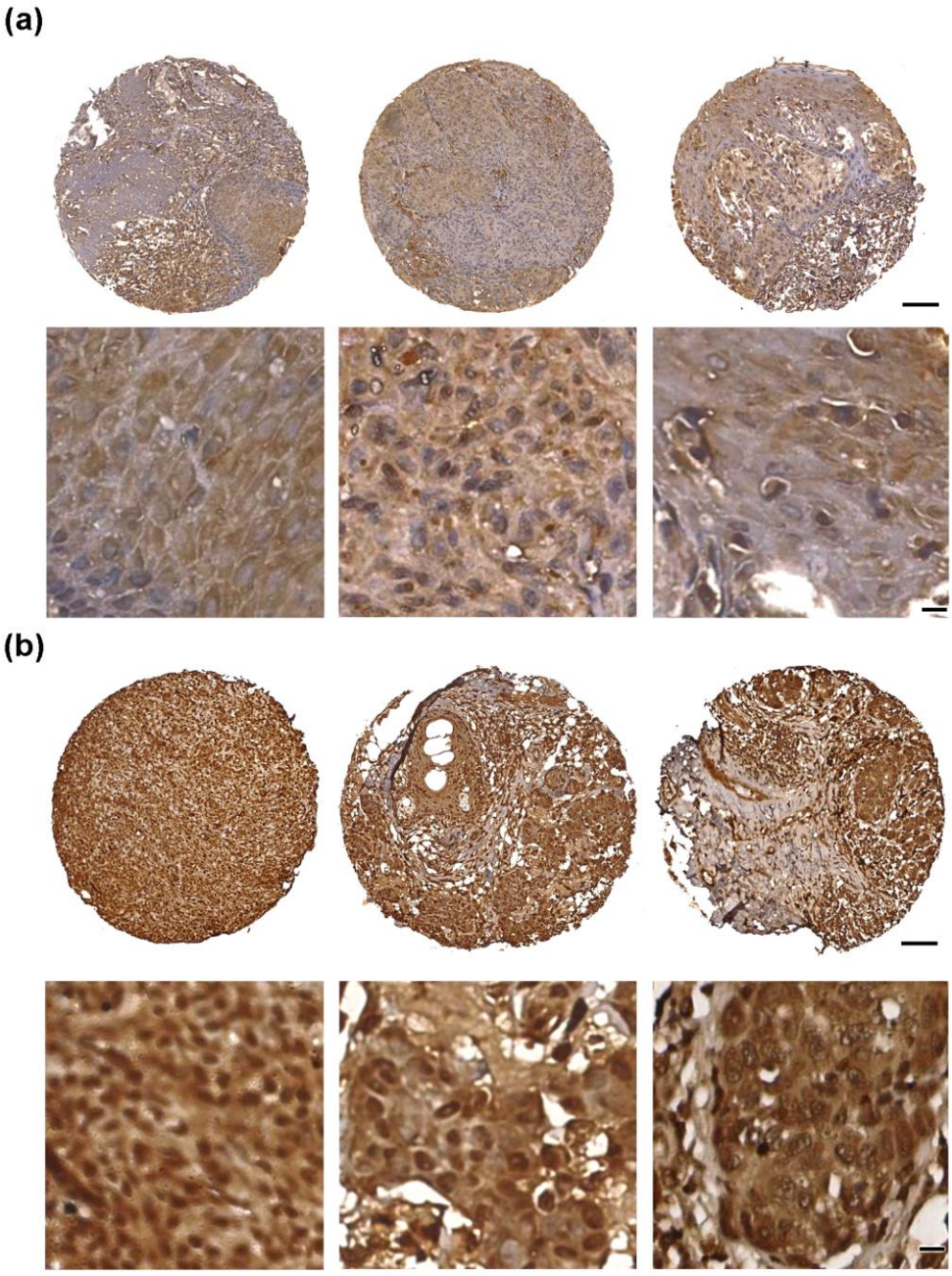

Figure 4.2. Images of BRAF expression in melanoma. (a) Representative images of BRAF immunohistochemistry staining in thin primary melanoma samples (≤2mm) (IRS:2-3). (b) Representative images of BRAF immunohistochemistry staining in thick primary melanoma samples (>2mm) (IRS: 6-8).

4.2.3. Correlation between BRAF expression and V600E mutation

Because of the old age of most tissue samples and fragile extracted DNA, sequencing result was not successful in all specimens. Out of 18 melanoma samples with low BRAF protein expression we found three V600E mutant cases, while nine out of 20 patients with high BRAF expression were positive for V600E mutation (7 heterozygous and 2 homozygous mutations). Although there was an obvious trend for increased number of V600E mutations in patients with high BRAF expression, the statistical evaluation did not find a significant correlation between the level of BRAF protein expression in our samples and V600E mutation status ($P = 0.063$; Spearman correlation test and $P = 0.06$; $\chi 2$ test) (Figure 4.3).

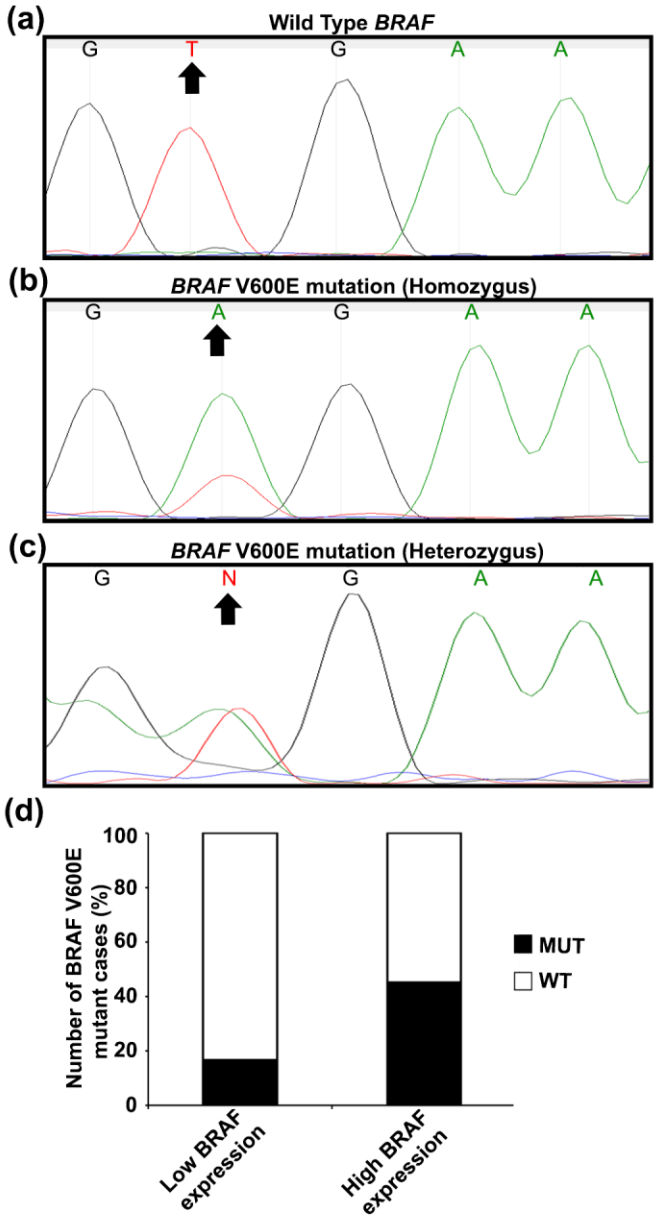

Figure 4.3. The BRAF exon 15 sequence and its correlation with protein expression level. (a) Wild type sequence: thymidine at nucleotide 1799 (arrow). (b) Homozygous mutant sequence: dominant single pick for adenine (arrow). (c) Heterozygous mutant sequence: a mix of signals for both thymidine and adenine. (d) In spite of an obvious trend for more prevalence of V600E mutations in high BRAF expression group, there was not a significant statistical correlation between V600E mutation and BRAF protein expression at same samples using immunohistochemistry staining (P = 0.06; $\chi 2$ test).

4.2.4 Correlation between BRAF expression and patient survival

We constructed Kaplan-Meier survival curves to assess the correlation between BRAF expression and 5-year overall and disease-specific survival of melanoma patients. Our data revealed that high BRAF expression was associated with poor overall (P = 0.001) and disease-specific 5-year survival (P = 0.001) (Figure 4.4.a). In addition, we used Cox proportional hazard regression model to estimate the crude hazard ratios of each clinico-pathological characteristics. Univariate Cox regression analysis of BRAF expression revealed a hazard ratio (HR) of 1.85 (95% CI, 1.28-2.65; P = 0.001) for overall survival and HR of 1.89 (95% CI, 1.28-2.79; P = 0.001) for disease-specific survival (Table 4.2).

For further statistical analysis, we divided patients into primary and metastatic melanoma. Kaplan-Meier survival analysis in primary melanoma patients revealed a significant correlation of high BRAF expression with poor overall and disease-specific survival (P = 0.009 and 0.007, respectively; Figure 4.4.b). Moreover, univariate Cox-regression survival analysis also showed that high BRAF expression in primary melanoma group is a risk factor for worse patient survival (HR, 2.08; 95% CI, 1.18-3.65; P = 0.011 for overall survival; HR, 2.39; 95% CI, 1.23-4.62; P = 0.009 for disease-specific survival; Table 4.2). This negative effect was persistently significant through our training and validation set analysis as well (Figure 4.5). However, BRAF expression did not correlate with patient survival in metastatic melanoma group (P > 0.05; Fig. 4.4.c). Furthermore, univariate analysis revealed that BRAF expression, thickness, ulceration, and AJCC stage were all significantly associated with overall and disease-specific 5-year survival in primary melanoma patients (Table 4.2). As expected, multivariate Cox regression analysis showed that AJCC staging and tumor thickness, but not BRAF expression, are independent

prognostic factors because BRAF expression is significantly correlated with AJCC stages and tumor thickness (Table 4.2).

