TISSUE MICROARRAY BASED BIOMARKER STUDY IN HUMAN CUTANEOUS MELANOMA

by

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Abstract

Cancer therapy recently experienced remarkable advances with better understanding of cancer pathogenesis and introduction of new intervention strategies. Biomarkers reflective of the presence of tumor cells, or linked with clinical outcomes, have potential to improve the management of cancers. The purpose of this thesis study is to identify novel biomarkers of human cancers based on tissue microarray (TMA) technology and to determine their value for clinical application in cancer management using melanoma as the model. Melanoma arises from uncontrolled proliferation of melanocytes. Although melanoma accounts for only 4% of all skin cancer, it is responsible for 80% of deaths related to skin malignancies.

To discover novel biomarkers of melanoma, we constructed a TMA using biopsies from 707 patients with various stages of melanocytic lesions. Using immunohistochemistry and TMA, multiple biomarker candidates were evaluated, and many were found to have significant prognostic value, including expression loss of Fbw7. To further improve the clinical value of these markers, various combinations of individual markers were evaluated, leading to the identification of KAI1 and p27 that together showed much stronger prognostic value than when used as individual markers. Moreover, since there has been a dearth of reliable prognostic markers to offer prognostic information on specific melanoma stages, we identified the AJCC-stage specific prognostic markers, including BRAF protein expression as a prognostic marker for thin melanomas.

In that significant prognostic value was found for Fbw7 protein in melanoma, we performed *in vitro* experiments on this protein in detail. Our data showed that the alpha isoform of Fbw7, located in the cell nucleus, was the dominant form expressed in melanoma. Knock-down of Fbw7α promoted melanoma cell migration, and the MAPK signaling

pathway was required for Fbw7 function in melanoma. These findings indicate loss of Fbw7 to be an independent melanoma prognostic marker, and important for the development of malignant behaviors of melanoma cells.

This study has demonstrated that the combination of TMAs of cancers with the corresponding clinical database represents a powerful technological platform for biomarker discovery. TMA/clinical database combination-based investigations should be applicable for the investigation of other types of human cancers as well.

Preface

Contributions

1. A version of chapter 1, paragraph 1.1.6 has been published [**Cheng Y**, Zhang G, Li G. Targeting MAPK pathway in melanoma therapy. *Cancer Metastasis Rev*. 2013 Dec]. I was the major investigator, responsible for the initiation, literature review and synthesis, and writing the first draft of the manuscript. Dr. G. Li was the supervisory author and was involved throughout the concept formation and manuscript revisions. Dr. G. Zhang was involved in the early stages of concept formation and contributed in manuscript revisions.
2. A version of chapter 1, paragraph 1.4 has been published [**Cheng Y**, Li G. Role of ubiquitin ligase Fbw7 in cancer progression. *Cancer metastasis Rev*. 2012 Jun]. I was responsible for project initiation, literature review and synthesis, and writing the first draft of the manuscript. Dr. G. Li. was the supervisory author and was involved throughout the concept formation and manuscript revisions.
3. Based on the established tissue microarray and clinical database described in Chapter 3, eleven research articles have been published in multiple journals (See Appendices), such as *J Invest Dermatol, Carcinogenesis* and *Brit J Cancer*. Additional studies are currently underway by several other researchers in our lab using the TMA and the clinical database on melanoma biomarkers, and likely will lead to more discoveries and manuscripts in the future. Dr. G. Li provided much of the financial support through research grants, infrastructure, and research materials for much of the thesis project. In addition, his guidance and expertise were essential during experimental design, data analysis and manuscript preparation stages of this project. Dr. M. Martinka provided clinical biopsies and contributed to sample selection, clinical data collection and assisted with the scoring

of tissue microarrays. Dr. Y. Zhou contributed to the update of the comprehensive clinical database, helped with some of experimental design and data analysis, and helped with manuscript preparation.

4. A version of chapter 4 has been published [**Cheng Y**, Chen G, Martinka M, Ho V, Li G. Prognostic significance of Fbw7 in human melanoma and its role in cell migration. *J Invest Dermertol*. 2013 Feb]. I was the major investigator, designed and performed most of the experiments, and prepared the manuscript. Dr. G. Li and Dr. V. Ho provided facilities, research materials, and contributed to experimental design and manuscript preparation. Dr. G. Chen contributed in the initiation of the project and manuscript edits. Dr. M. Martinka assisted with the scoring of tissue microarray.
5. A manuscript based on chapter 5 has been prepared and submitted [**Cheng Y***, Zhang G*, Tang Y, Cheng G, Safaee A, Rotte A, Martinka M, McElwee K, Zhou Y. Loss of tumor suppressors KAI1 and p27 identifies a unique subgroup with poor survival in primary melanoma patients]. Dr. G. Zhang and I conceived and performed the data analysis, and prepared the manuscript. Dr. Y. Zhou contributed to analysis design, database updating and manuscript preparation. Tang Y, Safaee A and Dr. Rotte A provided the raw data and contributed in the design of the study. Dr. M. Martinka assisted with the scoring of tissue microarray. Dr. K. McElwee provided the research facilities and materials.

   *Contribute equally to the study.
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G. Zhang and I performed the data analysis and prepared the initial draft of manuscript. Dr. G. Chen and Dr. A. Rotte and Safaee A provided the raw data. Dr. G. Li and Dr. K. McElwee provided the facilities and research materials.

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2. Zhang G*, Cheng Y*, Chen G, Safaee A, Rotte A, Martinka M, Li G, McElwee K, Zhou Y. Stage-specific prognostic biomarkers for melanoma. Submitted to *J Invest Dermatol*.

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6. Cheng Y, Li J, Martinka M, Li G. The expression of NAD(P)H:quinone oxidoreductase 1 is increased along with NF-κB p105/p50 in human cutaneous melanomas. *Oncology Reports*. 2010 Apr; 23 (4): 973-9.

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

Abbreviations	Definition
Akt	Thymoma viral proto-oncogene/ protein kinase B
ALK	Anaplastic lymphoma kinase
ALM	Acral lentiginous melanoma
AML	Acute myeloid leukemia
AP-2	Activating protein 2
APAF	Apoptosis protease activating factor
APC	Anaphase-promoting complex/cyclosome
ARF	Alternate open reading frame
ARID1	AT-rich interactive domain-containing protein 1
Arp2/3	Actin-related proteins 2/3
ATF	Activating transcription factor 2
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 -associated X protein
Bcl	B-cell lymphoma
Bcl-xL	B-cell lymphoma-extra large
BMI	Polycomb ring finger oncogene
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
BRMS1	Breast cancer metastasis-suppressor 1
C/EBP δ	CCAAT/enhancer binding protein-δ
Cdc42	Cell division control protein 42 homolog
CDK	Cyclin-dependent kinase
CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CEACAM-1	Carcinoembryonic antigen-related cell adhesion molecule 1
COX-2	Cyclooxygenase-2
CPD	Cdc phospho-degrons
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
CXCR4	C-X-C chemokine receptor type 4
CYLD	Cylindromatosis
DSS	Disease-specific survival
E1A	Early region 1A
EGFR	Epidermal growth factor receptor
EML4	Echinoderm microtubule-associated protein-like 4
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ETV1	ETS translocation variant 1
EZH2	Histone-lysine N-methyltransferase
Fak	Focal adhesion kinase
Fbw7	F-box and WD repeat domain-containing 7

FOXP3	Forkhead box p3
GAP	GTPases activating protein
GDP	Guanosine diphosphate
GEF	Guanine-nucleotide exchange factor
GNAQ	Guanine nucleotide-binding protein G(q) subunit alpha
GSK3	Glycogen synthase kinase 3
GTP	Guanosine-5'-triphosphate
HER2	Epidermal growth factor receptor 2
HIF1	Hypoxia-inducible factor 1
HLA	Human leukocyte antigens
HMGA2	High-mobility group AT-hook 2
hTERT	Human telomerase reverse transcriptase
IC50	Half maximal inhibitory concentration
ICAM1	Intercellular adhesion molecule 1
ID	Inhibitor of DNA-binding 1
IDH1	Isocitrate dehydrogenase 1 (NADP+) soluble
IDO	Indoleamine 2,3-dioxygenase
IHC	Immunohistochemistry
IRF4	Interferon regulatory factor 4
IRS	Immunoreactive score
JNK	Jun N-terminus kinase
KLF	Krüppel-like family
L1CAM	L1 cell adhesion molecule
LDH	Lactate dehydrogenase
LMM	lentigo meligna melanoma
LRR	Leucine-rich repeats
MAGE	Melanoma-associated antigen 3
MAPK	Mitogen-activated protein kinase
MART-1	Prostate-specific antigen
MC1R	Melanocortin-1 receptor
MCAM	Melanoma cell adhesion molecule
MDM2	Mouse double minute 2 homolog
MEK (MKK)	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MIA	Melanoma-inhibiting activity
MITF	Microphthalmia-associated transcription factor
ML	Metastasis-like
MLC	Myosin light chain
MLCP	Myosin-light-chain phosphatase
MMP	Matrix metalloproteinase
MRCK	Cdc42-binding kinase
MTOC	Microtubule-organizing center
mTOR	Mammalian target of rapamycin

NICD	Notch intracellular domain
NM	Nodular melanoma
NML	Non-metastasis-like
Notch1-IC	Notch1 intracellular domain
NRAS	Neuroblastoma RAS viral oncogene homolog
OS	Overall survival
PAK1	p21 protein-activated kinase 1
PCGF	Polycomb group RING finger protein
PCNA	Porliferating cell nuclear antigen
PD1	programmed cell death 1
PFS	Progression-free survival
PI3K	Phosphoinositide 3-kinase
PPP6C	Serine/threonine-protein phosphatase 6 catalytic subunit
PS	Presenilins
PSA	Prostate-specific antigen
PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase
RB	Retinoblastoma protein
Rb	Retinoblastoma protein
Rbx1	RING-box protien 1
Rho	Ras homolog gene family
ROCK	Rho-associated, coiled-coil containing protein kinase 1
RUNX3	Runt-related transcription factor 3
SCF	Skip1-Cul1-F-box protein
SGK1	Serum- and glucocorticoid-inducible kinase 1
Skp2	S-phase kinase-associated protein 2
SPARC	Secreted protein acidic and rich in cysteine
SRB	Sulphorhodamine B
Src	Proto-oncogene tyrosine-protein kinase Src
SREBP1	Sterol regulatory element-binding transcription factor 1
SSM	Superficial spreading melanoma
STAT	Signal transducer and activator of transcription
STK19	Serine/threonine-protein kinase 19
TA90IC	Tumor-associated antigen 90 immune complex
T-ALL	T-cell acute lymphoblastic leukaemia
TMA	Tissue microarray
TGFβ	Transforming growth factor-β
TGIF1	TGFβ-induced factor 1
TLR4	Toll-like receptor 4
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WASP/WAVE	Wiskott-Aldrich syndrome protein
WD40	Tryptophan-aspartic acid depeptide 40
WNT	Wingless-type MMTV integration site family

WT1	Wilms tumor protein
α-MSH	α-melanocyte stimulating hormone

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Dedication

To my beloved parents

Chapter 1: Introduction

1.1 Malignant Melanoma

Malignant melanoma is an aggressive form of skin cancer. Frequently resistant to traditional chemo- and radio-therapies, it is a notorious malignancy with high mortality (Inamdar et al, 2010). Although it accounts for only 4 percent of all dermatologic cancers, it is responsible for 80 percent of deaths arising from skin malignancy (Miller & Mihm, 2006). Melanoma arises from abnormal proliferation of melanocytes and can occur in any anatomic location containing melanocytes, such as cutaneous skin, the mucous membranes, eyes, meninges, and oesophagus (Houghton & Polsky, 2002). Therefore, melanoma can beassigned to three subtypes by the origin of melanocytes: cutaneous melanoma arising from melanocytes in the epidermis, mucosal melanoma from melanocytes located in the mucous membranes and uveal melanoma from melanocytes residing in the ocular structures. Because cutaneous melanoma is the most common subtype, accounting for more than 90% of cases of melanoma (Chang et al, 1998), it is the focus of this study.

1.1.1 Epidemiology of Cutaneous Melanoma

Melanoma has been reported as the 19th most common cancer globally, with estimated incidence rates of 2.8-3.1 per 100,000 (Ferlay et al, 2010). The incidence of melanoma varies between countries, with the highest rate of approximately 37 per 100,000 in Australia, and the lowest rate of 0.2 per 100,000 in south-Central Asia. The difference is mainly attributed to racial skin type, as well as exposure to ultraviolet radiation, suggesting that melanoma risk depends on genetic and environmental factors.

In the last few decades, the incidence of cutaneous melanoma has been rising faster than that of other malignancies in predominately fair-skinned populations, at rates of 2-3%

annually since 1960s (Eggermont et al, 2013; Kohler et al, 2011; Perlis & Herlyn, 2004). In Canada, melanoma is the eighth most common cancer in men and the seventh most common cancer in women (Canadian Cancer Society: Canadian Cancer Statistics 2013). According to Canadian Cancer Statistics, 2013, an estimated 6,000 new melanoma cases are expected to be diagnosed in Canada (13.4 cases per 100,000), while 1,030 patients are expected to die from this disease. In addition, unlike other cancers that affect elderly with a median age of 65, the majority of melanoma cases are diagnosed in younger patients, with a median age of 57 years (Erdmann et al, 2013). In Canada, it has been shown that melanoma is one of the most common causes of cancer deaths in the population aged 15 to 29 years (Canadian Cancer Society: Canadian Cancer Statistics 2013).