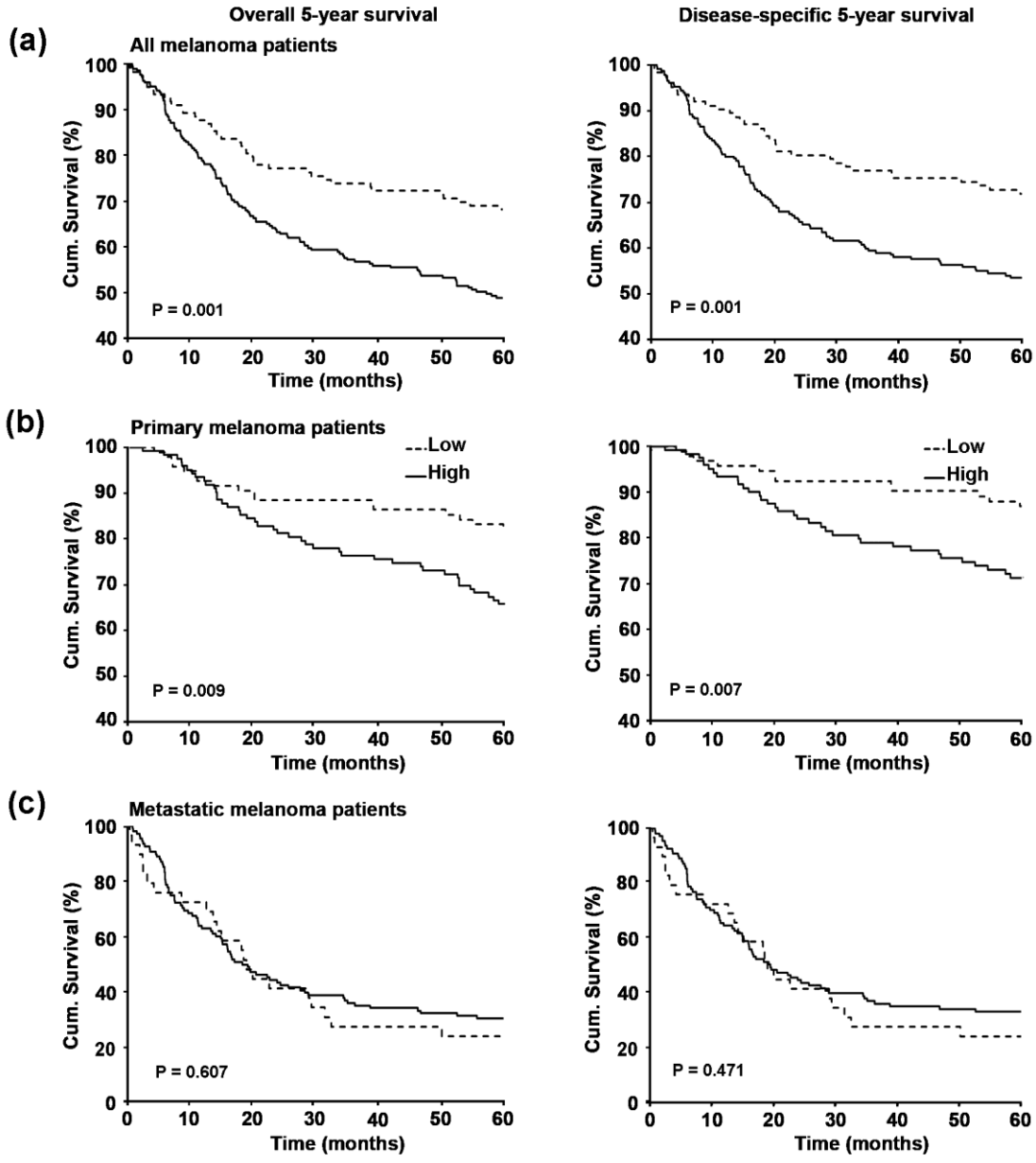

Figure 4.4. The correlation between BRAF expression and patient survival in all patients, primary and metastatic melanoma. (a) High BRAF expression is significantly correlated with poor overall (left) and disease-specific (right) 5-year patient survival in all melanoma patients (n=370). (b) High BRAF expression was also associated with poor overall (left) and disease-specific (right) 5-year patient survival in primary melanoma (n=232). (c) BRAF expression did not show correlation with patient survival in metastatic melanoma (n=138).

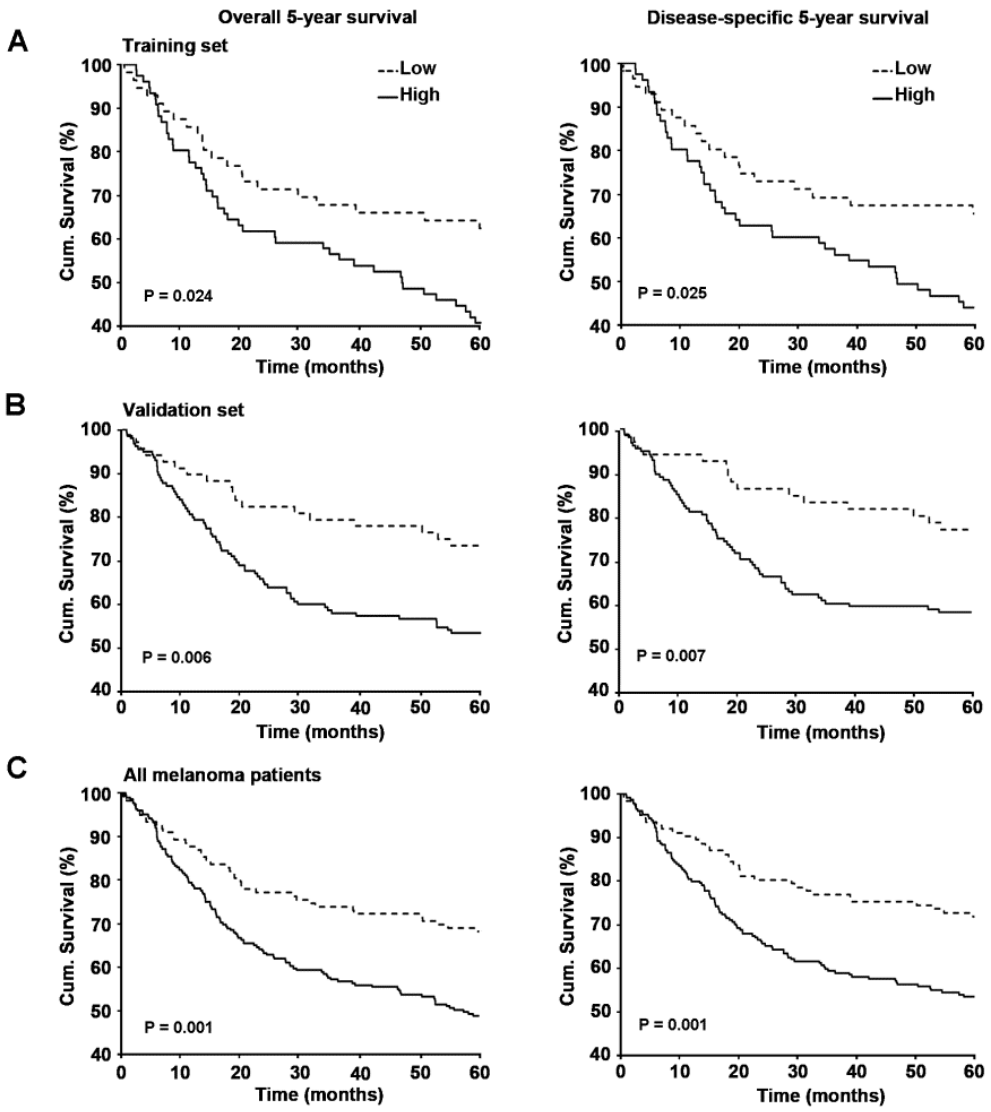

Figure 4.5. The correlation between BRAF expression and melanoma patient survival. High BRAF expression is significantly associated with poor patient overall (left) and disease specific (right) 5-year survival in training set (a), validation set (b), and all melanoma patients (c).

Table 4.2. Cox regression analyses on overall and disease-specific 5-year survival of melanoma patients

Variables [a]	Overall survival						Disease-specific survival					
	Univariate			Multivariate			Univariate			Multivariate		
	HR	**95% CI**	***P***	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
All melanoma (n=370)												
BRAF	1.85	1.28-2.65	0.001	1.06	0.72-1.57	0.747	1.89	1.28-2.79	0.001	1.06	0.72-1.57	0.747
Age	1.05	0.77-1.44	0.741	1.07	0.74-1.39	0.917	0.97	0.70-1.36	0.902	1.01	0.74-1.39	0.917
Sex	0.98	0.71-1.34	0.901	0.79	0.57-1.10	0.175	0.95	0.67-1.33	0.769	0.79	0.57-1.10	0.175
AJCC	5.41	3.26-8.97	<0.001	5.45	3.19-9.28	<0.001	6.94	3.83-12.5	<0.001	5.43	3.18-9.28	<0.001
Primary melanoma (n=232)												
BRAF	2.08	1.18-3.65	0.011	1.21	0.65-2.23	0.543	2.39	1.23-4.62	0.009	1.26	0.64-2.68	0.446
Age	1.91	1.12-3.23	0.016	1.41	0.80-2.48	0.230	1.61	0.89-2.90	0.109	1.27	0.60-2.14	0.685
Sex	0.70	0.42-1.17	0.180	0.69	0.40-1.17	0.173	0.64	0.35-1.15	0.139	0.62	0.35-1.19	0.680
Thickness	3.64	2.09-6.35	<0.001	2.86	1.53-5.33	0.001	4.56	2.35-8.81	<0.001	3.49	1.68-7.28	0.001
Ulceration	2.99	1.76-5.07	<0.001	1.60	0.88-2.90	0.122	3.55	1.97-6.40	<0.001	1.83	0.94-3.53	0.073
Location	0.96	0.54-1.70	0.892	0.93	0.51-1.69	0.815	0.71	0.35-1.44	0.356	0.73	0.34-1.50	0.390

[a] Coding of variables: Age was coded based on median age in patient groups: 1 (≤62 years) and 2 (>62 years) for all melanoma patients; and 1 (≤66 years) and 2 (>66 years) for primary melanoma. Sex was coded as 1 (male) and 2 (female). BRAF was coded as 1 (low) and 2 (high). Thickness was coded as 1 (≤2mm) and 2 (>2mm). Ulceration at the time of diagnosis was coded as 1 (no ulceration) and 2 (with ulceration). Location of lesions were coded as 1 (sun-protected area) and 2 (sun-exposed area). AJCC was coded as 1 (stage I) and 2 (stages II-IV).

NOTE: HR, hazard ratio; CI, confidence interval.

4.3. Discussion

A range between 36 to 45% has been reported for the prevalence of *braf* mutation in primary melanomas(Curtin et al. 2005, Liu et al. 2007, Thomas et al. 2007) and 42 to 55% in metastatic melanomas (Houben et al. 2004, Ugurel et al. 2007, Aguissa-Toure et al. 2011, Long et al. 2011). Interestingly, mutation of *braf* also occurs in nevocytic nevi, therefore it is thought to be involved in initiation of melanoma (Kumar et al. 2004). Recently, BRAF has been shown to be an important key player in melanoma cell invasion and tumor growth as well (Hoeflich et al. 2006, Arozarena et al. 2011). However, to our knowledge, so far there is no comprehensive study on the expression of BRAF at protein level and its possible correlation with tumor progression or patient survival of human melanoma. In this portion of the study, a total of 370 melanoma patients along with 49 nevi cases were analyzed for the expression of BRAF protein using TMA technology. Statistical analysis of TMA immunoreactive scores revealed that BRAF expression was significantly increased in primary melanoma compared with dysplastic nevi and further increased in metastatic melanoma compared with primary melanoma (Figure 4.1). These findings indicate the role of BRAF in the progression of melanoma from nevi to primary melanoma and further to metastatic melanoma. Furthermore, we found that BRAF expression was positively correlated with AJCC stage, tumor thickness and ulceration, all of which are prominent indicators of melanoma progression. In fact, this correlation highlights the important role of BRAF in melanoma progression toward more advanced stages and metastasis in addition to its already established role in the initiation of melanoma (Omholt et al. 2003, Dankort et al. 2009). The increased expression of BRAF protein in metastatic melanoma suggests that cancer cells at higher stage may have further increased their activity of BRAF and acquired the ability to metastasize to new sites. Interestingly, our observations are consistent with new studies

demonstrating the involvement of BRAF in progression of melanoma toward metastasis by enhancing its invasion and migration (Hoeflich et al. 2006, Arozarena et al. 2011).