1.1.2 Biology of Melanocytes and Melanoma

Melanocytes are melanin-producing cells located in human skin, retina of the eyes, sensorial epithelia of inner ears, and the leptomeninges. During embryonic development, melanocytes originate from neural-crest cells, which arise from the most dorsal point of the neural tube between the surface ectoderm and the neural plate (Ernfors, 2010). Neural-crest cells are transient multipotent, migratory cells unique to vertebrates. Besides melanocytes, neural-crest cells give rise to diverse other cell lineages, including peripheral and enteric neurons and glial cells, adrenal medulla, cardiac cells, and craniofacial tissue (Huang & Saint-Jeannet, 2004). The melanocyte precursors, also known as melanoblasts, migrate, proliferate and differentiate en route to their final destinations in the basal layer of the epidermis and hair follicles, and finally develop into functional melanocytes (Lin & Fisher, 2007; Slominski et al, 2004). In the epidermis of human skin, melanocytes are primarily in contact with keratinocytes at a ratio of about 1:36; thus they constitute 2-4% of the total epidermal cell

population (Vancoillie et al, 1999). Both melanocytes and keratinocytes express E-cadherin, the adhesion molecule that mediates interaction between melanocytes and keratinocytes at the epidermal/dermal junction, which in turn regulates proliferation of melanocytes (Hsu et al, 2000). When E-cadherin switches to N-cadherin, melanocytes are released from growth suppression and are able to proliferate and self-aggregate to form nevi (Li et al, 2001). Although melanocytes continuously differentiate and migrate, their replication and the normal melanocytic phenotype is delicately regulated by a multileveled mechanism orchestrated by undifferentiated, basal-type keratinocytes to achieve homeostatic balance (Tanimura et al, 2011). In contrast, melanoma cells are refractory to the keratinocyte-mediated regulatory mechanism, in concert with down-regulation of E-cadherin expression.

Mature melanocytes synthesize and package melanin within specialized organelles known as the melanosomes. Melanin is the main contributor to pigmentation of skin and hair, and serves several functions in humans, including photoprotection, trapping reactive oxygen species, capturing metal ions and so on (Riley, 1992; Riley, 1997). Two major types of melanin exist depending on the genotype of melanocortin-1 receptor (*MC1R*) gene: red/yellow phenomelanin and brown/black eumelanin (Rees, 2003). Phenomelanin and eumelanin are not only different in color, but also in the size, shape and packaging of their granules (Slominski et al, 2004). Both melanins develop from the tyrosinase-dependent pathway, with the same precursor, tyrosine. The absence or malfunction of tyrosine results in oculocutaneous albinism, which shows intact melanocytes but inability to make pigment (Oetting et al, 2003).

Ultraviolet (UV) irradiation causes genetic changes in the skin and induces DNA-damaging reactive oxygen species (Gilchrest et al, 1999; Thompson et al, 2005). The

defensive response to UV irradiation stimulates keratinocytes to release the α-melanocyte stimulating hormone (α-MSH). Binding of α-MSH to its receptor MC1R, in turn, stimulates intracellular signaling in melanocytes and increases tyrosinase expression, which is required for melanin production. Other signal transduction pathways, such as p38 mitogen-activated kinase pathway, are activated to enhance the activity and expression of tyrosinase, thereby inducing the synthesis of melanin by melanocytes (Corre et al, 2004). Increased melanin is then transferred to surrounding keratinocytes and distributed towards the surface of the epidermis or in hair shafts, where it absorbs and dissipates the UV energy, leading to the tanned appearance of the skin after sun exposure (Miyamura et al, 2007). Studies have shown that light-skinned and "redhead"-appearing people often carry germ-line polymorphisms of the *MC1R* gene, which weakens the activity of the receptor (Frandberg et al, 1998; Naysmith et al, 2004; Valverde et al, 1995). As a result, these people have reduced production of eumelanin, are more vulnerable to UV light effects, and have increased risk of developing skin cancers including melanoma (Kennedy et al, 2001).

The transformation from normal melanocytes to malignant melanoma can be summarized as described in the Clark model (Figure 1.1).

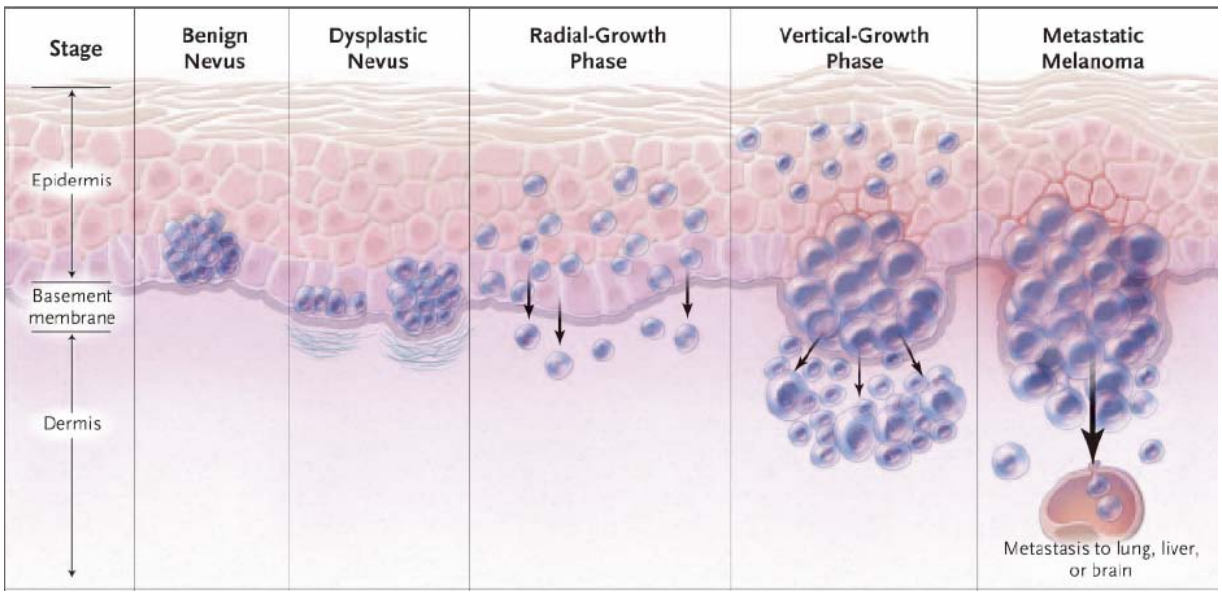

Figure 1.1 Biological events in the progression of melanoma described by the Clark model.

Modified from Miller *et al.* 2006, with permission to reprint.

The first event occurring in melanocytes is the development of benign nevi, which are formed by neval melanocytes. When UV or other factors cause genetic or epigenetic changes in essential growth regulatory genes, melanocytes can escape the tight regulation by keratinocytes (Haass et al, 2004). As a result, melanocytes can continuously proliferate, leading to the formation of a nevus (also called common mole). However, although the regulation of proliferation in neval melanocytes is disrupted, their growth potential is limited. Melanocytes in the common nevi can be restricted to the epidermis (junctional nevus), the dermis (dermal nevus) or overlapping components of both (compound nevus), which are generally benign and rarely progress to cancer (Clark et al, 1984). The next step toward melanoma is the development of dysplastic nevi, which may arise from pre-existing benign nevi or appear as new lesions. Such lesions histologically have random and discontiguous

cytologic atypia (Miller & Mihm, 2006). With additional genetic alterations, neval cells can progress to the radial growth phase (RGP) melanoma, where the cells acquire the ability to proliferate intraepidermally, or even penetrate the papillary dermis singly or in small nests. RGP cells are confined to the dermis, whereas tumors can enter the vertical growth phase (VGP) with nodules and nests of cells invading the dermis. The VGP cells are associated with increased malignancy because of invasive ability and metastatic potential.

1.1.3 Etiology of Melanoma

Malignant melanoma is a complex disease that involves numerous biologic events and molecular changes. In recent years, much has been revealed about the molecular basis and driver mutations of melanoma genesis. Compared to almost all other solid tumors, cutaneous melanomas exhibit markedly increased base mutation rates (Berger et al, 2012; Pleasance et al, 2010), with enrichment of cytidine to thymidine (C to T) transition that is typical of a UV-induced mutational signature. In addition, hotspot-activating mutations of melanoma genes such as STK19, Fbw7, and IDH1 due to UV-B mediated damage have been identified (Berger et al, 2012; Hodis et al, 2012). Compelling epidemiological and experimental evidences have established the direct causal role for UV exposure in melanoma pathogenesis. However, controversy still exists. For example, the oncogenic mutations of *NRAS* and *BRAF* genes are not the C to T transitions that are indicative of UV mutagenesis. Studies on familial melanoma cases and advanced genome or whole-exome sequencing have identified a series of gene mutations with variable degree of penetrance and prevalence in melanoma genesis (Tsao et al, 2012).

The best characterized high-penetrance gene is cyclin-dependent kinase inhibitor (CDKN2A), whose germline mutation occurs in about a third of melanoma affected families

(Borg et al, 2000; Thompson et al, 2005). CDKN2A encodes for two distinct proteins through alternative splicing: $p16^{INK4A}$ and $p14^{ARF}$ (Kamb et al, 1994; Nobori et al, 1994). INK4A is an inhibitor for CDK4 which blocks the cell cycle at the G1/S checkpoint by regulating the phosphorylation of the retinoblastoma protein (RB) (Sharpless & Chin, 2003). ARF inhibits the ubiquitination of p53 by binding to mouse double minute 2 (MDM2) and ARF-BP1/Mule ubiquitin ligase, thereby induceing cell cycle arrest and inhibiting tumorigenesis (Chen et al, 2005). Both INK4A and ARF act as brakes on the cell cycle, thus the loss-function mutation or epigenetics changes on these genes result in uninhibited cell growth and proliferation. Association of INK4A and ARF expression to melanoma progression and poor prognosis has been reported (Dobrowolski et al, 2002; Sanki et al, 2007). Notably, the downstream targets of INK4A, CDK4 and cyclin D1, are also found to be mutated in some melanomas (Sauter et al, 2002; Sotillo et al, 2001; Zuo et al, 1996). The cell cycle regulator RB1 is another high penetrance allele for melanoma. RB1 mutation carriers have a 4-80 fold elevated risk to develop melanoma in their lifetime (Braam et al, 2012).

The most frequent mutations in cutaneous melanoma occur in the ERK-MAPK signalling pathway, which is constitutively activated in up to 90% of human melanomas (Cohen et al, 2002). Activation of this pathway is the result of somatic mutations of *NRAS*, which are associated with about 15% of melanomas, or *BRAF*, which are found in approximately 50% of melanomas (Miller & Mihm, 2006). The majority of *BRAF* mutations (~80%) arise as a result of substitution of glutamic acid for valine at codon 600 (BRAF V600E) (Davies et al, 2002). A recent meta-analysis indicated that *NRAS* mutations occur more in nodular melanomas and melanomas caused by chronic sun-damage (Lee et al, 2011).

Moreover, patients presenting *NRAS* mutations demonstrated a worse clinical outcome as compared to patients with *BRAF V600E* mutations or without *NRAS* or *BRAF* mutations (Devitt et al, 2011). Interestingly, *BRAF* mutations occur in more than 80% of melanocytic nevi (Pollock et al, 2003; Yazdi et al, 2003), even a greater prevalence than in melanomas, suggesting that these genetic alterations are early events in melanoma development. Multiple lines of evidence showed that *BRAF* mutations alone are insufficient, and have to collaborate with other gene/protein alterations in order to transform melanocytes. Garraway et al found that ectopic MITF (microphthalmia-associated transcription factor) expression together with *BRAF V600E* mutation transformed primary human melanocytes (Garraway et al, 2005). Jane-Valbuena et al. demonstrated that ETV1 (ETS translocation variant 1, which is one of the ETS transcription factors) overexression in cooperation with *NRAS* or *BRAF* mutation was able to transform immortalizedmelanocytes, in the presence of MITF protein (Jane-Valbuena et al, 2010). By screening *BRAF* mutations in a series of melanoma samples, Tsao et al. raised the possibility for cooperation between BRAF activation and PTEN loss in melanoma tumorigenesis (Tsao et al, 2004). To characterize the functional impact of genetic alterations associated with melanoma, Chudnovsky et al. regenerated human skin *in vivo* on nude mice by inducing melanocytes selectively engineered to express specific mutations frequently observed in human melanoma (Chudnovsky et al, 2005a). They found that BRAF V600E failed to induce melanoma, but merely mild junctional melanocytic nesting. In contrast, both activated Ras and PI3K along with Rb (retinoblastoma protein)-p53 inhibition as well as hTERT (human telomerase reverse transcriptase) expression were able to yield invasive melanoma (Chudnovsky et al, 2005a). Unlike *BRAF* mutations, *NRAS* mutation with PI3K induction occur more frequently in melanoma than in benign nevi (Pollock et al, 2003;

Stahl et al, 2004; Wu et al, 2003), suggesting that BRAF, NRAS and PI3K activations represent events that can be triggered at different time points in melanoma development.

The phosphoinositide-3-kinase (PI3K)-Akt signaling pathway also plays an essential role in melanoma proliferation (Dai et al, 2005; Madhunapantula & Robertson, 2009). Akt is activated by phosphorylations at Thr308 and Ser473. Akt phosphorylates a number of substrates resulting in sustained cell proliferation and cell cycle progression, invasion, angiogenesis as well as reduced apoptosis (Chudnovsky et al, 2005b; Hsu et al, 2002; Smalley & Herlyn, 2005). The mechanism of activation of Akt in melanoma could be explained in part by the findings that mutation of PI3K, the upstream activator of Akt, is found in 3% of metastatic melanoma (Omholt et al, 2006), as are loss of function of PTEN, the negative regulator of Akt, in 5-20% of late stage melanoma (Wu et al, 2003), and the overexpression of Akt itself in up to 60% of melanoma (Stahl et al, 2004). However, the expression level of Akt does not necessarily correlate with its activity (Stahl et al, 2003). Therefore, there may be other mechanisms regulating Akt activation in melanoma.