Although some researchers did not find a significant effect of *braf* V600E mutation on melanoma patient survival (Shinozaki et al. 2004, Edlundh-Rose et al. 2006, Ugurel et al. 2007), a comprehensive meta-analysis report by our group (chapter 3) revealed a HR of 1.7 for this mutation as a significant risk factor for melanoma patients (Safaee Ardekani et al. 2012). Remarkably, our TMA data for the first time revealed the negative effect of high BRAF protein expression as well, as a significant risk factor for melanoma patient survival. This negative effect was persistently significant through our training and validation set analysis (Figure 4.5) as well as all our patient samples together (Fig. 4.2.4a). We did not observe a significant correlation between BRAF expression and patient survival in metastatic melanoma. Also, we did not find a significant difference in BRAF expression at higher AJCC stages (between stage III and IV). However, the BRAF protein expression level was significantly changed in a comparison of lower AJCC stages (between stage I and II), which implies a more functional role for BRAF at early phases of disease progression toward metastasis. In fact, the lack of obvious variation in BRAF expression level among higher AJCC levels (stage III and IV), which represent metastatic melanoma patients explains the observed non-significant effect of BRAF expression on metastatic melanoma patient survival. Our findings are indeed in line with previous studies which suggest the insufficiency of BRAF activity alone and the necessity of other genes' alteration (e.g. PTEN and CDKN2A) collaborate at the metastatic levels leading to melanoma progression (Dankort et al. 2009, Goel et al. 2009). We observed an increase in the prevalence of *braf* V600E mutation in patients with higher BRAF protein expression. However, the prevalence of V600E mutation in our low BRAF protein expression group was slightly lower than the

previous reports and the statistical analysis showed no significant correlation between the level of BRAF protein expression and V600E mutation. Although we selected the tumor parts of the tissue samples for DNA extraction, we think the contaminating DNA signals from surrounding cells (e.g. stromal cells and T-cell) would affect the sequencing results. Since the majority of low BRAF expression samples were from primary melanoma group, the chance of contamination with non-melanoma DNA was higher than metastatic melanoma samples, where most of high BRAF samples were located. Notwithstanding the fact that few other researchers were also not able to detect a significant correlation between BRAF protein expression and V600E mutation in thyroid cancer and melanoma patients (Tanami et al. 2004, Uribe et al. 2006, Kondo et al. 2007), there is a possibility that the number of patients in our analysis might affect the results. Considering the very high importance of *braf* mutation in melanoma treatment and the feasibility of immunohistochemistry analysis for BRAF expression, whether V600E mutation could definitely affect the BRAF protein expression needs a comprehensive prospective analysis with a large sample size. Despite the staggering amount of information regarding the function and regulation of BRAF activity, very little is known about the mechanisms by which the expression of BRAF mRNA and protein is regulated. One possible scenario would be the amplification of the genomic sequences encoding BRAF. However, overexpression of BRAF has been suggested not to be strictly a result of gene amplification in melanoma cells (Tanami et al. 2004, Uribe et al. 2006). Another possible mechanism would be BRAF protein's enhanced stability conferred by activating mutations or the expression of BRAF splice variants (which is discussed in greater detail in next chapter). Nevertheless, our findings implicate the prominent role of BRAF protein expression in melanoma biology and evidently highlight its prognostic value in patient outcome assessment for primary melanoma. However, further studies are required to explain the

mechanism by which BRAF protein expression, as a significant risk factor in melanoma patients, is increased.

In conclusion, we revealed that BRAF protein expression is significantly increased in primary melanomas compared with nevi samples and further increased in metastatic melanomas. Moreover, we found that high BRAF protein expression is correlated with poor overall and disease-specific five-year survival while the correlation of BRAF protein expression with V600E mutation was not statistically significant. Although the exact mechanism responsible for the increased BRAF protein expression in melanoma patients needs further studies, our observations suggest a new method in melanoma patient outcome evaluation. We showed that BRAF protein expression analysis through immunohistochemistry could be used as a cheap and feasible method to predict melanoma patients' outcome.

Chapter 5. Kinase dead splice variants of BRAF: the expression pattern and effect on melanoma progression and patient survival

5.1. Background

Despite much recent advancement in melanoma treatment, the prognosis for metastatic melanoma patients remains poor and they may only survive for a few months. BRAF, a key upstream activator of mitogen-activated protein kinase (MAPK) pathway, is highly mutated in melanoma patients. Since the first report of high BRAF mutation prevalence in melanoma by Sanger institute (Davies et al. 2002), the clinical approach to disease treatment and patient prognosis has been revolutionized. An activating BRAF mutation is reported in more than 50% of melanoma cases; among which, BRAF V600E mutation (a T to A substitution at codon 1799) is the most prevalent one (Davies et al. 2002). As a result, FDA approval for the BRAF inhibitor vemurafenib (PLX4032/ Zelboraf) in late 2011 was a breakthrough in the treatment of patients carrying BRAF V600E mutations (Chapman et al. 2011). However, the hope for a revolutionary melanoma treatment did not last for long due to the rapid emergence of acquired resistance to BRAF inhibitors (Wilson et al. 2012).

Over-activation of alternative pathways, CRAF activation, and emergence of new BRAF spliced variants (BRAFsv), which are independent of upstream signals, are a few potential mechanisms by which BRAF inhibitor resistance may occur (Montagut et al. 2008, Emery et al. 2009, Nazarian et al. 2010, Poulikakos et al. 2011, Corcoran et al. 2012, Prahallad et al. 2012, Su et al. 2012). The reported BRAFsv are usually missing some parts of conserved region (CR) 1 and 2 (Ras binding domain and cysteine rich domain), which in turn leads to a constant BRAF

activity independent of upstream RAS signals (Poulikakos et al. 2011). By contrast to the previously reported BRAFsv, here we describe the characterization of novel splice variants, which are missing parts of the kinase domain (CR3), thus giving rise to a dominant negative suppression of BRAF activity. We found that the expression of these variants significantly correlates with a better prognosis and prolonged patient survival.

5.2. Results

5.2.1. Alternative splicing of BRAF in melanoma patients and cell lines

Total RNA was extracted from six melanoma patients who had not received any BRAF inhibitor treatment before. After cDNA synthesis, full length BRAF coding region was amplified in search for expression of alternative splice variants. All of the patients expressed at least two main shorter variants of BRAF along with full length BRAF (Figure 5.1. A). Nine melanoma cell lines (PMWK, MMRU, MMLH, MMAN, A375, SK110, SK93, RPEP, and RPMI) were further investigated for verification of BRAFsv. Full-length *braf* coding region amplification in these melanoma cell lines also revealed multiple variant expressions along with full-length *braf* in all cell lines. The main dominant variants in most cell lines were sized around 1.2 kp (Figure 5.1B).

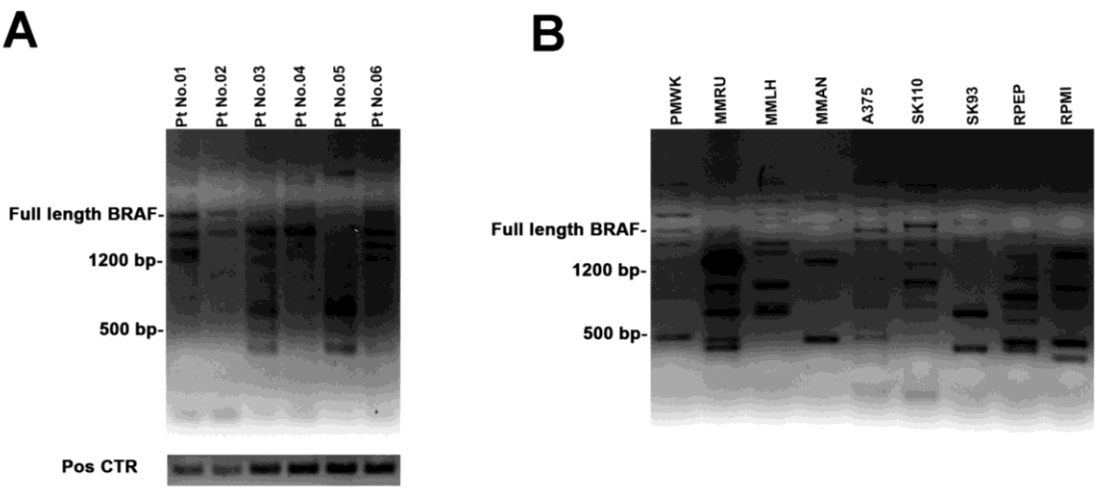

Figure 5.1. The expression pattern of braf gene variants in melanoma patient samples and cell lines. Results of PCR for whole braf gene amplification in (A) 7 melanoma patient samples and (B) 9 melanoma cell lines. RNA was extracted from samples and PCR was performed on the constructed cDNA using a pair of primers amplifying the whole length of braf gene. Interestingly, full length braf gene (2.3kb) was not fully expressed in all patient samples and cell lines and multiple braf variants were expressed in each sample. Although each patient had a unique pattern of braf variant expression, the 1200pb variant was consistently expressed in most of the cell lines and the patient samples.

To further identify the BRAF variants in more detail, eight pairs of primers were designed to break down the *braf* coding sequence into smaller segments of 200-400 bp (Table 2.3). PCR amplification of *braf* in smaller segments revealed that MMAN and SK110 melanoma cell lines dominantly express shorter variants of *braf* missing a large segment of the kinase domain (Figure 5.2A). Each successfully amplified variant of *braf* was extracted and cloned into a plasmid vector for sequencing. Sequencing results revealed two new splice variants of BRAF missing exon 4-17 (BRAF ΔEx 4-17), exon 11-17 (BRAF ΔEx 11-17) (Figure 5.2B).

To evaluate the protein expression of these splice variants in melanoma cell lines, total cell lysate extract was assessed through western blotting. Two separate BRAF antibodies targeting N-terminal or C-terminal BRAF protein (BRAF 12-156 and BRAF 431-445 respectively) were

used to differentiate between kinase dead splice variants and full-length BRAF expression. As it is viewed in Figure 5.2C, D, MMAN and SK110 cell lines which dominantly expressed kinase dead *braf* sv at the DNA level, also expressed a lower amount of full length BRAF at the protein level when probed with BRAF 12-156 antibody. In addition, BRAF 431-445 was not able to detect the full-length BRAF in MMAN and SK110 cell lines while a shorter variant around 43kDa was detected by BRAF 12-156 antibody.

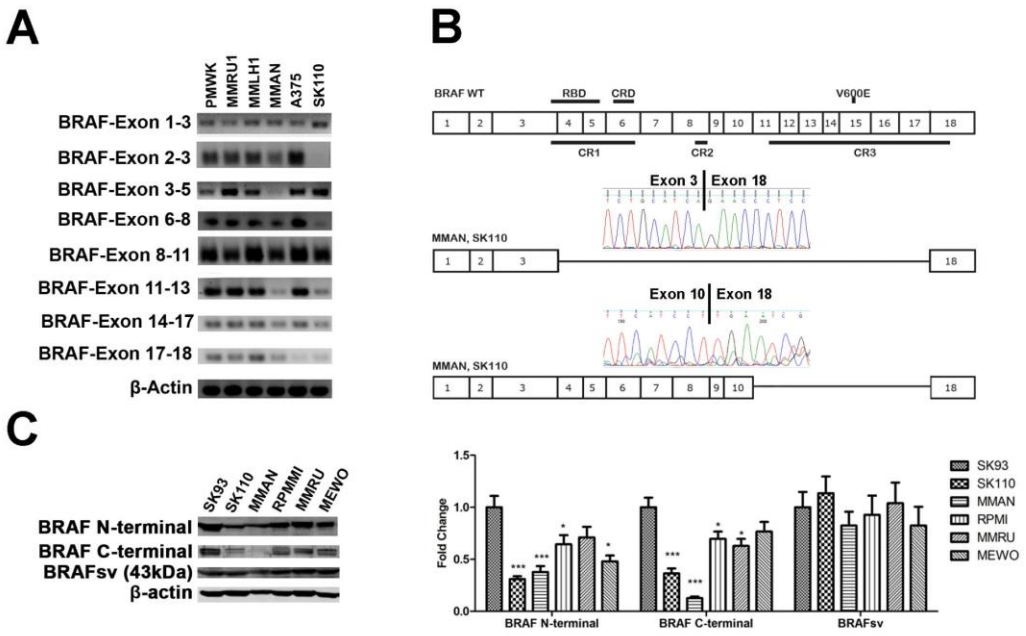

Figure 5.2. The *braf* gene expression analysis in melanoma cell lines. (A) Full length braf gene was divided into eight smaller segments and the results of conventional PCR was visualized on 1.5% agarose gel. Two melanoma cell lines (MMAN and SK110) have very low expression of exons 4-6 and 11-17 that contain most parts of the kinase domain. (B) Each variant was separately cloned into a plasmid vector and sequenced accordingly. Most prevalent splice variants of braf gene found in melanoma cell lines are depicted in a schematic view. (C) The difference in protein expression patterns of melanoma cell lines in immunoblot assay. Cell lines that are missing exons 4-17 (SK110 and MMAN) express lower levels of full length BRAF protein expression with both BRAF N-terminal (BRAF 12-156) and C-terminal (BRAF 431-445) while they have a significant expression of a smaller variant 47kd (detected by BRAF N-terminal antibody). Bar chart shows mean±SD of three independent quantifications of western blots. Asterisks show the statistical difference between cell lines.

5.2.2 Dominant negative effect of BRAFsv

BRAF ΔEx 4-17 (strongly expressed in metastatic melanoma cell lines MMAN and SK110), was sub-cloned into a 3×Flag-CMV-10 expression vector. Over expression of this BRAFsv in MMRU cell line showed a significant decrease in phosphorylation of downstream factor pERK/MEC (P = 0.029) (Fig 5.3A). Further cell survival analysis using MTS assay (which is an indirect indicator of mitochondrial activity) showed that over expression of BRAF ΔEx 4-17 in MMRU cell lines leads to a decrease in the melanoma cell survival after 48 hours (P = 0.024) (Fig 5.3 B). This finding is another evidence in favor of the dominant negative effect of BRAF ΔEx 4-17 on full length BRAF.

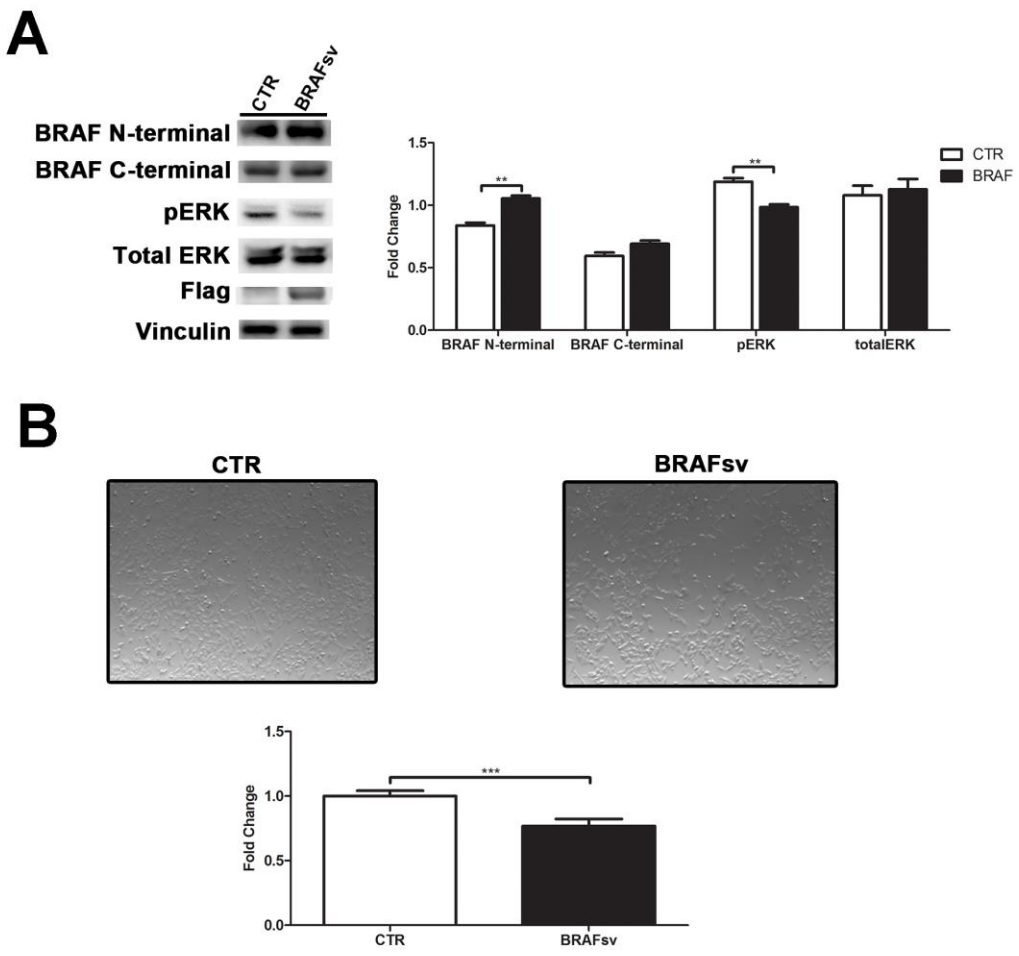

Figure 5.3. Dominant negative effect of BRAFsv on full length BRAF in melanoma cell lines. Phosphorylation of MAPK (ERK1/2) was significantly decreased after over expression of BRAF Ex Δ4-17 in melanoma cell lines (P = 0.029) (A) while the amount of total ERK expression was remained unchanged. The bar graph shows the quantification of three independent measurements. (B) MTS assay indicates that BRAF Ex Δ4-17 over expression significantly decreases the viability of metastatic melanoma cell lines after 48hrs (P = 0.024). Microscopic pictures and the bar graph are representative of three separate biological replicates of the same experiment.

5.2.3. Clinicopathologic and demographic features of the patients

In the beginning 638 tissue samples from melanoma patients at different stages were used for this study. However, because of core loss and insufficient tumor cells in some cores, 438 melanoma and 49 nevi samples were used for immuno-histochemical assessment. We lost 23 patients during follow up and 257 primary melanoma cases, 158 metastatic melanoma, 25 normal nevi, and 24 dysplastic nevi samples were stained for BRAFsv using BRAF 12-156 antibody (Figure 2.1). BRAF 431-445 antibody staining was used to further exclude the patients expressing full-length BRAF. Sixty-five primary and 27 metastatic cases expressing C-terminal spliced BRAF variants were used to evaluate the effect of BRAFsv on melanoma patient survival.

Of 232 primary melanoma cases, age ranging from 7 to 95 years (median 66), 104 were female and 128 male. One hundred thirty six cases of primary melanoma were $\leq$2 mm (low-risk), and 96 were thicker than 2 mm (high-risk). Ulceration was detected in 46 cases of primary melanoma patients. For 138 metastatic melanoma patients, 45 were female and 93 male, age ranging from 23 to 94 years (median 59). We also used American Joint Committee on Cancer (AJCC) stage to categorize the patients: 126 cases were at AJCC stage I, 106 at stage II, 59 at stage III, and 79 at stage IV at the time of diagnosis (Table 5.1).

Table 5.1. BRAF N-terminal epitope expression and clinicopathologic characteristics of melanoma patients

Variables	Neg. BRAF No. (%)	Pos. BRAF No. (%)	Total	P value
All melanoma (n= 431)				
Age, y				
≤ 62	65 (27.9)	168 (72.1)	233	.326
> 62	47 (23.7)	151 (76.3)	198	
Sex				
Male	67 (25.3)	198 (74.7)	256	.674
Female	45 (27.1)	121 (72.9)	166	
AJCC stage				
I	38 (27.9)	98 (72.1)	163	<.0001
II	15 (11.5)	116 (88.5)	131	
III	27 (37.5)	45 (62.5)	72	
IV	32 (34.4)	60 (65.6)	92	
Primary melanoma (n= 272)				
Age, y				
≤ 62	28 (20.1)	111 (79.1)	139	.902
> 62	26 (19.5)	107 (80.5)	133	
Sex				
Male	29 (18.4)	129 (81.6)	158	.466
Female	25 (21.9)	89 (78.1)	114	
Tumor thickness (mm)				
≤ 1.0	29 (37.2)	49 (62.8)	78	<.0001
1.01 -2.00	11 (15.5)	60 (84.5)	71	
2.01 -4.00	3 (5.2)	55 (94.8)	58	
>4.00	11 (16.9)	54 (83.1)	65	
Ulceration				
Absent	44 (20.8)	168 (79.2)	212	.483
Present	10 (16.7)	50 (83.3)	60	
Subtype				
Lentigo maligna	12 (26.7)	33 (73.3)	45	.178
Superficial spreading	20 (21.1)	75 (78.9)	95	
Nodular	5 (9.6)	47 (90.4)	52	
Unspecified	17 (21.3)	63 (78.8)	80	
Site [a]				
Sun-protected	15 (21.7)	54 (78.3)	69	.649
Sun-exposed	39 (19.2)	164 (80.8)	203	
Metastatic melanoma (n= 159)				
Age, y				
≤ 62	37 (39.4)	57 (60.6)	94	.364
> 62	21 (32.3)	44 (67.7)	65	
Sex				
Male	38 (35.5)	69 (64.5)	107	.717
Female	20 (38.5)	32 (61.5)	52	