1.1.4 Staging and Subtypes of Melanoma

The current widely adopted standard staging system for melanoma is the American Joint Committee on Cancer (AJCC) system (Balch et al, 2009). The system divides patients into four categories, based on the evaluation of primary tumor thickness (T), the presence or absence of regional lymph nodes metastases (N), and the distant metastases (M). According to this system, tumor thickness, mitotic rate and ulceration are the three most dominant prognostic factors. In the 2009 version of the AJCC system, mitotic activity was added to the assessment of thin melanoma, and elevated serum LDH level was proven as an independent predictor of clinical outcome among stage IV melanomas. Moreover, the AJCC Melanoma

Staging Committee recommends the sentinel lymph node biopsy (SLNB) should be required for stage IB or II melanomas to assist the design of adjuvant therapy. SLNB is a procedure in which the sentinel lymph node is identified, removed, and examined to determine whether cancer cells are present (Balch & Cascinelli, 2006).

Histopathologic and molecular data indicate that melanoma is comprised of biologically distinct subtypes. The World Health Organization (WHO) distinguishes four main subtypes of melanomas: superficial spreading melanoma (SSM), nodular melanoma (NM), acral lentiginous melanoma (ALM) and lentigo meligna melanoma (LMM) (Clark et al, 1969). SSM is by far the most common type of melanoma, accounting for about 70% of all diagnosed melanoma cases (Gray-Schopfer et al, 2007). This subtype is characterized by an irregular border, flat appearance and intra-epidermal component at the edge, and is usually associated with sunburn in early ages. Nodular melanoma is the most aggressive subtype, which consists of raised nodules without a significant flat portion (Clark et al, 1984). NM accounts for about 15% of all melanomas, and often tends to grow more rapidly in thickness; therefore the prognosis is often worse. ALM is the subtype of melanoma commonly found on the palms of the hands, the soles of the feet or in the nail bed, indicating that it is not associated with UV exposure. ALM accounts for 5% of all melanomas, but accounts for about 50% of melanomas in non-Caucasian patients (Kuchelmeister et al, 2000). LMM typically occurs on sun-exposed skin in elderly people, especially on the face. So this subtype is considered to be caused by chronic sun exposure. LMM appears as a spreading, flat patch with irregular borders and variable colors of brown, and accounts for about 5-10% of all melanomas (McKenna et al, 2006).

1.1.5 Targeted Therapy for Melanoma

Current clinical treatments for melanoma are generally dictated by whether the tumor is local or metastases have occurred. Most melanomas can be completely cured by surgical excision if early diagnosis occurs, but patients with metastatic melanoma have to face a very poor prognosis with less than 15% of patients surviving for three years (Balch et al, 2009). Dacarbazine (DITC), which is a DNA alkylating agent, has long been used as the first choice of chemotherapeutic drug in patients with unresectable or metastatic melanoma, although the response rate was marginal (10% to 20%) and patient long-term survival benefit has rarely been achieved (Bedikian et al, 2006; Lui et al, 2007). The immune-based therapies, including interferon (IFN)-α, high dose interleukin 2 (HD IL-2), generated similar response rate (6% to 10%) with severe adverse effects, and also failed to improve the overall survival for metastatic melanoma patients (Eggermont & Schadendorf, 2009). The resistance of melanoma to conventional chemo-therapy highlights the necessity of developing new strategies for treating metastatic melanomas.

Breakthroughs have recently occurred in targeted therapy for metastatic melanoma. As discussed above, MAPK signalling cascades are critical and constitutively activated in melanoma. Therefore numerous therapeutic strategies targeting this pathway have been intensely tested (Figure 1.2).

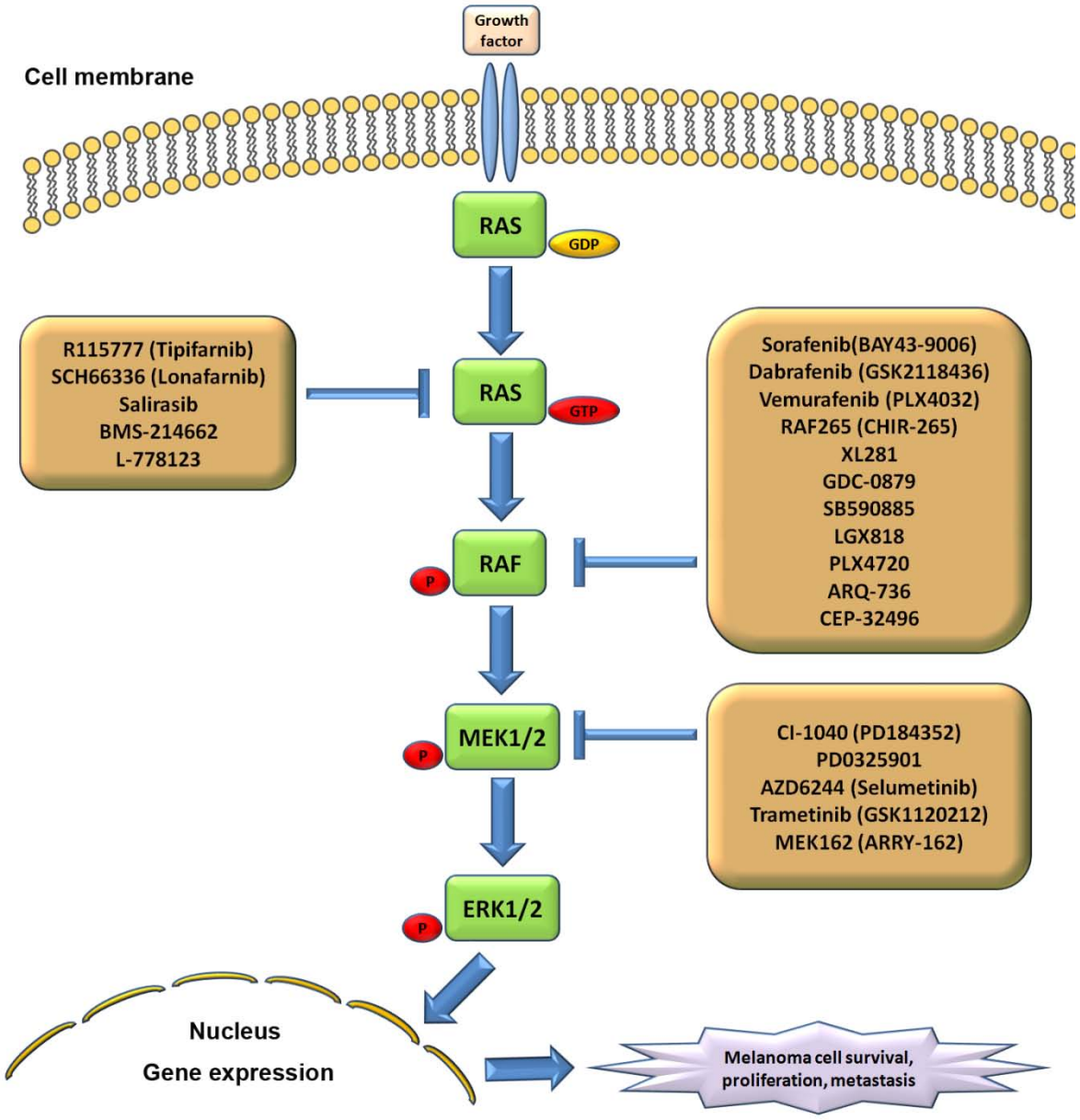

Figure 1.2 Schematic diagram of the MAPK-ERK signalling pathway and selected inhibitors.

In 2011, the FDA approved two small molecule drugs for metastatic melanoma treatment: an immune stimulatory agent, iplilimumab (Yervoy), and a BRAF V600E inhibitor, vemurafenib (Zelboraf) (Finn et al, 2012). Then in 2013, two additional agents: BRAF inhibitors dabrafenib (Tafinlar) and MEK inhibitor trametinib (Mekinist) reached the market. These drugs improve clinical response rates, progression-free survival and overall survival in metastatic melanoma patients with *BRAF* V600 mutations, as compared with chemotherapy agents. However, the clinical benefits of single-agent BRAF inhibitor treatment have been significantly limited by the rapid development of resistance. Therefore, multiple strategies have been tested and evaluated to overcome the drug resistance.

Vemurafenib, an potent, oral inhibitor of mutated BRAF, was the first drug approved for the treatment of metastatic melanoma with *BRAF* V600 mutations (Bollag et al, 2010; Bollag et al, 2012). Early *in vitro* studies revealed that vemurafenib inhibited ERK-MAPK signaling in melanoma cell lines harboring *BRAF* V600E/K mutations, but activated the ERK pathway promoting cell migration and proliferation of cells with wild type *BRAF* (Halaban et al, 2010). A phase III randomized clinical trial compared vemurafenib with dacarbazine in 675 patients with previously untreated, metastatic melanoma with *BRAF* mutation (Chapman et al, 2011a). The trial results showed that vemurafenib improved overall survival (84% vs 64%) and response rate (48% vs 5%) of patients as compared to those treated with dacarbazine (Chapman et al, 2011a). Thereafter, these compelling results led to the approval of vemurafenib by the FDA. Although vemurafenib has shown a significant improvement in the treatment of metastatic melanoma, severe adverse effects and drug resistance have also been observed. Secondary non-melanoma skin cancers, including cutaneous squamous cell

carcinoma and keratoacanthomas occurred in 15-30% patients treated with vemurafenib, usually within 2-3 months of therapy (Flaherty et al, 2010).

Dabrafenib, the second BRAF kinase inhibitor approved by the FDA after vemurafenib, is a reversible, ATP-competitive inhibitor that selectively inhibits $BRAF^{V600E}$ kinase activity both *in vitro* and *in vivo (*Falchook *et al, 2012)*. It has been shown possess significant antitumor activity in human tumor cell lines and xenografts (Wilhelm et al, 2006; Wilhelm et al, 2004). In a phase III trial comparing dabrafenib usage to dacarbazine usage in 733 patients with *BRAF V600E* mutant metastatic melanoma, dabrafenib demonstrated improved response rate (53% vs 19%) and progression free survival (5.3 vs 2.7 months) as compared to dacarbazine (Hauschild et al, 2012). Dabrafenib has shown similar clinical efficacy as vemurafenib, but with relatively mild and manageable toxicity. Therefore, the FDA approved dabrafenib for the treatment of patients with BRAF V600E positive metastatic melanoma in 2013 (Gibney & Zager, 2013).

Trametinib is potent orally available, selective MEK1/2 inhibitor and the first MEK inhibitor approved by the FDA (Gilmartin et al, 2011). Unlike other MEK inhibitors, trametinib has been shown to be well tolerated, and to have a relatively long circulating half-life. In the clinical trial that led to its FDA approval, trametinib significantly improved PFS compared with chemotherapy drugs (4.8 months vs 1.5 months) in 322 metastatic melanoma patients with V600E or V600K *BRAF* mutations (Flaherty et al, 2012b). Notably, there were no cutaneous squamous-cell carcinomas observed in patients who received trametinib, suggesting that MEK inhibitors may have different molecular effects on wild-type BRAF (Flaherty et al, 2012a).

The success of BRAF and MEK inhibitors in metastatic melanoma constituted an oncogene-targeted strategy in cancer treatment. However, disease progression due to drug resistance was observed in approximately 50% of patients, 6-7 months after initial treatment (Hauschild et al, 2012; Sosman et al, 2012). Multiple mechanisms responsible for the BRAF/MEK inhibitor resistance development have been described. For example, tumor cells overexpress molecules, such as CRAF (another isoform of RAF, other than BRAF), or MAP3K8 (mitogen-activated protein kinase kinase 8, another MEK kinase), thereby bypassing BRAF or MEK to restore the ERK-MAPK pathway (Johannessen et al, 2010; Montagut et al, 2008). Alternatively, tumor cells can compensate MAPK signaling by activation of other pathways, such as the PI3K-Akt pathway, to maintain cell proliferation and progression (Nazarian et al, 2010; Villanueva et al, 2010). To overcome the resistance of a single agent therapy, intense investigations and clinical studies have been designed for combination regimens, including combination of BRAF and MEK inhibitors, combination of BRAF/MEK and PI3K inhibitors (Temsirolimus and Everolimus), combination of BRAF/MEK inhibitors and immune regulatory antibodies.

1.2 Cancer Migration, Invasion and Metastasis

The ability of tumor cells to spread from the primary site to secondary discontiguous organs and to metastasize is the most lethal feature of cancer. To date, metastasis is the major cause of 90% of deaths from solid tumors (Gupta & Massague, 2006). Therefore, understanding the nature and mechanisms of this process is crucial in cancer research and to the development of new treatment strategies. Metastasis is a complex, dynamic process including a sequence of discrete steps. First, tumor cells break away from the primary tumor and invade adjacent tissue. By overcoming the extracellular matrix (ECM) barriers and gaining increasing

invasiveness and motility, tumor cells enter nearby blood and lymphatic vessels (intravasation), and travel via the circulatory system. Then, following escape from the lumina of vessels (extravasation), the neoplastic cells relocate to the parenchyma of distant organs, proliferating and establishing vascularization. After the formation of small nodules (micrometastases), the micrometastatic lesions can develop into a macroscopic tumor, and finally achieve "colonization" (Kopfstein & Christofori, 2006; Talmadge & Fidler, 2010). To date, the contribution and timing of each molecular event in metastasis pathogenesis remains unclear.

1.2.1 Cell Migration

Cell migration is a fundamental component in many biological processes, such as embryonic development, skin and intestines renewal, tissue repair, and immune surveillance (Ridley et al, 2003). Abnormal cell migration is characteristic of several troublesome pathological processes, including vascular disease, osteoporosis, chronic inflammatory diseases, as well as cancer invasion and metastasis (Friedl & Wolf, 2009). Efficient and essential cell migration requires coordinated regulation of cytoskeletal protrusion, adhesion, proteolysis and contraction (Friedl & Wolf, 2009; Lauffenburger & Horwitz, 1996). A basic two-dimensional (2D) model of cell migration has been established using isolated cells that cross ECM-coated surfaces (Lauffenburger & Horwitz, 1996). However, most cells in the mammalian body migrate within three-dimensional (3D) tissues, in which the cell morphology and actin-rich protrusions engaging with the ECM are quite different. Some migration models are *in vivo* 3D tissue environments, while others are dedicated exclusively to 2D environments. (Keren et al, 2008).