5.2.4. Correlation between BRAFsv expression and clinicopathologic parameters

Using the monoclonal antibody against N-terminal epitope (BRAF 12-156) TMAs were stained and scored as described. A significant difference in BRAF staining was observed between dysplastic nevi and primary melanoma as well as between primary and metastatic melanoma ($P = 0.001$ and <0.0001, respectively; Kruskal-Wallis test). However, no significant difference was detected between normal nevi and dysplastic nevi samples ($P > 0.05$; Kruskal-Wallis test, data not shown). Similarly, $\chi 2$ test confirmed an increase in positive expression of BRAF (N-terminal epitope) in primary melanoma compared with dysplastic nevi (87.9% and 74.14% respectively, $P = 0.004$; Figure 5.5A, B, C). Interestingly, metastatic melanoma cases lost the expression of BRAF (N-terminal epitope) and showed a lower expression level (67.04%) compared to primary melanoma ($P < .0001$; Figure 5.4A, B, C). We also found the same trend in BRAF (N-terminal epitope) expression in correlation with AJCC staging. The expression increased from 72.1% to 88.5% from stage I to II but then decreased to an average of 64% in metastatic melanoma stages (AJCC III and IV) ($P < .0001$; Table 5.1). In addition, we found a positive correlation between BRAF (N-terminal epitope) expression and lesion thickness in primary melanoma patients while very thick lesions >4mm lost the expression and did not follow the trend ($P < .0001$). However, we did not find any significant correlation between BRAF (N-terminal epitope) expression and ulceration, melanoma subtypes, the location of the lesions or other clinicopathologic variables such as age and gender in primary or metastatic melanoma patients (Table 5.1).

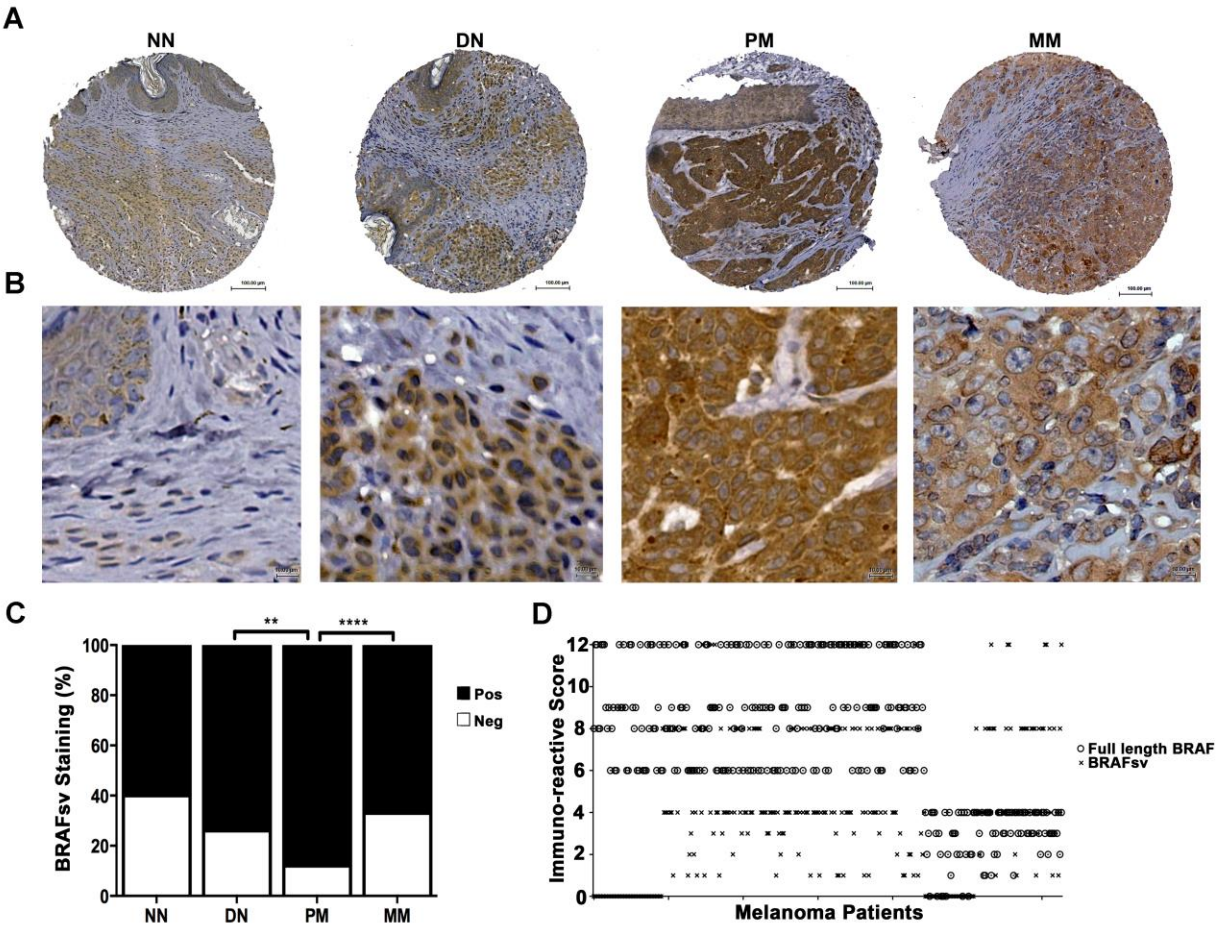

Figure 5.4. Correlation between BRAFsv expression and melanoma progression. (A, B) Representative images at low (A) or high (B) magnification of cytoplasmic BRAFsv immunohistochemistry staining which shows a gradual increase in BRAFsv protein expression from nevocytic nevi (NN) samples (Immuno-reactive Score IRS: 2) to dysplastic nevi (DN) (IRS:4) and then to primary melanoma (PM) (IRS: 12). However, mean IRS score decreased in metastatic melanoma (MM) (IRS: 8). Bar = 100 µM in core pictures and 10 µM in high magnifications. (C) Bar graph shows the increasing pattern in positive expression of BRAFsv protein from NN, to DN, and further up to PM, while MM patients had lower levels of BRAFsv expression. The number of negative cores for BRAFsv staining is significantly higher in MM cases compared with PM. (D) Graph shows the correlation of full length BRAF and BRAFsv protein expression. Patients with high levels of BRAF full length expression (usually representing advanced PM or MM cases) show significantly lower levels of BRAFsv expression. However there is a positive correlation between full length BRAF and BRAFsv in patients with low to moderate levels of full length BRAF expression.

In next step we stained the same TMAs for C-terminal BRAF epitope using BRAF 431-445. Because BRAF C-terminal is only expressed in full length BRAF, not the kinase dead splice variants, we were able to indirectly evaluate BRAFsv protein expression and its correlation with full length BRAF. As we reported previously, full length BRAF protein expression was significantly increased with melanoma disease progression (Safaee Ardekani et al. 2013). The protein expression of kinase dead BRAFsv was evaluated by deducting tissue samples expressing C-terminal epitope from our patient database who positively expressed BRAF N-terminal epitope (Figure 2.2). BRAFsv expression followed the same pattern of progression in melanoma progression as BRAF N-terminal epitope, however the difference was more obvious (Figure 5.5A, B Mean rank for PM:4.60 to MM:1.96; P= .002; Kruskal-Wallis test). Furthermore, our correlation analysis indicated that patients with high levels of full length BRAF expression (mostly advanced stages of primary or metastatic melanoma)(Safaee Ardekani et al. 2013) showed a low level of BRAFsv expression while low to medium expression of full length BRAF was positively correlated with BRAFsv expression (Figure 5.4D).

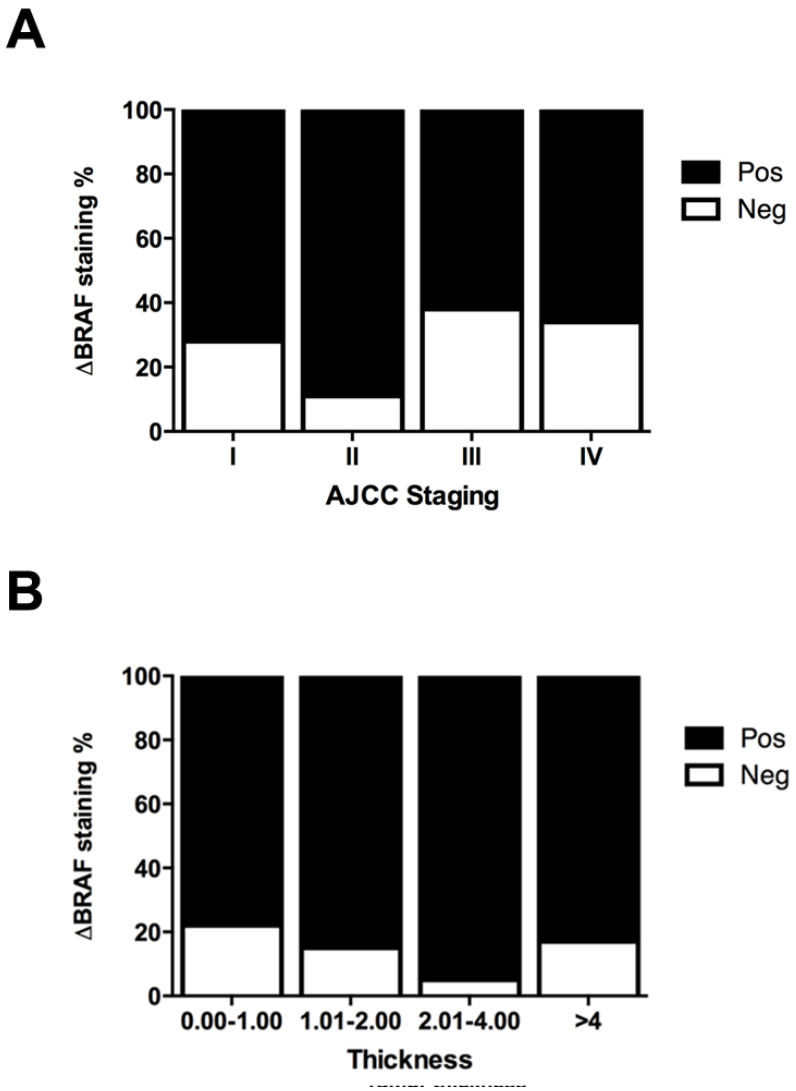

Figure 5.5. Progression pattern of BRAFsv in melanoma. (A) The BRAFsv protein expression increased from normal nevi to dysplastic nevi and primary melanoma while metastatic melanoma patients had lower expression compared with primary melanoma. Because BRAF-N-Terminal staining is able to detect both full length BRAF and BRAFsv, it could be concluded that the effect of BRAFsv is very strong at the presence of full length BRAF as both analysis showed a similar progression pattern while the difference is more obvious in BRAFsv analysis. (B) BRAFsv also showed the similar pattern of expression with tumor thickness as it had with AJCC staging. Very thick primary melanoma tumours that are considered in stage III of AJCC staging and mostly are metastatic showed lower BRAFsv expression compared to thinner tumours at earlier stages.