Cells can migrate collectively or individually, depending on the structure and molecular factors of both tissue environment and cell behavior (Friedl et al, 1998; Lammermann & Sixt, 2009; Thiery, 2002). The two patterns of cell migration share most of the steps and mechanisms, except that cells remain attached to each other in the collective migration pattern. Collective migration and single-cell migration serve mutually exclusive purposes during normal physiological and pathological conditions. Collective migration is critical in formation of complex tissues, such as epithelia, ducts, glands, and vessels, but also contributes to cancer progression by providing motility and local invasion (Alexander et al, 2008; Friedl & Wolf, 2009). Single cell migration enables cell integration into tissues (neural crest cells) or travel from one site to another (immune cells) (Friedl, 2004; Teddy & Kulesa, 2004). This capability can be recapitulated by tumor cells during cancer metastasis to distant sites (Thiery, 2002).

In general, the process of cell migration can be described in a 5-step model composed of cell polarization and actin-driven leading edge protrusion (step I), integrin-mediated focal adhesion of the leading edge to the substrate (step II), proteolytic degradation of ECM rearward of the leading edge (step III), followed by actomysin-mediated cell contraction (step IV) and rear-end retraction and forward sliding of the cell body (step V) (Friedl & Wolf, 2009). At the initiation stage, normal poorly polarized cells require the impulse of various stimuli to start the migration, such as chemokine- or adhesion receptor-mediated signalling (Ridley et al, 2003). As a result, protrusions of leading pseudopods can be formed in the direction of migration. The protrusions could be large lamellipodia or spike-like filopodia, both of which enable the cells to push the plasma membrane. Lamellipodia and filopodia are both formed by polarization of actin filaments, but the organization of

filaments depends on the type of protrusion. Actin filaments organize as a branching network in lamellipodia, whereas they form into long parallel bundles in filopodia (Welch & Mullins, 2002).

1.2.2 Two Modes of Cell Migration

For single cells, two modes of mutually interchangeable migration exist: the amoeboid mode and the mesenchymal mode. Aamoeboid migration is named after the action of amoeba, characterized by repeated expansion and contraction of the cell body mediated by the cortically localized actin and myosin (Yumura et al, 1984). This mode of migration is commonly adopted by rounded or ellipsoid cells that lack mature focal adhesions and stress fibers, such as leukocytes and certain types of tumor cells (Friedl et al, 2001; Lammermann & Sixt, 2009; Yumura et al, 1984). The amoeboid movement can be divided into two subtypes: the first is propulsive, pushing migration of the rounded, blebby cells that do not adhere or pull on substrate; while the second occurs in more elongated cells that have actin-rich filopodia at the leading edge (Fackler & Grosse, 2008; Sanz-Moreno et al, 2008). Amoeboid migration is regulated by the Rho/ROCK signaling pathway (Sahai & Marshall, 2003; Wyckoff et al, 2006).

Alternatively, the mode of Individual cells with increased attachment and contractility is mesenchymal migration, characterized by focal cell-matrix interaction and fibroblast-like spindle shaped morphology (Grinnell, 2008; Maaser et al, 1999). Besides fibroblasts, keratinocytes, and endothelial cells, some tumor cells also use this migration mode. Cells initiate mesenchymal migration by the action of the filopodia and lamellipodia at the leading edge, usually driven by actin polymerization and the small GTPase from the Rho family, primarily by Rac and Cdc42 (Nobes & Hall, 1995; Ridley & Hall, 1992). Adhesive

interaction with the ECM and the contractile actin stress fibers attached to the cells generate traction forces (Sheetz, 1994). The key components providing focal contacts with ECM are integrins, transmembrane receptors. The activation and co-clustering of integrins recruit several adaptor proteins (paxillin, vinculin) and signaling proteins (FAK, Src) to stabilize the adhesions to the ECM (Zaidel-Bar et al, 2004), and then further recruit extracellular proteolytical enzymes, such as matrix metalloptoteinases (MMPs), to perform pericellular ECM remodeling and to generate a path for cell migration (Brooks et al, 1996).

1.2.3 Signaling Pathways Regulating Cell Migration

The cell migration cycle is a complex process that is regulated by several signaling pathways. The central regulators of cell migration are the Rho family small GTP-binding proteins (GTPases), which function as the molecular switches, responsible for maintaining actin cytoskeleton and controlling of the formation of lamellipodia and filopodia. The regulation is achieved by conformational change from switching binding of Rho proteins to GTP or GDP: when bound to GTP, they are active and thereby stimulate their downstream target proteins, including protein kinases, lipid-modifying enzymes, and activators of the Arp2/3 complex (Etienne-Manneville & Hall, 2002). However, the Rho family proteins have intrinsic phosphatase activity that hydrolyze the GTP to GDP, turning the protein “off” (Heasman & Ridley, 2008). Interaction with Guanine-nucleotide Exchange Factors (GEFs) facilitates the exchange of GDP to GTP and activates Rho GTPase, whereas GTPase Activating Proteins (GAPs) do the opposite. Notably, Rac, Cdc42 and RhoG are the GTPases required for protrusion of lamellipodia and filopodia (Ridley et al, 2003). The major downstream effector of Rho is the Rho-associated serine-threonine protein kinase (ROCK), which directly phosphorylates the myosin II light chain (MLC2) and results in more efficient interactions

between MLC2 and actin filaments that finally increase cell contractility (Mierke et al, 2008). ROCK can also inactivate myosin-light-chain phosphatase (MLCP), which dephosphorylates MLC2, thus indirectly activating MLC2.

The other important protein involved in cell contractility control is Cdc42, the master regulator of cell polarity in eukaryotic organisms (Itoh et al, 2002). Cdc42 functions are exerted through the downstream effector myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), the inhibitor of MLCP. Similar to Rho/ROCK, the Cdc42/MRCK signalling suppresses MLCP activity, thus activating MLC2 and determining the level of actomyosin contractility. Cdc42 also restricts the direction where lamellipodia form and orients the microtubule-organizing center (MTOC) and Golgi apparatus in front of the nucleus towards the leading edge. It has been found that reorganization of MTOC was more important for the migration of slow-moving cells, since MTOC is usually located behind the nucleus in fast-moving cells, such as neutrophils and T cells (Serrador et al, 1999). PAK1 is another downstream target of Cdc42 that is able to mediate Cdc42 activation, forming a positive feedback loop between Cdc42 and PAK1 (Li et al, 2003). Integrins are also involved in feedback loops that contribute to maintaining local Cdc42 activation (Etienne-Manneville & Hall, 2001).

WASP/WAVE family proteins are the major downstream effectors for the Rho family members, Rac and Cdc42, and play important roles in actin polymerization of protrusions. WASP proteins are scaffolds that transmit upstream signals to activate the Arp2/3 complex, which is crucial for the reorganization of the actin cytoskeleton at the cell cortex (Takenawa & Suetsugu, 2007). Rac can activate WAVE proteins, thereby leading to lamellipodia extension (Cory & Ridley, 2002), while Cdc42 activates WASP proteins and induces

dendritic actin polymerization (Welch & Mullins, 2002). However, WASPs may not be responsible for the filopodia formation, since cells lacking WASPs are still able to induce filopodia (Snapper et al, 2001). On the other hand, WAVE/WASP proteins can regulate the activities of Rac and Cdc42 by binding to GAPs and GEFs (Cory et al, 2002; Soderling et al, 2002). Other signals, such as the Src family kinases and phosphoinositides, can also regulate WAVE/WASP proteins (Cory et al, 2002; Suetsugu et al, 2002).

The ERK/MAPK signal pathway plays a well-known role in tumor cell survival, proliferation and stress response, but it has also been proven to be essential to cell migration. The MAP kinases are characterized by a common Thr-x-Tyr motif in the kinase domain, and can be classified into three groups based on the differences of motifs within their activation loops: extracellular-signal-regulated protein kinase (ERK/MAPK), p38 and Jun N-terminus kinase (JNK) (Inamdar et al, 2010). Several cytoskeleton-associated proteins and adaptor proteins have been identified as JNK substrates, such as microtubule-associated proteins (MAPs), the actin binding protein spir and the focal adhesion adaptor paxillin (Chang et al, 2003; Huang et al, 2003; Otto et al, 2000). Multiple studies have demonstrated that p38 is involved in growth factor and cytokine-induced cell migration (Huang et al, 2004). The ERK-MAPK pathway has been linked to migration of various cell types. The ERK-MAPK signaling inhibitors PD98059 and U0126, which can selectively inhibit MEK, suppress the migration of diverse cell types in response to different stimuli, such as fibronectin, collagen, VEGF, insulin and uPA (Anand-Apte et al, 1997; Eliceiri et al, 1998; Jo et al, 2002; Shono et al, 2001). Moreover, several substrates of ERK/MAPK are involved in ERK-mediated cell migration, including MSK1 (mitogen- and stress- activated protein kinase-1), MLCK, the protease calpain, paxillin and FAK (focal adhesion kinase) (Deak et al, 1998; Fukunaga &

Hunter, 1997; Glading et al, 2004; Klemke et al, 1997; Liu et al, 1999). Recent studies have found that ERK/MAPK signaling regulates paxillin-FAK interaction, which is involved in focal adhesion disassembly (Hunger-Glaser et al, 2003). In addition, ERK/MAPK also regulates cell migration by suppressing the activities of integrins, but the precise molecular mechanism remains to be elucidated (Chou et al, 2003).

1.3 Protein Degradation

Almost all the proteins within mammalian cells are continually being degraded to amino acids and replaced by newly synthesized proteins. The protein degradation process is highly selective and precisely regulated by the cell's degradative machinery (Glickman & Ciechanover, 2002). The rates of individual protein's destruction differ widely, with half-lives ranging from several minutes to weeks (Bohley, 1995; Burger & Seth, 2004). The protein degradation program also serves as a quality-control system to eliminate mutated, mis-folded, or damaged proteins, whose accumulation would be harmful to normal cell function and viability (Goldberg, 2003). The protein degradation is irreversible and is involved in the regulation of cell division, gene expression and other key biological processes (Goldberg et al, 1995). Therefore, aberrations in protein degradation systems cause a variety of pathological phenotypes. Cancer can develop from abnormal increase in oncogenic protein degradation, or from destabilization of tumor suppressors.

1.3.1 Protein Degradation Pathways

Cellular proteins can be degraded by two major mechanisms: lysosomal and proteasomal degradation pathways. Lysosomal degradation is responsible for cell surface proteins that are taken up by endocytosis, and accounts for 10-20% of protein turnover (Tanaka et al, 2004). However, about 80% of cellular proteins are degraded by the proteasome in the cytoplasm

and nucleus (Glickman & Ciechanover, 2002). Most proteins destined for degradation are labelled by ubiquitin in an energy-required process and then subjected to digestion and further destruction in the large proteolytic complex, 26S proteasome. Notably, with a few exceptions, ubiquitination could also lead to lysosomal degradation, such as of the extracellular and membrane proteins (Glickman & Ciechanover, 2002; Rock & Goldberg, 1999).

Proteasomes are multicatalytic complexes localized in the nucleus and cytosol providing the major proteolytic activity in eukaryotes. There are two major types of proteasomes, 26S proteasome that assembles from ring-like 19S and 20S particles, and 20S proteasome consisting of four stacked rings. Both 26S and 20S proteasomes are composed of numerous polypeptide subunits. The 20S is an ATP-independent protease that digests peptides and unfolded proteins; whereas the 26S is an ATP-dependent proteasome that degrades ubiquitin-tagged proteins (Gerards et al, 1998).

1.3.2 Ubiquitin-Proteasome System

The ubiquitin proteasome system (UPS) is responsible for the destruction of regulatory proteins within eukaryotic cells and is thus crucial for maintaining cellular homeostasis (Crusio et al, 2010). Most proteins targeted by the 26S proteasome are tagged by the small molecule ubiquitin (Welchman et al, 2005). Ubiquitin is a 76 amino acid protein containing seven lysine residues that can be used for attachment to other ubiquitins, thereby allowing the formation of polyubiquitin chains. Various cellular processes are controlled by the UPS, including cell cycle, proliferation, differentiation, stem cell quiescence, transcription, DNA damage repair and apoptosis (Schwartz & Ciechanover, 2009).

The process of ubiquitination involves an enzymatic cascade that consists of E1, the ubiquitin-activating enzyme, E2, the ubiquitin-conjugating enzyme and E3, the ubiquitin ligase (Schwartz & Ciechanover, 2009). In brief, the E1 enzyme activates ubiquitin in an ATP-dependent manner, and then conjugates the activated ubiquitin to E2. In cooperation with the E3 ubiquitin ligase, the E2 enzyme transfers the ubiquitin to substrate proteins, and followed by multiple cycles of ubiquitination, the polyubiquitinated substrates are destined for destruction by the 26S proteasome (Nandi et al, 2006). It is important to note that the ubiquitination process can be reversed by a family of deubiquitinating enzymes, which can remove ubiquitin from the substrates or free polyubiquitin chains. The E3 ubiquitin ligase provides the specificity of UPS targeted substrate proteins. To date, several hundred E3 ligases have been identified in the human genome; however, only very few of them have been well characterized (Pickart & Eddins, 2004). E3s have been classified into three types, the HECT (homologous to E6AP C-terminus) domain types, the single-subunit RING-finger type and the multi-subunit RING-finger type E3 ligases (Weissman, 2001). HECT-domain E3s bind to ubiquitin first and then transfer it to substrate proteins, whereas RING-domain E3s directly transfer activated ubiquitin. Within the multi-unit RING-finger E3s, two sub-classes have been identified, the SCF E3 ligases that responsible for G1-S transition in cell cycle progression, and the anaphase-promoting complex/cyclosome (APC) that functions at the end of mitosis.

1.3.3 SCF E3 Ubiquitin Ligases

Among the RING-finger E3 ligases, the SCF (Skip1-Cul1-F-box protein) E3 ligase is one of the best characterized. In the SCF complex, Cul1 acts as a molecular scaffold that simultaneously interacts with a catalytic RING-finger protein Rbx1 (also known as Roc1 or

Roc2) and a specific E2 ubiquitin-conjugating enzyme at the C-terminus, and with the crucial adaptor subunit Skp1 at the N-terminus. Skp1 binds to the F-box motif of one of the F-box proteins, which function as the substrate recognition component and interact directly with the substrates (Ang & Wade Harper, 2005).

The F-box proteins are defined by the presence of the Skp1-binding F-box motif that consists of approximately 40 amino acids, and the term F-box was named after the first identified F-box protein, Cyclin F (Cardozo & Pagano, 2004). Based on the substrate binding domain in the C-terminus, F-box proteins are classified into three groups: FBWs with the presence of WD40 repeats, FBLs with leucine-rich repeats (LRR), and FBXs without WD40 repeats or LRRs but often with different protein-protein interaction domains (Ho et al, 2006). In general, substrate recognition by F-box proteins requires a post-translational modification of the target protein in a degradation sequence (degron) (Skaar et al, 2009b). The most common form of post-translational modification involved in the substrate targeting is phosphorylation, but other modifications, such as glycosylation, have been reported. In addition, a single F-box protein can recognize diverse substrates, enables the SCF ubiquitin ligases have multiple target and numerous biological functions (Ho et al, 2006). To date, 69 human F-box proteins have been identified; however, most of them are not well-characterized (Skaar et al, 2009a).

1.4 Novel Tumor Suppressor Fbw7

The F-box and WD repeat domain-containing 7 (also known as Fbw7, Fbxw7, CDC4, AGO and SEL10) is one of the well-studied members of F-box protein family. Fbw7 serves as a substrate adaptor for the Skp1-Cul1-F-box protein-Rbx1 (SCF) ubiquitin ligase complex and mediates the recognition and binding of the substrate proteins. SCF^{Fbw7} degrades several

proteins with important roles in cell growth, proliferation, differentiation, and survival (Welcker & Clurman, 2008). Some of the Fbw7 substrates are extensively studied proteins in cell biology, including c-Myc, cyclin E, Notch, c-Jun, mTOR and Mcl-1 (Inuzuka et al, 2011; Mao et al, 2008; Nateri et al, 2004; Oberg et al, 2001; Strohmaier et al, 2001; Yada et al, 2004). Notably, most Fbw7 substrates have been shown as oncogenes in multiple types of human cancer (Tan et al, 2008). Mounting evidence indicates that Fbw7 is a tumor suppressor in multiple cancers.

1.4.1 Gene Location and Splicing Variants

In mammals, the *Fbw7* gene is located within chromosome 4 and encodes three transcripts (Fbw7α, β and γ) as a result of alternative splicing of their first exons (Figure 1.3) (Ho et al, 2006). These three isoforms have their own promoter and generate three protein isoforms with unique N termini, determining the expression pattern, subcellular distribution, and the functions of the protein (Welcker & Clurman, 2008).

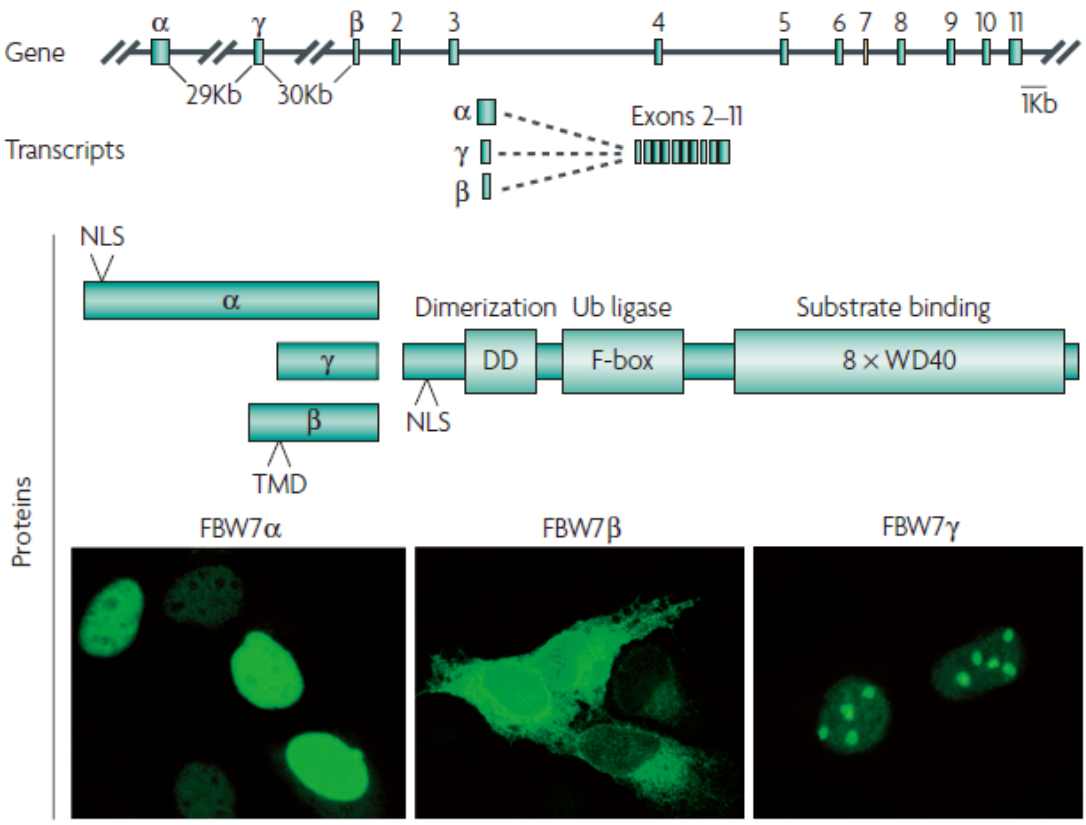

Figure 1.3 The organization of the *FBW7* gene and its protein isoforms.

These localization patterns, here shown for overexpression isoforms in U2OS cells, are typical for most cell lines. NLS, nuclear localization signal; TMD, trans-membrane domain. This figure was from Welcker *et al*, 2008, reprinted with permission.

The Fbw7α localizes to the nucleus and is predominantly expressed in adult mouse tissue. Compared to theother two isoforms, Fbw7α mRNA expression is much higher in most human cell lines and primary cells (Spruck et al, 2002). Grim et al. established isoform-specific Fbw7-null mutations in human cells and found that the Fbw7α isoform is responsible for the degradation of most Fbw7 substrates, including cyclin E, c-Myc and SREBP1 (Grim et al, 2008). Fbw7β is primarily a cytoplasmic protein and most abundant in the brain and testis (Spruck et al, 2002). A recent study showed that Fbw7β contains a putative

transmembrane domain in the NH2 terminus region, and Fbw7β deficient cells have higher sensitivity to oxidative stress (Matsumoto et al, 2011). Expressed primarily in heart and skeletal muscle, Fbw7γ shows a nucleolar distribution (Spruck et al, 2002). It has been reported that the Fbw7γ isoform localizes with c-Myc in the nucleolus and regulates its turnover (Welcker et al, 2004). Although one study showed that Fbw7 protein isoforms interact with each other and regulate cyclin E degradation (Zhang & Koepp, 2006), more investigations are needed to confirm the cooperation status of each isoform when SCF^{Fbw7} exerts its functions. It is likely that the transcription of each isoform is differentially regulated, and each isoform is responsible for different substrates and functions during protein degradation. However, the exact physiological role and regulation mechanism of each isoform remains unclear.

The structure of Fbw7 has been well studied, and several of its domains were found to be essential for Fbw7 functions. The F-box domain, which is the common feature of all F-box proteins, offers direct interaction with Skp1 in the SCF complex (Bai et al, 1996). Eight WD40 repeats are protein-protein interaction domains that provide physiological connections with the substrate. The WD40 repeats form an eight-bladed barrel-shaped β propeller structure termed phospho-degron binding pockets, which recognize and bind to substrates after they have been phosphorylated within conserved phosphor-degron motifs, defined as CPD (Cdc phosphor-degrons) (Hao et al, 2007; Nash et al, 2001; Orlicky et al, 2003; Perkins et al, 2001). That all Fbw7 substrates contain at least one conserved CPD sequence (T/S) PXX(S/T/E), in which the T/S residue can be phosphorylated by glycogen synthase kinase 3 (GSK3) has been revealed in research (Welcker & Clurman, 2008). The motif sequence localizing just before the F-box region, called D domain, is required for Fbw7 dimerization

(Welcker & Clurman, 2007). Recent studies indicate that F-box proteins regulate substrate binding and ubiquitylation by forming homodimeric or heterodimeric complexes (Tang et al, 2007; Welcker & Clurman, 2007). The dimerization might largely depend on the degron strength and is substrate-specific and provides a possibility of precise control of substrate degradation.

1.4.2 Fbw7 and Cell Cycle Regulation

Substantial evidence supports the suggestion that Fbw7 serves as a tumor suppressor by controlling the degradation of several important oncogenic proteins having key roles in cell cycle progression, proliferation and cell division (Figure 1.4).

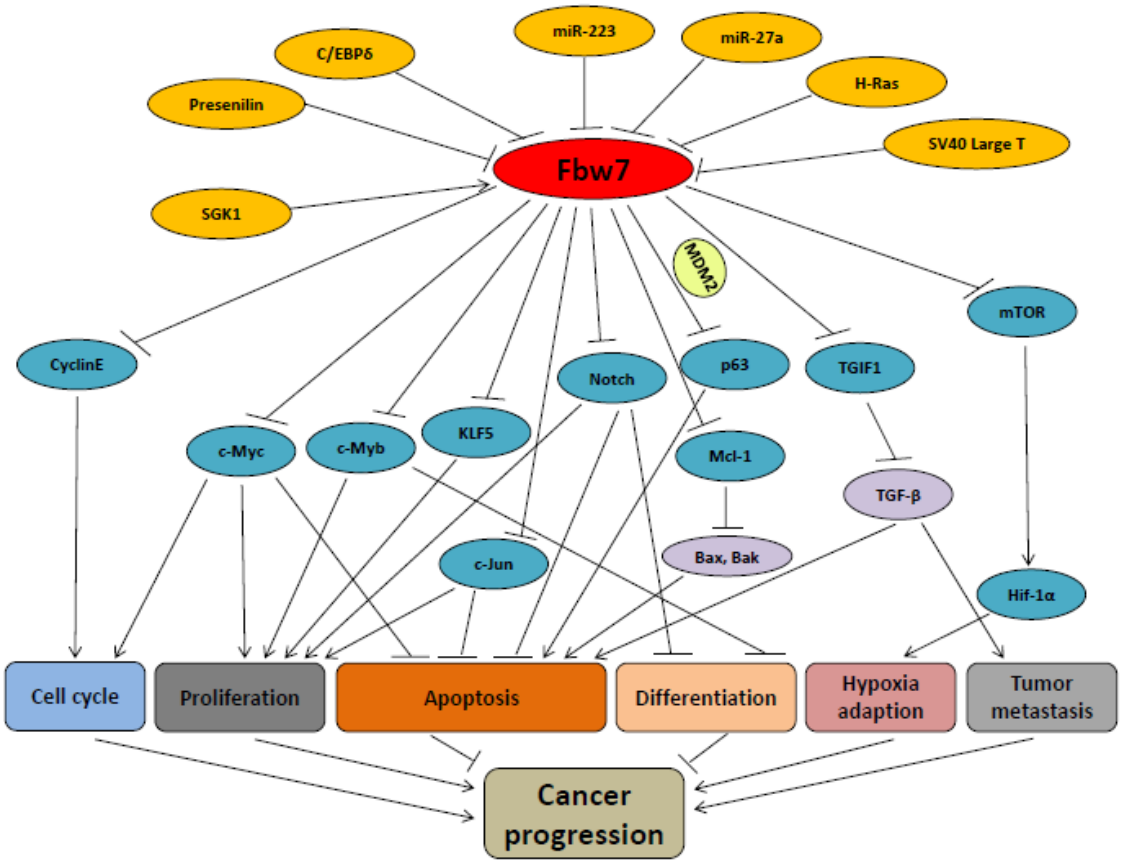

Figure 1.4 Schematic diagram of the functions and regulation networks of Fbw7 in connection with cancer progression.

Fbw7 primarily targets various proteins controling cell cycle progression, such as cyclin E, c-Myc, c-Jun, Notch and KLF5, thereby regulating cell proliferation (Cheng et al, 2013). Cyclin E binds to G1 phase cyclin-dependent kinase Cdk2 and controls the G_1 to S phase transition in the cell cycle, which is the rate-limiting step for proliferation (Clurman et al, 1996; Won & Reed, 1996). As the key regulator of cell cycle machinery, cyclin E, its amount and kinase activity is tightly controlled by ubiquitin-mediated proteolysis. Deregulation of cyclin E has been frequently found in cancer, and elevated expression of cyclin E leads to genomic instability and tumorigenesis (Spruck et al, 1999). That the p53 and Fbw7 pathways cooperatively protect cells against cyclin E-induced genomic instability in primary human cells has also been determined. In addition to cyclin E, c-Myc is another key regulator of exit and re-entry into the cell cycle. At least four ubiquitin ligases have been identified that ubiquitylate c-Myc and regulate its turnover. Besides Fbw7, little is known about the recognition of c-Myc by the other ubiquitin ligases. Degradation of c-Myc by Fbw7 has been shown in controlling c-Myc stability in G1 phase (Sears et al, 2000). The AP1 transcription factor c-Jun is degraded by SCF^{Fbw7} as well. The Jun N-terminal kinase (JNK) signalling pathway leading to c-Jun phosphorylation stimulates cell proliferation, and Fbw7 is found to antagonize c-Jun function. In addition, the regulation of c-Jun by Fbw7 is dependent on JNK phosphorylation of the c-Jun N terminus (Nateri et al, 2004).