5.2.5. Correlation of BRAFsv and full length BRAF expression with patient survival

Utilizing Kaplan-Meier survival curves, first we assessed the correlation of BRAF full-length expression and 5-year overall and disease-specific survival of melanoma patients. Our analysis showed the same negative effect of BRAF full-length expression with patient outcome as we reported before (Safaee Ardekani et al. 2013). High expression of full-length BRAF was correlated with poor overall (P = 0.001) and disease-specific 5-year survival (P = .001) (Figure 5.6A). However, Kaplan-Meier analysis of BRAF N-terminal expression revealed a positive effect on patients' survival. Melanoma patients with positive expression of BRAF N-terminal showed a better survival rate compared with patients who did not express BRAF N-terminal (P = 0.045 overall, and P = .047 disease specific 5-year survival) (Figure 5.2.6B). Accordingly, patients with positive expression of BRAFsv showed even more significant higher survival rates compared with those who did not express BRAFsv (P = .027 overall, and P = .011 disease specific 5-year survival) (Figure 5.6C).

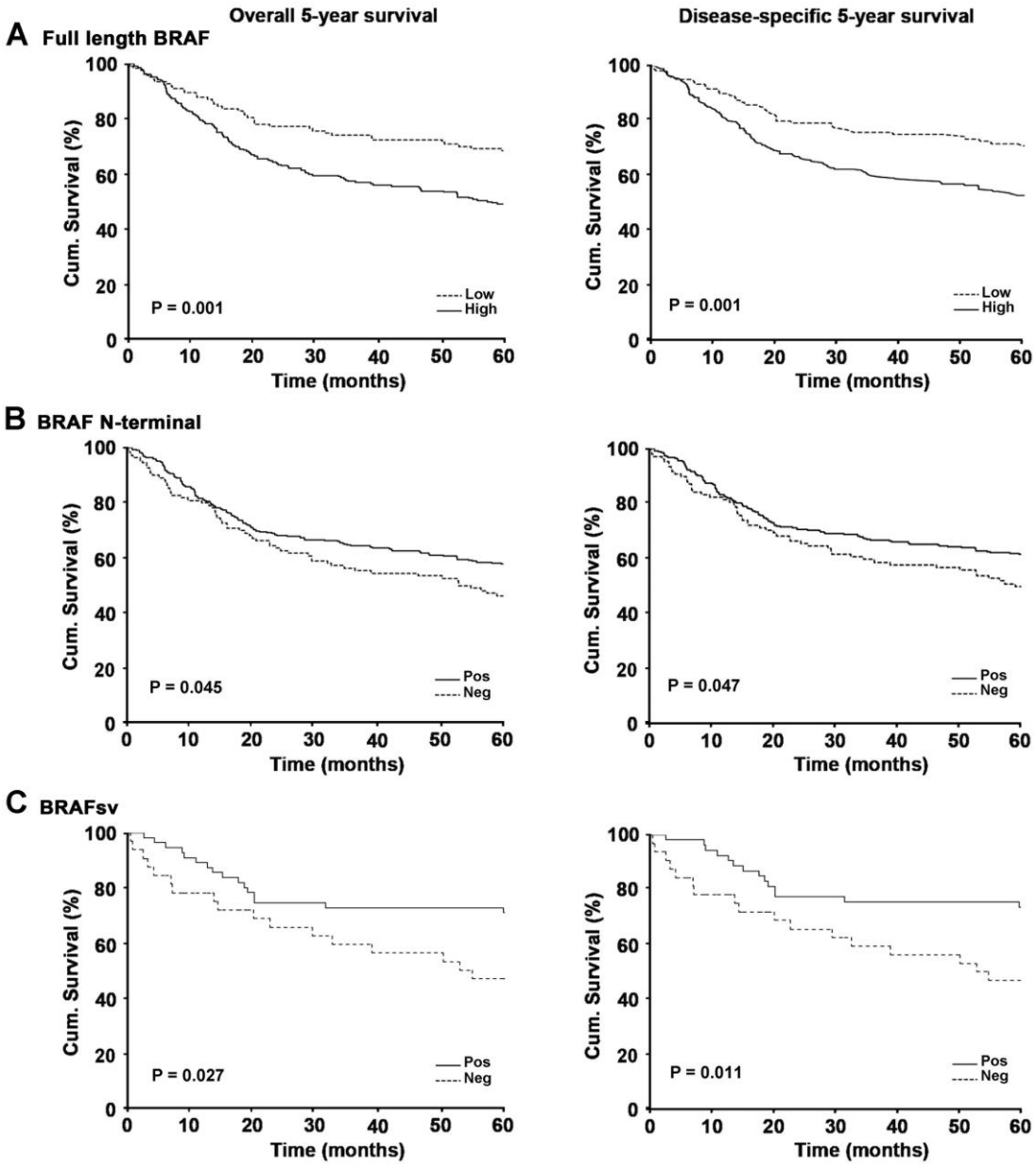

Figure 5.6. The correlation between whole BRAF and BRAFsv protein expression and melanoma patients' survival. (A) High expression of full length BRAF is significantly correlated with poor overall (left) and disease-specific (right) 5-year patient survival in melanoma patients (P=.001 both). (B) Positive expression of BRAF N-terminal is associated with a significantly better overall (left) and disease-specific (right) 5-year patient survival in melanoma patients (P=.045 and P=.047 respectively). (C) Positive expression of BRAFsv is associated with a significantly longer overall (left) and disease-specific (right) 5-year survival in melanoma patients (P=.027 and P=.011 respectively).

In addition, we used Cox proportional hazard regression model to estimate the crude hazard ratios of each clinicopathological characteristic. Univariate Cox regression analysis of BRAFsv expression revealed a hazard ratio (HR) of .47 (95% CI, .23-.97; P = 0.031) for overall survival and HR of .41 (95% CI, .20-.83; P = 0.014) for disease-specific survival (Table 5.2). As expected, multivariate Cox regression analysis showed that BRAFsv expression is not an independent prognostic factor as it is significantly correlated with AJCC staging (Table 5.2).

Table 5.2. Cox regression analyses on overall and disease-specific 5-year survival of melanoma patients

Variables [a]	Overall survival						Disease-specific survival					
	Univariate			Multivariate			Univariate			Multivariate		
	HR	**95% CI**	***P***	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Melanoma (n=92)												
BRAFsv	.47	.23-.97	.031	1.19	.52-2.80	.679	.41	.20-.83	.014	.97	.40-2.35	.963
Age	1.56	.79-3.10	.196	1.96	.95-4.03	.066	1.38	.68-2.81	.361	1.67	.79-3.51	.172
Sex	1.19	.59-2.40	.618	1.07	.51-2.26	.842	1.16	.56-2.39	.682	1.03	.48-2.21	.931
AJCC	5.03	2.49-10.1	<.0001	5.97	2.57-13.8	<.0001	5.21	2.53-10.7	<.0001	5.46	2.29-13.0	<.0001

Furthermore, patients were divided into four categories based on the level of BRAF full length and BRAFsv expression to further evaluate the effect of BRAF protein expression on patients' survival in more detail. We found that patients expressing BRAFsv along with low levels of BRAF full-length, had the best survival rate. While those patients who had a high level of full-length BRAF with no BRAFsv showed the worst survival rate among all four groups (P = .018 overall, and P = 0.022 disease specific 5-year survival) (Figure 5.7). This observation is in line with what we have seen at cellular level. The patients who are expressing the full length BRAF (with kinase activity) have the worst survival and those who are expressing the kinase dead BRAFsv (which has showed a negative dominant effect at cellular level) have the best survival rates.

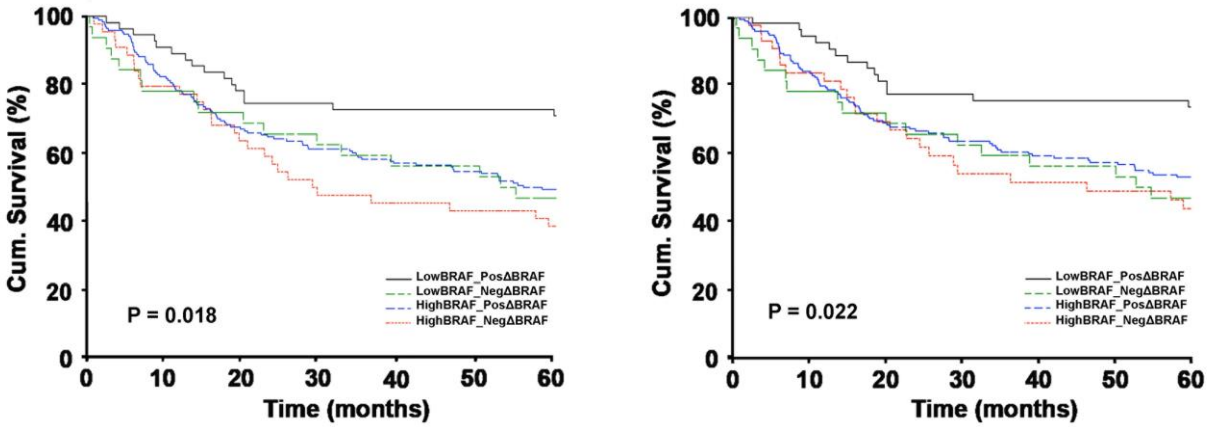

Figure 5.7. Combined effect of BRAF full length and BRAFsv protein expression on melanoma patient survival. Melanoma patients are divided into four groups based on the level of full length BRAF and BRAFsv expression. Survival analysis revealed that patients with positive expression of BRAFsv and low expression of full length BRAF have the best 5 year survival, and those with negative BRAFsv and a high level of full length BRAF expression have the worst outcome in 5 years of follow up (overall (P=.018) and disease specific (P=.022) 5 year survival).

5.2.6. Effect of BRAFsv on BRAF inhibitor treatment in melanoma cell lines

To evaluate the effect of BRAFsv on BRAF inhibitor treatment, a total number of 1.4×10^4 MMRU (a *braf*V600E mutant metastatic melanoma cell line) were seeded and treated with DMSO or different concentrations of PLX4720 (5μM and 10μM). BRAF Ex Δ4-17 was over expressed in one additional group with PLX4720 (10μM) treatment. All samples were followed for up to 72hrs while media was changed after 48hrs. MTS assay was used to compare the viability of cells in different groups (data was normalized and depicted as fold change compared to the control group). As it is shown in the Figure 5.8, PLX4720 was effective in blocking melanoma cell growth in a dose dependent manner (one-way ANOVA: $P < 0.0001$; CI: 95%). In addition, over expression of BRAF Ex Δ4-17 in melanoma cell lines was shown to increase the efficacy of PLX4720 by 18% ($P = 0.009$) (Figure 5.8).

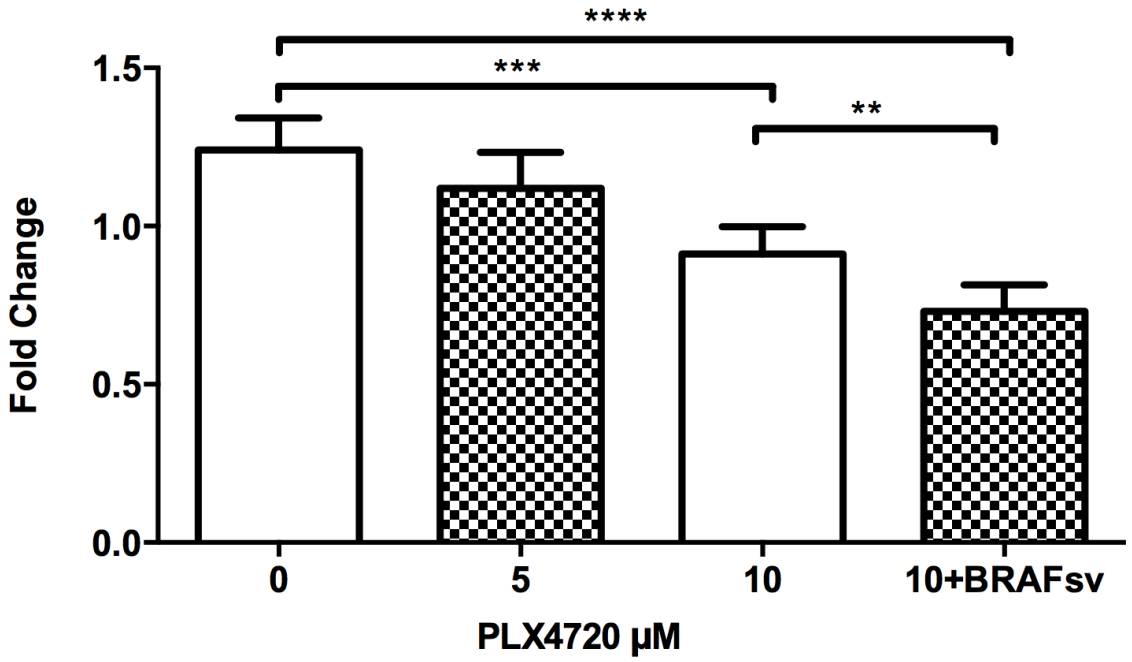

Figure 5.8. Effect of BRAFsv over expression on BRAF inhibitor treatment. MMRU cell line (a BRAF-V600E mutant metastatic melanoma) was seeded and treated with DMSO or different concentrations of PLX4720 (5µM and 10µM). BRAF Ex Δ4-17 was over expressed in one additional group with PLX4720 (10µM) treatment. Samples were followed for up to 72hrs, and were analyzed for cell viability using MTS assay (data was normalized and showed as fold change compared to the control group). As it is shown in the graph over expression of BRAF Ex Δ4-17 in melanoma cell lines increased the efficacy of PLX4720 in blocking melanoma cell growth by 18% (P=0.009).

5.3. Discussion

BRAF inhibitors were introduced as one of the most promising drug treatments for improving progression-free and overall survival in melanoma patients carrying this mutation (Flaherty et al. 2010, Chapman et al. 2011). Although the early response to BRAF inhibitor treatment was very dramatic, almost all patients become resistant to treatment in a few months (Chapman et al. 2011, Wagle et al. 2011, Sosman et al. 2012, Wilson et al. 2012, Ascierto et al. 2013). A number of mechanisms including RAS activation or CRAF mutation, activation of parallel pathways like PI3K/AKT or receptor tyrosine kinase, COT expression have been proposed to explain the resistance to BRAF inhibitor treatment (Emery et al. 2009, Nazarian et al. 2010, Villanueva et al. 2010, Poulikakos et al. 2011, Lito et al. 2012, Shi et al. 2012). Accordingly, Poulikakos and co-workers (Poulikakos et al. 2011), reported a truncated variant of BRAF in resistant melanoma cell lines and patient samples that lack exon4-8 (containing the RAS-binding-domain). This splice variant shows an enhanced dimerization activity independent of upstream RAS signaling that induces resistance to BRAF inhibitors.

A variety of other types of BRAFsv were also reported in other diseases including thyroid carcinoma and rheumatoid arthritis (Baitei et al. 2009, Weisbart et al. 2013). The majority of the reported variants in these two reports were observed to induce MAPK activity even though some were missing parts of the kinase domain (e.g. BRAF ExΔ4-13)(Heidorn et al. 2010, Weisbart et al. 2013). It was postulated that the altered open reading frame for C-terminal BRAF, and conservation of required C-terminal sequence for CRAF dimerization in these kinase dead BRAFsv, leads to a hetero-dimerization with CRAF and consequent MAPK activity (Weisbart et al. 2013). However more recently, Hirschi and colleagues (Hirschi et al. 2013) reported a significantly low kinase activity for new splice variants of BRAF missing parts of the kinase

domain (e.g. BRAF ExΔ14-15) in colorectal cancer. They reported the alternative transcripts to be derived from both wild type and BRAF-V600E mutant parental alleles, while the kinase dead BRAF variants disrupt the overall cellular activity of BRAF and MAPK.

In this chapter we described novel kinase dead splice variants of BRAF in melanoma patients' samples and cell lines that have suppressing activity of the MAPK pathway (Figure 5.3 A) and reduce melanoma cell viability *in-vitro* (Figure 5.3 B). We found a simultaneous expression of BRAFsv along with full length BRAF in melanoma cell lines and patients' samples. Reverse transcription and subsequent molecular cloning was chosen over differential melting curve analysis to isolate the BRAF variants as it has been shown to be a more sensitive method (Hirschi et al. 2013). The most expressed variant in cell lines and patient samples, BRAF ExΔ4-17, was successfully cloned into MMRU cell lines and phosphorylation of erk1/2 was measured to show the dominant negative effect of BRAFsv (Figure 5.3). The detection of discrete signals at the predicted protein size is an indication of gene product stability and no signs of degradation were detected. Therefore, as discussed previously (Lewis et al. 2003), we think changes in antibody binding in western blot assay after aberrant protein folding, induced the observed deviation of signal intensity rather than nonsense-mediated mRNA decay.

Previously, inhibition of BRAF activity by ATP-competitive small molecule inhibitors (like Vemurafenib) was shown to paradoxically increase the overall BRAF kinase activity through dimerization with active variants (Baitei et al. 2009, Poulikakos et al. 2010). However, none of the kinase dead variants of BRAF in colorectal cancer (Hirschi et al. 2013), and in melanoma samples from our study, were able to re-activate the full length BRAF kinase (Figure 5.3 A). The contradictory observations are most probably due to the difference in the mechanism of BRAF inactivation. While ATP-competitive small molecule inhibitors do not cause extensive

steric changes in BRAF structure and conserve the non-kinase functions, deletion of C-terminal motifs is highly likely to induce an aberrant spatial structure.

To evaluate the effect of BRAFsv expression on melanoma disease progression and patient survival, we used TMA study as it was shown to be a sensitive test for screening (Ehsani et al. 2014). We analyzed 454 melanoma patients along with 49 nevi samples as our controls. Our analysis revealed that BRAFsv expression is significantly increased in primary melanoma compared with dysplastic nevi samples while metastatic melanoma cases had lost this expression compared with primary melanoma (Figure 5.4 A,B,C, and Figure 5.5 A,B). These findings suggested a different role for BRAFsv (compared with full length BRAF), since loss of expression occurs in metastatic melanoma. Accordingly, we showed that over expression of BRAF ExΔ4-17 in metastatic melanoma cell lines significantly decreases the viability of these cells *in vitro* (Figure 5.3B). Furthermore, we found that BRAFsv expression is positively correlated with AJCC staging I and II, and lower tumor thicknesses, while higher stages of AJCC (III and IV; indicative of metastatic stages of melanoma) and very thick primary melanoma tumors (>4mm), had lost the expression of kinase-dead BRAFsv. In fact, this observation highlights a new role for BRAFsv in melanoma progression, as a protective factor, in spite of an already established role for BRAF in the initiation of melanoma (Omholt et al. 2003, Dankort et al. 2009). The decreased expression of BRAFsv protein in metastatic melanoma, along with the dominant negative effect of BRAFsv *in-vitro*, suggests that cancer cells at higher stage may maintain their tumorigenic activity by loss of expression of BRAFsv.

Previously, we have reported a negative effect of *braf*V600E mutation and BRAF protein expression on melanoma patient survival (Safaee Ardekani et al. 2012, Safaee Ardekani et al. 2013, Bhandaru et al. 2014). We used the same set of patients and were able to reproduce our

previous observation (Figure 5.6A), while here we found a novel protective role for BRAFsv protein expression on melanoma patient survival (Figure 5.6 C). Further detailed analysis revealed that patients who have low expression of full length BRAF along with positive expression of BRAFsv have the best survival rate among all melanoma patients (Figure 5.7). These data, for the first time, have revealed a protective effect for BRAFsv protein expression in melanoma disease progression, which correlated with prolonged patient survival.

In addition, our preliminary investigations showed that over expression of BRAFsv enhances the effect of BRAF inhibitor (PLX4720) in *braf*V600E mutant melanoma cell lines (Figure 5.8). Further studies are required to evaluate the effect of BRAFsv expression on BRAF inhibitor treatment response in melanoma patients. Novel techniques could be used to enhance BRAFsv expression in melanoma patients as a possible therapeutic approach for melanoma patients with wild type BRAF status or as a complimentary modality to BRAF inhibitor treatment regimens in patients with mutant BRAF. However, further mechanistic and clinical studies are required to evaluate the role of BRAFsv expression in the context of BRAF inhibitor resistance in melanoma. Nevertheless, our findings revealed novel kinase-dead spliced variants of BRAF in melanoma that express a protective effect on patients' survival. Our results also showed the co-expression of both BRAFsv and full length BRAF in melanoma patients and highlighted the critical importance of antigenic site for BRAF antibodies, especially in prognosis studies.