Other than those well-known substrates related to cell cycle and cell proliferation regulated by Fbw7, some novel molecules have been identified recently. KLF5 (also known as IKLF, and BTEB2) is a member of Krüppel-like family (KLF) transcription factors playing important roles in multiple physiological and pathological processes, including stemness, inflammation, and atherogenesis (Liu et al, 2010). Multiple studies demonstrate

that KLF5 promotes cancer cell proliferation and serves as an oncogenic transcription factor (Chen et al, 2006; Nandan et al, 2005; Zheng et al, 2009). Zhao et al. found that the KLF5 transactivation domain contains two putative evolution-conserved CPD motifs, 303SPPSS and 323TPPPS, which could recruit Fbw7 upon phosphorylation. GSK3β kinase is involved in KLF5 S303 phosphorylation that is required for Fbw7-mediated KLF5 degradation (Zhao et al, 2010).

1.4.3 Fbw7 in DNA Damage and Cellular Apoptosis

Fbw7 ubiquitin ligase has been shown to be vital in maintaining chromosome stability, and crucial to cell differentiation and apoptosis through degradation of the downstream substrates (Mao et al, 2004). A well-known tumor suppressor protein, p53 also plays an essential role in conserving genome stability after DNA damage (Kern et al, 1991). It has been demonstrated that p53 regulates the expression of the cytoplasmic isoform Fbw7β (Kimura et al, 2003). Fbw7 might also be positioned upstream of p53 in a signalling axis that activates the tetraploidy checkpoint in response to mitotic inhibitors (Finkin et al, 2008). Mao et al. have found that radiation-induced lymphomas from $p53^{+/-}$ mice, but not those from $p53^{-/-}$ mice, have frequent loss of heterozygosity and a 10% mutation rate of *Fbw7* gene. $Fbw7^{+/-}$ mice have greater susceptibility to radiation-induced tumorigenesis, indicating that Fbw7 is a p53-dependent, haplo-insufficient tumor suppressor gene (Mao et al, 2004).

Transforming growth factor-β (TGFβ) signalling pathway regulates multiple cellular processes, including apoptosis and cell differentiation. Dysfunction of TGFβ signalling has been implicated in various human disorders ranging from vascular diseases to cancer (Massague & Wotton, 2000; Miyazono et al, 2003). TGFβ signalling is negatively regulated by the transcriptional repressor TGFβ-induced factor 1 (TGIF1), which has also recently

been reported as a target of degradation by Fbw7 in a phosphorylation-dependent manner (Bengoechea-Alonso & Ericsson, 2010). By inactivating TGIF1, Fbw7 enhances TGFβ-dependent transcription. Accordingly, inactivation of Fbw7 results in the accumulation of phosphorylated TGIF1 and attenuation of TGFβ-dependent gene transcription, cell proliferation and migration. Thus, Fbw7 could be an important regulator of TGFβ signalling by targeting the transcriptional repressor TGIF1 for degradation. Moreover, Fbw7 can control cellular apoptosis through regulating TGFβ signalling.

Most recent studies revealed more mechanisms of how Fbw7 affects cell survival by regulating Mcl-1 turnover. Mcl-1 is a pro-survival Bcl-2 family member, inhibiting apoptosis by blocking the cell death mediators Bax and Bak (also known as Bak1) (Willis et al, 2007). In 2011, two groups independently found that Mcl-1 is a novel substrate of Fbw7. Inuzuka et al. showed that Fbw7 governs cellular apoptosis by targeting Mcl-1 for ubiquitylation and destruction upon phosphorylation by GSK3 (Inuzuka et al, 2011). They found in T-ALL cell lines, loss of Fbw7 resulted in Mcl-1 overexpression. At the same time, T-ALL cell lines with defective Fbw7 are sensitive to the multi-kinase inhibitor sorafenib but resistant to the Bcl-2 antagonist ABT-737. Moreover, both Fbw7 reconstitution and Mcl-1 depletion restores sensitivity to ABT-737, indicating that Mcl-1 as a relevant bypass survival mechanism to enable Fbw7-deficient cells to evade apoptosis. Similarly, another group showed that the degradation of Mcl-1 was blocked in Fbw7-deficient or loss-of-function mutated tumor cells, and Mcl-1 regulated the sensitivity of tumor cells to anti-tubulin chemotherapy through Fbw7 (Wertz et al, 2011). These data indicate that Fbw7 regulates cellular response to DNA damage, as well as the sensitivity to anti-cancer agents, suggesting promising combination chemotherapy for cancer treatment.

1.4.4 Fbw7 Mutations

As a major regulator of a set of oncoproteins, *Fbw7* were found lost or mutated in various human cancers (Tan et al, 2008). The *Fbw7* gene localizes to *4q31.3*, which is deleted in ~30% of human cancers (Knuutila et al, 1999). By using a mammalian genetic screen, Mao et al. found that Fbw7 loss cooperated with p53 to promote tumor formation in mice, indicating Fbw7 to be a p53-dependent, haploinsufficient tumor suppressor gene (Mao et al, 2004). Moreover, one study identified mutations in *Fbw7* in both human colorectal cancers and their precursor lesions that result in chromosomal instability (Rajagopalan et al, 2004). These findings together also suggest the tumor suppressor role of Fbw7 in human cancers.

Fbw7 status has been examined in numerous primary human tumors. A genetic study of primary tumors showed that the overall mutation rate in *Fbw7* is approximately 6%, but the mutation rates are quite tumor type dependent (Akhoondi et al, 2007). Cholangiocarcinoma and T-cell acute lymphoblastic leukaemia (T-ALL) harbour the highest frequency of mutations at 35% and 31%, respectively (Akhoondi et al, 2007). While in tumors of the stomach, colon, pancreas, and endometrium, only 9-15% of tumors contained *Fbw7* mutations (Akhoondi et al, 2007; Calhoun et al, 2003; Rajagopalan et al, 2004; Spruck et al, 2002). Interestingly, many tumor types including acute myeloid leukemia (AML), breast, bladder, bone, liver, lung, and melanoma show rare (≤4%) or no mutations in the *Fbw7* gene (Akhoondi et al, 2007; Kwak et al, 2005; Nowak et al, 2006; Woo Lee et al, 2006; Yan et al, 2006). Notably, 6% of *Fbw7* mutations are isoform-specific, suggesting each isoform may play a distinct role in human tumorigenesis. It remains to be determined whether Fbw7 isoforms function cooperatively or separately in Fbw7-mediated protein ubiquitination. Akhoondi et al. also identified the two mutational hotspots within the WD40

repeats domain: Arg^{465} and Arg^{479}. These two residues account for approximately 40% of all tumor related mutations in *Fbw7* (Akhoondi et al, 2007). The point mutations occur in key residues disturb the formation of substrate binding interfaces, therefore resulting in failure of substrate binding.

The *Fbw7* mutation has been most extensively studied in T-ALL compared to other tumor types. The Notch pathway plays the central role in T-ALL development; 56% of T-ALL cases harbour *Notch* mutations (Weng et al, 2004). The majority of mutations found in the *Notch* locus are located in two regions: the HD (heterodimerization domain 26%) and the PEST (12.5%) domain. Mutations in the HD domain induce ligand-independent proteolytic cleavage of Notch, leading to activation of Notch, while mutations in the PEST domain increase the half-life of NICD by blocking Fbw7 interaction. *Fbw7* mutations were identified in 31% of T-ALL patients, which are the second frequent genomic lesions in T-ALL (Maser et al, 2007). O' Neil et al. showed that *Fbw7* mutated T-ALL cell lines cannot bind to the Notch intracellular domain (NICD), thereby causing NICD accumulation (O'Neil et al, 2007). NICD accumulation in turn stabilizes another Fbw7 substrate c-Myc, both of which contribute to leukemia transformation. It seems that disturbed Notch degradation by Fbw7 loss-function mutation is the major mechanism responsible for T-ALL pathogenesis.

Since GSK3 is the major priming kinase mediating the phosphorylation of Fbw7 substrates, interruption of GSK3 activity affects Fbw7 substrate stability. Several activated signalling pathways, such as the Wnt and the PI3K–Akt signalling pathways, as well as reduced GSK3 activity were found in many cancers (Fresno Vara et al, 2004). In fact, PTEN (phosphatase and tensin homologue), which is an antagonist of the PI3K pathway, is one of the most frequently inactivated tumor suppressor genes, especially in T-ALL (Maser et al,

2007). One comprehensive study of 47 T-ALL patients indicated 26 cases with *PTEN* mutations and merely 5 cases with *Fbw7* mutations (Larson Gedman et al, 2009). Interestingly, these researchers also found that PTEN transcripts positively correlated with c-Myc transcript levels, Suggesting that the relationship between PTEN and Fbw7 substrates still needs further investigation.

1.4.5 Regulation of Fbw7 Expression

Although much is known about the Fbw7 ubiquitin ligase mediated protein degradation pathway and a number substrates of Fbw7 only identified in the last decade, the regulation and upstream regulators of Fbw7 itself are poorly understood. Several Fbw7 regulators reported in recent literature are demonstrated in Figure 1.4.

Balamurugan et al. reported that the transcription factor CCAAT/enhancer binding protein-δ (C/EBP δ) directly inhibits Fbw7 gene expression and induces Fbw7 oncogenic targets mTOR and Aurora A (Pawar et al, 2010). ChIP assay with MCF-7 breast cancer cells showed the endogenous C/EBP δ binds to the Fbw7α promoter. Consequently, C/EBP δ enhances the PI3K/Akt/mTOR pathway and promotes HIF-1α translation and activity, which is necessary for hypoxic adaptation and tumor metastasis (Balamurugan et al, 2010). This 2010 report is the first showing a correlation between Fbw7 and tumor metastasis, suggesting a potential value of clinical application of Fbw7 or mTOR inhibitors (Strimpakos et al, 2009) to prevent metastatic disease. Another regulator of Fbw7 is E1A (early region 1A), a product of human adenovirus type 5 (Isobe et al, 2009). The Isobe et al study for the first time showed E1A perturbs SCF^{Fbw7}-mediated protein turnover through the inhibition of Fbw7 ubiquitin ligase. However, the mechanism by which E1A inhibits the activity of Fbw7 and the major determinant of the specificity remain unclear.

Presenilins (PS), are the catalytic components of γ-secretase, required for Notch1 activation (De Strooper et al, 1999; Koo & Kopan, 2004). It has been reported that Fbw7α targets the Notch1 intracellular domain (NICD) for degradation and positively regulates EGFR by affecting ubiquitylation and stability of EGFR. At the sametime, PS negatively modulates Fbw7 transcription, thus positively and negatively regulating EGFR and Notch signalling, respectively (Rocher-Ros et al, 2010). Using a novel epidermal conditional PS-deficient mouse model deleting PS in keratinocytes of the basal layer of the epidermis, researchers found the mice developed epidermal hyperplasia associated with increased expression of both EGFR and Fbw7 and reduced NICD level in keratinocytes. This study, the first to show a molecular regulation mechanism of Fbw7 expression at the transcriptional level, indicated a crosstalk between PS, EGFR and Notch signalling through Fbw7 during cell growth and skin carcinogenesis. Fbw7 appears to serves as a central mediator of the upstream PS and downstream EGFR and Notch signalling. In contrast with its previously established tumor suppressor role, Fbw7 could also function as an oncogene as it promotes EGFR stability and signalling in epidermal cells. More study is required to determine the exact role of Fbw7 in epidermal cell transformation and to determine whether Fbw7 affects EGFR degradation directly.

Another potential group of Fbw7 regulators are microRNAs. MicroRNA are short RNA molecules, averaging 22 nucleotides long, which bind to complementary sequences on target mRNAs resulting in translational repression of target degradation and gene silencing (Kim & Bartel, 2009). Xu et al. found that overexpression of miR-223 could increase endogenous cyclin E protein and activity levels, and increase genomic stability by significantly reducing Fbw7 mRNA level (Xu et al, 2010). This finding provided first

evidence that microRNA can directly regulate the activity of SCF^{Fbw7} ubiquitin ligase. Recently, another group identified miR-27a as a suppressor of Fbw7 and as a resultant inhibitor of ubiquitylation and degradation of the key Fbw7 substrate cyclin E (Lerner et al, 2011). More importantly, their data demonstrated that miR-27a is the only suppressor of Fbw7 during G1-S phase transition. By inhibition Fbw7, miR27a overexpression leads to abnormal cell cycle progression and DNA replication stress. These data confirm the transcriptional regulation of Fbw7 by a different type of regulators, a phenomenon which could affect Fbw7 substrate degradation and functions.

Besides the negative regulators of Fbw7 we already mentioned, one positive Fbw7 regulator SGK1 (serum- and glucocorticoid-inducible kinase 1) has been identified (Mo et al, 2011). SGK1 is an important regulator of multiple cellular processes, including metabolism, cell proliferation, cell volume, cell survival and differentiation (BelAiba et al, 2006; Kinugawa et al, 2001). SGK1 is an inhibitor of γ-secretase (Mo et al, 2011), required for Notch1 activation by releasing the Notch1 intracellular domain (Notch1-IC) (De Strooper et al, 1999). Mo et al. found that Notch-IC was able to form a trimeric complex with Fbw7 and SGK1, with SGK1 promoting the protein degradation of Notch1-IC through the Fbw7-dependent pathway. Furthermore, activated SGK1 appeared to induce Notch1-IC protein degradation and ubiquitylation through phosphorylation of Fbw7 at serine 227 (Mo et al, 2011). These data indicate that activation of SGK1-mediated Fbw7 phosphorylation could serve as a therapeutic strategy for developing anti-leukemia drugs in T-ALL cells. Interestingly, another study also found that serine 227 was phosphorylated in a PI3K-dependent manner, in particular the Fbw7 alpha isoform (Schulein et al, 2011). This PI3K-dependent phosphorylation reduces Fbw7 stability but promotes ubiquitylation of its two

substrates, c-Myc and cyclin E. Taken together, these evidences suggest that several kinases serve as isoform-specific regulators of Fbw7 protein, and that the activity of Fbw7 can be regulated at the post-translational level.