Chapter 6. Conclusion

6.1. Summary of findings and implications

Melanoma is the most lethal type of skin cancer, which is responsible for the majority of death due to skin cancer. It is very well managed with a simple excisional biopsy at the early stages, however there is no effective treatment available when it has metastatized to a new site (Garbe et al. 2009, Linos et al. 2009, Nikolaou et al. 2014). Genetic instability is very prevalent in melanomas, some of which induce tumor invasion, motility, angiogenesis, proliferation, and evasion of apoptosis (Chin et al. 2006). Among all mutations in non-inherited type of melanoma, BRAF (an upstream factor in the MAPK pathway) has the highest rate, which leads to over activation of the pathway (Garnett et al. 2004, Forbes et al. 2011, Holderfield et al. 2014). Although thus far *braf*V600E point mutation is the most common site for oncogenic mutations in melanoma, there is no consensus on the effect of *braf*V600E mutation on the cancer patient survival. We are the first group who utilized the systematic review and meta-analysis, as the most reliable research method that produces level-one medical evidence, to address this concern. We combined the results of four published studies on melanoma that met our inclusion criteria and analyzed a pool of 674 patients. We found that *braf*V600E mutation increases the risk of death in melanoma patients almost two times higher compared with the patients with wild type *braf*; HR= 1.7 (95% CI, 1.37- 2.12). We also found the same negative effect of *braf*V600E on colorectal cancer patients outcome (Safaee Ardekani et al. 2012). Then we further studied the role of BRAF protein expression in melanoma progression and investigated whether the negative effect of *braf*V600E mutation on melanoma patients is translated to the protein level. We found that compared with nevi samples, BRAF expression was remarkably increased in primary

melanomas and further increased in metastatic melanomas (P=1.8×10^{-11}). High BRAF expression was significantly correlated with the known negative prognostic markers including thicker tumors, ulceration and higher AJCC stages (P=1.5×10^{-7}, 1.5×10^{-5}, 3.6×10^{-13}, respectively). We also found that in primary melanoma cases, patients with high BRAF protein expression had significantly worse overall (P=0.009) and disease-specific five-year survival (P=0.007). This effect was similar to our previous finding of *braf*V600E effect on patient survival (HR=2.08 overall survival; HR=2.39 disease-specific survival). However, metastatic melanoma patients did not show the same negative effect for BRAF protein expression. We also investigated the correlation between BRAF protein expression and *braf*V600E in our melanoma database. Although we found a trend for higher prevalence of *braf*V600E mutation in patients with high BRAF protein expression, no significant correlation was observed between protein expression and *braf*V600E mutation. So far we have shown the negative effect of *braf*V600E mutation on patients' survival and highlighted the importance of BRAF protein expression in melanoma progression. Also we showed that BRAF protein expression could be used as a cheap and feasible prognostic marker in melanoma patients independent from *braf*V600E mutation status. To study the molecular mechanism behind BRAF protein expression we searched for all possible hypotheses and we found co-expression of novel spliced variants of BRAF along with full length BRAF in melanoma patients and cell lines. We further showed that as a result of having lost portions of the kinase domain, over expression of the BRAFsv decreases MAPK activity and melanoma cell line viability *in-vitro*. In our melanoma patient database, we found that the expression of BRAFsv is increased in primary melanoma patients compared with normal nevi samples but the metastatic melanoma patients had lower BRAFsv expression compared with early stages of primary melanoma. Accordingly, we found that more aggressive types of

melanoma (thick primary melanoma and higher AJCC stages) had lower expression of BRAFsv indicating an inhibitory role for BRAFsv in melanoma progression and aggressive behavior. Further studies on melanoma patients' outcome revealed that patients with positive BRAFsv expression and low levels of full length BRAF expression had the best survival rate over five years.

Our data highlights the prognostic role of BRAF in melanoma both at the gene and protein levels and investigates the correlation of BRAF protein expression and *braf*V600E mutation in melanoma. We also revealed the co-expression of kinase-dead BRAFsv with the full length BRAF in melanoma patients and reported a unique tumor suppressor effect for BRAFsv.

6.1. Study limitations and future directions

In the first part of our research (in which we investigated the effect of *braf*V600E mutation on patient survival) only four published papers on melanoma had enough data to meet the criteria for meta-analysis. As we discussed previously, more clinical trial and cohort studies are required for a more comprehensive assessment of the topic. For BRAF protein expression evaluation we chose IHC and used BRAF431-445 and BRAF12-156 antibodies. We chose these two antibodies because of their commercial availability and the fact that one of them (BRAF12-156) targets the N-terminal BRAF (Exons 1-3) and the second one (BRAF431-445) targets the beginning of the kinase domain at the C-terminal (Exons 10-11 junction). First we used BRAF431-445 (targetting the kinase domain) to detect full length BRAF and investigate its role in melanoma progression. Later we used BRAF12-15 antibody to detect all the variants (including the kinase dead variants which are not detectable with BRAF431-445 antibody). Substracting the positive samples for BRAF431-445 from positive samples for BRAF12-15 antibody we indirectly assessed the

expression of kinase dead splice variants of BRAF. As an alternative, more quantitative tools like a Nanostring probe against the new splice sites or against various *braf* exons could be used to re-confirm the findings. The problem with this method however is the low quality of RNA extracts from FFPE patients' samples. Next generation target specific sequencing or any digital sequencing techniques also would be a more quantitative method to detect the copy number of each variant. However, due to the same problem with Nanostring technology (low quality RNA extracts) and budget limitations this experiment was postponed to the future phase of the study.

As for the future direction of this project, we still need to reconfirm our findings with another set of patient samples, ideally from a different centre. A very immediate goal would be to study the effect of kinase-dead BRAFsv on melanoma invasion, migration and tumor growth with the possibility of a potential use for these variants as a stand alone treatment in BRAF wild type melanoma patients, who do not have any hope for a promising treatment option right now. Also over expression of kinase dead BRAFsv has the potential to be used in combination with BRAF inhibitors in patients with mutant BRAF status. Then we could study the role of BRAFsv in the context of BRAF inhibitor resistance. Resistance to BRAF inhibitor treatments is perhaps the biggest challenge in melanoma treatment currently. While the role of BRAF-p61 splice variant in inducing BRAF inhibitor resistance has been reported previously (Poulikakos et al. 2010) it would be worthwhile to investigate whether our new kinase dead splice variants of BRAF could play any role in that process. However, considering our preliminary results of enhancing PLX4720 (Vemurafenib) effect in case of BRAFΔEx4-17 over expression, unlike BRAF-p61, we think the kinase dead BRAFsv should play a protective role against BRAF inhibitor resistance. Finally we believe a comprehensive expression analysis of kinase dead splice variants of BRAF in all major cancers in which *braf*V600E mutation is prevalent may add

to our knowledge of the mechanism responsible for their expression and their role in cancer progression.

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Appendix

List of refereed journal publication

1. **Ardekani GS** (FA), Jafarnejad M, Mousavizadeh SR. Khosravi Sh, Martinka M, Zhou Y. McElwee KJ, Duronio V. New Kinase Dead Splice Variant of BRAF Identified in Melanoma that is Correlated with Improved Patient Prognosis and Survival

(Manuscript is under review by the Journal of Investigative Dermatology, the highest ranked dermatology journal (by Science Watch); Impact factor: 6.372. I designed the study, performed the experiments, analyzed the data and prepared the final manuscript.)

2. Cheng Y, Zhang G, Tang Y, Chen G, **Ardekani GS** (CA), Martinka M, Rotte A, Li G, McElwee KJ, Zhou Y. Stage-Specific Prognostic Biomarkers for Melanoma. Oncotarget. 2015 Feb 28;6(6):4180-9.

(Impact Factor: 6.63. I helped with research plan, IHC, scoring the pathology slides and the final data analysis)

3. Khosravi Sh, Tam KJ, **Ardekani GS** (CA), Martinka M, McElwee KJ, Ong CJ. eIF4E is an adverse prognostic marker of melanoma patient survival by increasing melanoma cell invasion. J Invest Dermatol. 2015 Jan 6. doi: 10.1038/jid.2014.552.

(Journal of Investigative Dermatology (the highest ranked dermatology journal); Impact factor: 6.372. I helped with the study design, IHC, Western blot experiments and data analysis.)

4. Khosravi Sh, Wong R, **Ardekani GS** (CA)**,** Zhang G, Martinka M, Li G. Role of EIF5A2, a downstream target of Akt, in promoting melanoma cell invasion. Br J Cancer. 2014 Jan 21;110(2):399-408. doi: 10.1038/bjc.2013.688

(Impact Factor: 4.814. I helped with research plan, experimental design, scoring the pathology slides and the final data analysis)

5. Bhandaru M, **Ardekani GS** (CA), Zhang G, Martinka M, McElwee KJ, Li G, Rotte A. A combination of p300 and Braf expression in the diagnosis and prognosis of melanoma. BMC Cancer. 2014 Jun 3;14:398.

(Impact Factor: 3.32. I helped with IHC, scoring the pathology slides and the final data analysis)

6. Sjoestroem C, Khosravi S, Cheng Y, **Ardekani GS** (CA), Martinka M, Li G. DLC1 expression is reduced in human cutaneous melanoma, and correlates with patient survival. Mod Pathol. 2014 Feb 21. Mod Pathol. 2014 Sep;27(9):1203-11.

(Impact Factor: 6.364. I helped with research plan, IHC, scoring the pathology slides and the final data analysis)

7. Cheng Y, Zhang G, Tang Y, Chen G, **Ardekani GS** (CA), Martinka M, Rotte A, McElwee KJ, Zhou Y. Loss of tumor suppressors KAI1 and p27 identifies a unique subgroup with poor survival in primary melanoma patients. (Accepted for publication in Oncotarget)

(Impact Factor: 6.63. I helped with research plan, IHC, scoring the pathology slides and the final data analysis)

8. **Ardekani GS** (FA), Jafarnejad M, Martinka M, Ho V, Li G. Disease Progression and Patient Survival Are Significantly Influenced by BRAF Protein Expression in Primary Melanoma. Br J Dermatol. 2013 Aug;169(2):320-8.

(Br Journal of Dermatology is one of the top three dermatology journals. Impact Factor: 4.1. I planned the research protocol, executed the experiments, analyzed the data and prepared the final manuscripts.)

9. **Ardekani GS** (FA), Jafarnejad M, Tan L, Saeedi A, Li G. The Prognostic Value of *BRAF* Mutation in Colorectal Cancer and Melanoma: A Systematic Review and Meta-analysis. PLoS One. 2012;7(10):e47054. doi: 10.1371/journal.pone.0047054.

(This article has been cited more than 77 times in less than 3 years which shows its great value to the research community. I planned the research protocol, performed the data search, reviewed all the manuscript and extracted the data, helped with statistical analysis and prepared the final manuscripts.)

10. Jafarnejad SM, **Ardekani GS** (CA), Ghaffari M, Martinka M, Li G**.** Sox4 mediated Dicer expression is critical for suppression of melanoma cell invasion. Oncogene. 2013 Apr 25;32(17):2131-9 doi: 10.1038/onc.2012.239

(Impact Factor: 8.559. I helped with research plan, IHC, Western blot, scoring the pathology slides and the final data analysis)

11. Jafarnejad M, Sjoestroem C, **Ardekani GS** (CA), Li G. Reduced Expression of nuclear Dicer Correlates with Melanoma Progression. Pigment Cell Melanoma Res. 2013 Jan;26(1):147-9. doi: 10.1111/pcmr.12039. Epub 2012 Nov 21 2012 Oct 19. doi: 10.1111/pcmr.12039.

(Pigment Cell Melanoma Res is the second highest ranked dermatology journal, Impact Factor: 5.839. I helped with research plan, IHC, scoring the pathology slides and the final data analysis)

12. Jafarnejad M, **Ardekani GS** (CA), Ghaffari M, Li G. Pleiotropic function of SRY-related HMG box transcription factor 4 in regulation of tumorigenesis. Cell Mol Life Sci. 2013 Aug;70(15): 2677-96. doi: 10.1007/s00018-012-1187-y.

(Impact Factor: 5.856. I helped with the study plan, wrote the section on the role of Sox4 in development and helped with the final manuscript)