1.4.6 Fbw7 and Tumor Metastasis

By regulating the expression of substrate proteins, Fbw7 suppresses tumor metastasis in certain types of cancer, such as breast cancer (Balamurugan et al, 2010). Recent studies showed that c-Myc can regulate the epithelial-to-mesenchymal transition (EMT) necessary for cellular invasion and migration by promoting TGFβ-mediated activation of the SNAIL transcription factor (Wolfer & Ramaswamy, 2011). Based on the previous reports, the role of TGFβ is variable in different stages of carcinogenesis. In the early stages of tumor formation, TGFβ functions as a tumor suppressor (Massague et al, 2000), but it can promote EMT and metastasis during late stages of tumor development (Bengoechea-Alonso & Ericsson, 2010). This finding is contradictory to the notion that Fbw7 promotes the degradation of negative regulator of TGFβ, TGIF1. Additionally, Kuppusamy et al. reported that C/EBPδ directly inhibits Fbw7 gene expression, thus promoting the mTOR/Akt/HIF-1 pathway, contributing to cell migration and metastasis (Balamurugan et al, 2010). These studies demonstrate the complexity of the Fbw7-mediated signalling network and of the exact role of Fbw7 in tumor metastasis

1.5 Biomarkers of Melanoma

A biomarker, as defined by the US Food and Drug Administration, is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. The introduction of

biomarkers to cancer diagnosis and treatment has revolutionized the practice of oncology. The detection and evaluation of biomarkers provide information about the disease that cannot be gathered by clinicians in standard procedures. Biomarkers can be proteins, RNA transcripts, DNA, epigenetic modifications of DNA, and they can be detected in patient tissue samples, usually obtained by surgical resection, or bodily fluids, such as blood or urine. The ideal biomarker for clinical use should be safe, easily used, sensitive and specific, capable of improving decision-making ability in combination with clinicopathological parameters. Although the fundamental mechanisms of cancer are now better understood and the technologies of discovering useful biomarkers have been meaningly significantly improved, only a handful of robust cancer biomarkers have been validated and translated into clinical practice.

Several notable examples of useful cancer biomarkers have emerged in recent years. For example, overexpression of the human epidermal growth factor receptor 2 (HER2) in breast cancer, and gene fusion between echinoderm microtubule-associated protein-like 4 (EML4) and the anaplastic lymphoma kinase (ALK) gene (EML4-ALK) in lung cancer, are effective predictive biomarkers to identify the patients who will most likely benefit from therapies targeting those genetic alterations (Kwak et al, 2010; Slamon et al, 2001). Another example is the protein biomarker prostate-specific antigen (PSA), whose serum expression level is commonly used for managing treatment of patients with hormone-naive prostate cancer (Lilja et al, 2008). In melanoma, recent remarkable advances in the targeted therapies are closely associated with the discovery of mutations in BRAF V600 codon. The presence of The BRAF V600E mutation identifies the individual most likely to respond positively to BRAF inhibitors (vemurafenib and dabrafenib) or MEK inhibitors (trametinib). However, in

general melanoma has lagged behind other cancers in the development of clinical-grade molecular signatures. Gould Rothberg and colleagues have conducted a systematic review of the prognostic tissue biomarkers that have been reported before January, 2008. Among 387 protein biomarkers they identified, only five were found significantly correlated with melanoma patient survival according to the REMARK criteria (Gould Rothberg et al, 2009). For tissue biomarkers in melanoma, the mitogenicity marker Ki67 is the only one that has been occasionally used in clinical setting so far, but more are expected to follow in the next few years.

1.5.1 Necessity of Biomarker Study in Melanoma

Currently, the management of melanoma is largely based on the staging and classification defined by morphologic and morphometric criteria. The AJCC staging system is the widely adopted standard in clinics globally, using tumor thickness, presence of ulceration and mitotic figures as the major prognostic factors. However, these criteria based on wide population studies are not able to give accurate individual information or predict the risk of metastasis for a specific individual. For example, at least 5% of thin melanomas (with thickness less than 1 mm) relapse, and a third of patients diagnosed with metastatic melanoma do not present with regional lymph node involvement (Carlson et al, 2003). Moreover, 10% of patients who undergo successful surgical removal of the primary tumor were found to recur with metastatic tumors without clinical evidence of dissemination (Scott et al, 2011). In addition, current guidelines usually suggest clinical stage II (thicker than 2.00 mm) melanoma patients take adjuvant treatment. However, only 50% of these patients will develop metastatic disease later on; therefore, thousands of melanoma patients are actually over-treated (Balch et al, 2001b; Gschaider et al, 2012). These facts indicate an urgent need

to find useful biomarkers to provide detailed information to be used to individualized melanoma diagnosis, prognosis and treatment.

1.5.2 Tissue Biomarkers

Tissue biomarkers can be used for melanoma diagnosis to help distinguish melanoma from other types of cancers. These tissue biomarkers include S100 calcium-binding protein-β (S100B), melanocyte lineage-specific antigen gp100 (also known as HMB-45 antigen and PMEL17), and melanA protein (also known as MART-1). Most of these biomarkers belong to the melanocyte pigmentation machinery, and show high sensitivity for melanoma. However, the specificity of these markers to distinguish melanoma from nevi is low, since they can be detected in melanocytic nevi as well. Although most melanomas can be adequately diagnosed using histological criteria, some suspicious cases still exist. For example, dysplastic nevi and Spitz nevi share overlapping histological features with melanomas, making the diagnosis difficult. A recent study has demonstrated the ability of a panel of five tissue biomarkers to distinguish benign melanocytic lesions from melanoma with 95% of specificity and 91% sensitivity (Kashani-Sabet et al, 2009).

The most widely used tissue biomarkers in melanoma diagnosis belong to S100 proteins. S100 proteins are small, acidic molecules involved in multiple cellular functions, including leukocyte chemoattraction, macrophage activation, and cell proliferation. At least 20 members of this family have been identified, while several of them are found associated with the p53 pathway and apoptosis. S100B is widely distributed in human tissue and different types of tumors, including melanoma. S100B is the best-studied melanoma biomarker and a widely used as a tissue biomarker of pigmented skin lesions. It has been demonstrated that S100B directly interacts with p53 and suppresses its function, resulting in tumor progression. In the 1980s, S100B was found expressed in melanoma cell lines, suggesting its value in diagnosis of melanoma. However, a subsequent study of 126 patients

found similar expression of serum S100B in healthy patients and in those with benign skin lesions, and elevated in 1.3%, 8.7% and 73.9% of patients with stage I/II, III, and IV melanoma, respectively (Guo et al, 1995). Therefore, S100B is deemed not to be a useful tool for detecting early melanoma.

Tissue biomarkers are also of prognostic value. In order to get a general picture for the currently identified tissue biomarkers, especially those of prognostic value in cutaneous melanoma, we searched the Medline medical database on Nov 17, 2013, using the queries showed in Table 1.1.

Table 1.1 Search strategy for prognostic tissue biomarker in cutaneous melanoma reported between 2003 and 2013

Step	Query	Hits
1	exp *Melanoma	58478
2	cutaneous melanoma.ti.	2009
3	Or/1-2	58577
4	exp *Tumor Markers, Biological	92943
5	tissue biomarker*.ti,ab.	298
6	or/4-5	93126
7	(prognos* or surviv* or gene* or protein*).ti,ab.	5739718
8	3 and 6 and 7	887
9	uvea*.mp.	8564
10	sentinel.mp.	21572
11	or/9-10	30125
12	8 not 11	754
13	limit 12 to english language	729
14	limit 13 to humans	702
15	limit 14 to yr="2003 -Current"	478

According to the Reporting recommendations for tumor MARKer prognostic studies (REMARK) published in 2005 (McShane et al, 2005a; McShane et al, 2005b; McShane et al, 2005c; McShane et al, 2005d; McShane et al, 2005e), we selected the highest quality studies

based on the inclusion criteria: 1) Cohort study design with a clearly defined source population (non-Caucasian cohorts were not counted in this study); 2) Primary cutaneous tumor specimens were included and assayed; 3) Clear description of IHC methods and the way of handling tissue samples; 4) The primary antibody was well-validated; 5) Multivariate proportional hazards model was analyzed by statistics. Finally, 79 manuscripts reporting 106 tissue biomarkers were identified as shown in Figure 1.5, Table 1.2. Due to the large number of biomarkers identified, the tissue biomarkers were grouped by the protein functions accordded to the six acquired biological capabilities of cancer as defined by Hanahan and Weinberg (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).

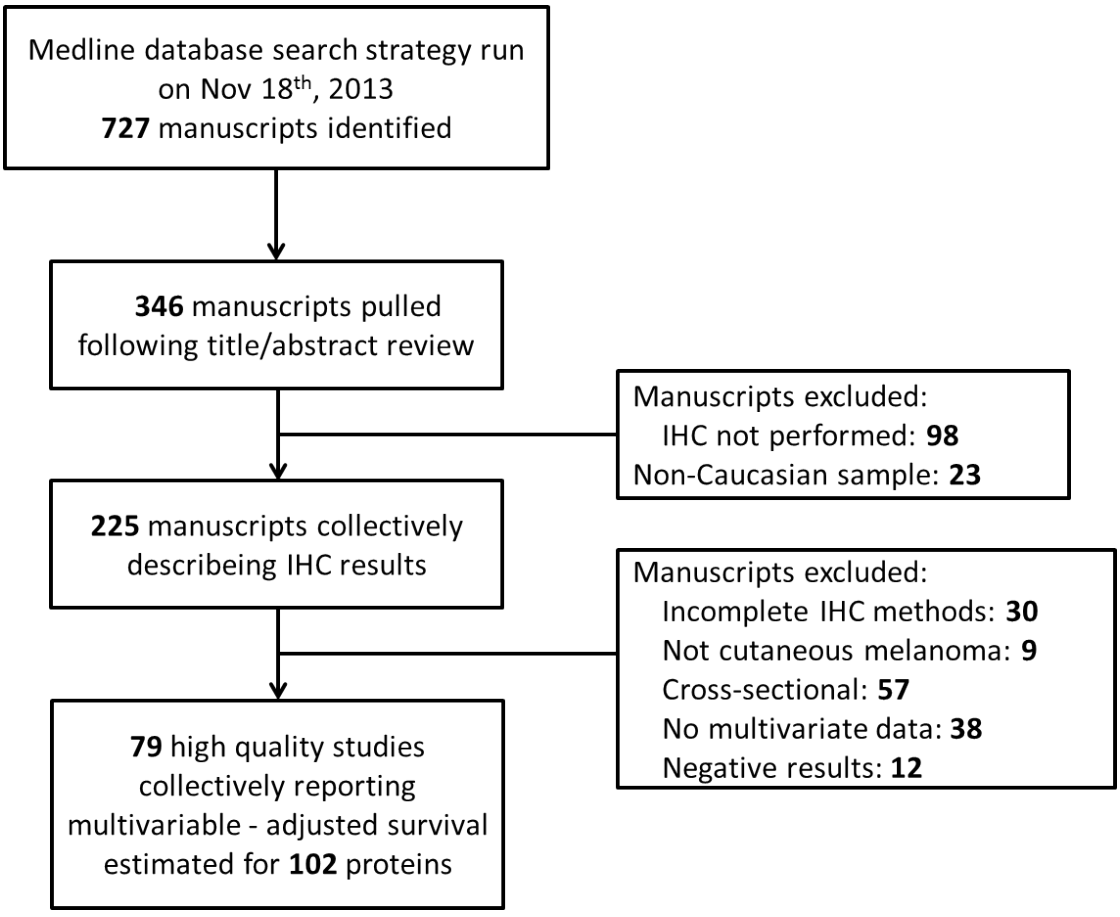

Figure 1.5 Flow diagram of the literature search.

Table 1.2 Summary of tissue biomarkers with significant prognostic value in human cutaneous melanoma reported between 2003 and 2013

Protein	Association with worse prognosis
Altered immunocompetence	
CCR7	Increased expression
CCR10	Increased expression
CD163	Expression
COX-2	Increased expression
FoxP3	Increased expression
MHC class I (HLA-A, -B, -C)	Expression
MHC class II (HLA-DR, -DP, -DQ)	Nuclear expression
MAGE-3	Loss of expression
STAT-1	Loss of nuclear expression
TLR4	Increased expression
Evading apoptosis	
APAF-1	Decreased expression
Bak	Decreased expression
Bax	Decreased expression
Bcl-2	Increased expression
Bcl-xL	Increased expression
p-Akt	Increased expression
SNF5	Loss of expression
Survivin	Increased expression
Insensitivity of antigrowth signals	
AP-2	Loss of nuclear expression
Bcl-6	Expression
BMI-1	Decreased expression
CYLD	Decreased expression
EGFR	Increased expression
ERK	Loss of cytoplasmic expression
HER3/ERBB3	Increased expression
HMGA2	Increased expression
ID1	Increased expression
Mel-18 (PCGF2)	Decreased expression
Nestin	Increased expression
p16/INK4a	Decreased expression
p21/WAF1	Increased expression
p27/KIP1	Increased nuclear expression/decreased cytoplasmic expression
p-ERK	Activation
pP300	Decreased nuclear expression
pRb	Inactivation due to protein phosphorylation
RUNX3	Loss of expression
Limitless replicative potential	
Cyclin A, B, D, E	Increased expression
CDK-1, -2	Decreased expression
CDK-6	Increased expression
c-met	Expression

Protein	Association with worse prognosis
c-Myc	Increased expression
Fbw7	Decreased expression
Geminin	Increased expression
Ki-67	Increased expression
Ku70	Loss of expression
Ku80	Loss of expression
MCM3	Increased expression
MDM2	Increased expression
Metallothionein	Increased expression
Microtubule-associated peotein-2	Decreased expression
nm23	Decreased expression
p53	Increased expression
PCNA	Increased expression
PHH3	Increased expression
RBM3	Increased expression
Skp2	Increased expression
Topoisomerase II	Decreased expression
Melanocyte differentiation	
gp100/HMB45	Increased expression
MelanA/MART-1	Increased expression
Tyrosinase	Increased expression
Self-sufficiency in growth signals	
AP-2α	Increased cytoplasmic/nuclear AQUA score ratio
ATF-2	Increased nuclear and decreased cytoplasmic AQUA score
c-Kit	Decreased expression
Glypican-3	Increased expression
ING3	Decreased expression
ING4	Decreased expression
Mum-1/IRF4	Increased expression
MFG-E8	Increased expression
NCOA3/AIB-1	Increased expression
Protein kinase C-α	Increased expression
Protein kinase C-β	Decreased expression
Sustained angiogenesis	
iNOS	Increased expression
D2-40	Increased expression
CD34	Increased expression
STAB1	Increased expression
Tissue invasion and metastasis	
αVβ3 integrin	Increased expression
B7-H3	Increased expression
BRMS1	Decreased expression
Caveolin	Decreased expression
CD44	Expression
CEACAM-1	Increased expression
CXCR4	Expression
E-cadherin	Loss of expression
Galectin-3	Loss of expression
L1-CAM	Increased expression

Protein	Association with worse prognosis
Maspin	Increased expression
MMP2	Increased expression
MMP9	Decreased expression
MCAM/MUC18	Expression
N-cadherin	Increased expression
Osteonectin/SPARC	Increased expression
Osteopontin	Increased expression
P-cadherin	Loss of expression
Tenascin-C	Expression
Tissue plasminogen activator	Increased expression
Others	
CD10	Increased expression
MTAP	Loss of expression
WT1	Increased cytoplasmic expression

*Organized according to Hanahan-Weinberg functional capabilities

Multiple studies have shown that expression of some cell adhesion molecules (CAMs) are elevated in melanoma and associated with the clinical outcome of melanomas. The list of CAMs includes MCAM, L1CAM, vascular CAM (VCAM1), intercellular adhesion molecule 1 (ICAM1) and carcino-embryonic antigen-related CAM 1 (CEACAM1) (Haass et al, 2005). In the meta-analysis of 62 identified tissue biomarkers before January 2008, MCAM appeared to be best of the five significant biomarkers to correlate with patient survival (HR, 16.34; 95% CI, 3.80-70.28). MCAM induces homologous and heterologous adhesions between melanoma cells and endothelial cells, respectively. Expression of MCAM increases along with melanoma progression and is highest in metastatic melanoma. Numerous studies have shown that MCAM serves as an independent prognostic biomarker inversely correlated with patient survival in melanoma (Lehmann et al, 1989; Pacifico et al, 2005).

Cadherins and catenins are another group of biomarkers implicated in melanoma. E-cadherin is the most important cadherin, expressed on the cell surface of both keratinocytes and melanocytes. E-cadherin is the major molecule mediating cell-cell interaction in the epidermis, and is lost in most melanoma cells. In contrast, N-cadherin is commonly up-

regulated in melanoma cells. A study with 144 primary melanomas, 53 metastatic melanomas, and 8 benign nevi showed that E-cadherin expression was significantly correlated with the tumor thickness, but not correlated with clinical outcome (Andersen et al, 2004). This conclusion was confirmed by several follow-up studies (Nishizawa et al, 2005). The catenin family proteins are also implicated in melanoma prognosis. β-catenin is the typical member in this family. Its role of in adhesion is not clear, but it plays an important role in the WNT signaling pathway. Functioning as a transcription factor, β-catenin activates several genes involved in melanoma pathogenesis. It has been reported that nuclear β-catenin expression was related to tumor thickness and found to be an independent prognostic factor for better survival (Bachmann et al, 2005). Other signaling transduction molecules also commonly serve as prognostic biomarkers. Important signaling pathways, including ERK-MAPK, PI3K/Akt/mTOR, and NFκB pathways, often show an aberrant activation attribute to expression change or gene mutations. As shown in Table 1.2, multiple signal transduction molecules have been listed. However, very few of them are currently accepted in clinical practice, or even close to being translated into the clinical usage.

With the development of multiple small molecules that inhibit signaling pathways in melanoma treatment, biomarkers that can predict responses to the targeted therapies are urgently needed. A very recent study has shown that reduced phosphorylation of ribosomal protein S6 (P-S6) predict cell death induced by BRAF inhibitors or MEK inhibitors, and is associates with improved PFS in patients with relevant mutations (Corcoran et al, 2013). Moreover, P-S6 change can be monitored in real time by serial fine-needle aspiration biopsies, making P-S6 a promising biomarker to guide treatment in *BRAF*-mutant melanoma.

1.5.3 Serum Biomarkers

Lactate dehydrogenase (LDH) has been used as an important serum biomarker, ever since the elevated expression of LDH in the serum of melanoma patients was detected (Hill & Levi, 1954). LDH is an enzyme functioning in the conversion of pyruvate to lactate when oxygen level is low, thus its increased expression is the sign of melanoma cells expanding their blood supply (Balch et al, 2001a). Studies have shown that serum LDH expression is the strongest independent prognostic biomarker in metastatic melanoma, and one associated with liver metastases (Balch et al, 2009). LDH has been included in the AJCC staging system, in which patients with elevated LDH levels and distant metastases are considered stage IV M1c (Balch et al, 2009).

Although S100B is commonly used as a tissue biomarker, multiple studies have found the elevated serum S100B level correlates with more advanced melanoma and worse survival (Abraha et al, 1997; Henze et al, 1997). In a study with 1339 serum samples from 412 melanoma patients, the overall survival time of patients with serum S100B value lower than 0.2μg/ml was significantly longer, as compared to that for patients with elevated S100B level (Hauschild et al, 1999). A meta-analysis involving a total of 3393 melanoma patients across all stages showed that serum S100B negatively correlated with patient survival (Hazard Ratio, 2.23; 95% confidence interval, 1.92-2.58; $P < 0.01$) (Mocellin et al, 2008). Due to the limited sample size and heterogeneity among studies, the potential of S100B as a prognostic biomarker needs further evaluation. Moreover, the elevated serum S100B level has also been found in patients with kidney and liver injury, and inflammatory or infectious diseases (Molina et al, 2002). Some European countries have recommended determination of

serum S100B of patients with thickness > 1mm lesions every 3-6 months, but conflict concerning the prognostic value of S100B still exists (Gogas et al, 2009).

Another important serum biomarker is Melanoma-inhibiting activity (MIA), which is highly expressed in melanoma cells, but not in benign skin lesions (Bosserhoff et al, 1996). MIA is a soluble 11 kDa protein and found to inhibit cell growth. Increased serum MIA level has been associated with aggressive melanoma and poor prognosis (Bosserhoff et al, 1997; Meral et al, 2001). MIA can be also used to monitor melanoma treatment, since MIA serum expression is found to be correlated with response to therapy (Bosserhoff & Buettner, 2002). However, a study comparing MIA, S100B, LDH in 373 melanoma patients, showed that MIA was less sensitive than S100B, and less specific than both LDH and S100B (Krahn et al, 2001).

Other serum biomarkers have been investigated to predict clinical response and monitor disease progression, including Tumor-associated antigen 90 immune complex (TA90IC), YKL-40, vascular endothelial growth factor (VEGF), Tyrosinase, 5-S-cysteinyldopa, and L-DOPA/ L-tyrosinase (Gogas et al, 2009). However, none of them has been found superior as biomarkers to S100B or already established LDH.

1.5.4 Genetic Biomarkers

The advances in large-scale genomic screening and sequencing study of melanoma have revealed the heterogeneity of this disease, indicating that melanomas can be further sub-grouped, according to oncogenic mutation patterns and gene expression profiles. This subgrouping would allow for the designing of individualized therapy based on the prediction of the response to targeted therapy.

With the successful development of BRAF V600E inhibitors, *BRAF* mutations have become the most important biomarkers of melanoma. *BRAF* mutations are detected in about half of all melanomas, and approximately 80% of these mutations are V600E substitutions (Davies et al, 2002); whereas V600K and V600D mutations accounts for 16% and 3%, respectively (Long et al, 2011). It has been reported that V600E mutations are more frequently occurring in younger patients in sites with moderate sun exposure, but V600K mutations are associated with higher amounts of cumulative sun damage (Curtin et al, 2005; Menzies et al, 2012). A highly specific real-time PCR Cobas (Roche Molecular Diagnostics) 4800 assay has been approved by the FDA for detection of *BRAF* V600E mutations. It should be noted that melanomas with negative results using this assay should be screened for V600K mutations, since little cross-reactivity for non-V600E profiles can be detected (Halait et al, 2012). Meanwhile, the V600E-specific VE1 antibody can be used to detect this signature biomarker as well, with 97% sensitivity and 98% specificity (Long et al, 2013). Although limitations have been shown in the BRAF inhibitor treatment, it is a promising therapeutic approach. In combination with the MEK inhibitor, it has already shown improved PFS from 5.8 to 9.4 months (Flaherty et al, 2012a). Therefore, we can foresee *BRAF* mutation screening becoming routine for advanced melanoma patients in near future. In addition, other mutations that activate MAPK pathways have been found in 20% of cutaneous melanomas (in *NRAS*) and in 83% of uveal melanomas (in *GNAQ* or *GNA11*) (Platz et al, 2008; Van Raamsdonk et al, 2009).

Another important genetic change that predicts response to therapy is the *KIT* mutation. A small subgroup of melanomas (approximately 2 - 10%) harbor activating *KIT* mutations. The *KIT* mutations preferentially occur in those patients with acral and mucosal

melanomaariseing from skin with chronic sun damage (Curtin et al, 2006). Clinical studies have shown that melanomas with one of the two most common activating mutations L576P and K642E are sensitive to KIT inhibitor imatinib (Carvajal et al, 2011). Moreover, the same study indicated that the presence of an activating mutation in *KIT* is predictive of response, rather than the KIT receptor expression. *KIT* mutation may affect multiple signaling pathways, and, therefore requires further work to identify the exact role of diverse *KIT* mutation in melanoma.

A rapidly enlarging number of mutated genetic biomarkers have emerged in recent years, including *PPP6C*, *STK19*, *FBW7*, *RAC1*, *PREX2* and *TRPAP* (Berger et al, 2012; Hodis et al, 2012; Krauthammer et al, 2012; Wei et al, 2011). Additionally, a series of chromatin remodeling proteins have been shown altered in melanoma, such as ARID1A, ARID1B, ARID2, and SMARCA4 that in SWI/SNF family (Hodis et al, 2012), and EZH2, JARID1B and BMI-1 that belong to polycomb protein family (Bachmann et al, 2008; Fan et al, 2011; Roesch et al, 2010). Since these mutations account for relatively small population in melanoma patients and their functional roles are largely unclear, additional studies are required.

1.5.5 Immunological Biomarkers

Recent clinical development of two anti-CTLA-4 antibodies, tremelimumab and ipilimumab has led to intensive studies on predictive and prognostic immunological biomarkers. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is an inhibitory T cell receptor that is up-regulated by T cell activation. Specific antibodies bind with CTLA-4, and release the “brake” of T cells, resulting in enhanced immune response. Ipilimumab has been approved by FDA after the clinical trial showing improved overall survival of stage III or IV

melanoma patients (6.4 months vs 10.0 months) (Hodi et al, 2010). Further immunohistochemical studies of tumor biopsies from stage IV melanoma patients treated with ipilimumab demonstrated that patients with high levels of (indoleamine 2,3-dioxygenase) IDO and FoxP3 showed higher rate of response (Hamid et al, 2011). Interestingly, both of IDO and FoxP3 are immune suppressors. The ability of these two biomarkers to predict response for immune-modulatory therapy needs further validation.

Similar to ipilimumab, antibody blocking the programmed cell death 1 (PD1) protein has shown encouraging results in recent clinical trials (Topalian et al, 2012). Besides CTLA-4, PD1 is another inhibitory receptor expressed by T cells suppressing T-cell activity upon binding with its ligand, B7-H1 (also known as PD-L1). The same clinical study demonstrated that B7-H1 expression on tumor cells is associated with response, and follow-up investigations on PD1 and B7-H1 in melanoma are ongoing.

Some other cell surface molecules related to T cell function have shown promise to predict response to immunotherapy, such as CD200, B7-H3, B7-H4, galectin 3 and 9, and so on (Sznol, 2011). A comprehensive study on the correlation between expression of these molecules and clinical data may give more insights into these candidate biomarkers.

1.5.6 Current Challenges and Barriers for Translating Biomarker Studies into Clinic Usage

So far, apart from mutational signatures, such as BRAF and RAS mutations, there is no reliable single expression biomarker or multi-biomarker signature that is highly sensitive and specific, or has been widely used in routine clinical practice. The reasons for the lack of significant clinical translation are multiple. One likely major reason is the lack of independent verification by diverse centers, making it difficult for the clinicians to assess the

relative merits or technical feasibility of individual markers. Biomarker expression pattern can be varied in populations with distinct characteristics, such as age, race and sun exposure intensity. Therefore, one biomarker discovered and validated on one population may not maintain the prognostic value in another population. However, for practical reasons (cost, labor load, connection), smaller and heterogeneous patient sets are often employedd and many candidate biomarkers will not be validated.

For melanoma, a unique obstacle in biomarker study is the limited tissue availability. The primary melanoma is commonly a few millimeters in size, and after diagnosis only very little or no residual tissue is left for further examination, whereas high-throughput genomic screening often requires relatively large amount of fresh tissue. In this regard, proteomics appears to be an ideal choice for the biomarker study in melanoma, especially with the development of tissue microarray technology. But there are several disadvantages in TMA as well. Firstly, the small core of tissue used in construction of TMA may not be able to represent the whole section, due to the high heterogeneity of melanoma (Quintana et al, 2010). Secondly, the formalin-fixed paraffin-embedded (FFPE) tissues are refractory to proteomic examination, because of the high level of covalently-linked proteins arising from formalin fixation (Rezaul et al, 2008). Thirdly, the results from TMA sections can be greatly affected by stroma, necrotic tissue, serum proteins and blood cells within the sample. For this reason, S-100 staining TMAs in parallel can be useful to reduce the interference by other types of cells.

Moreover, the heterogeneity in the experiment design and procedures also influences directions, statistical significance and thus affects the consistency of studies. In IHC-based tissue biomarker studies, these factors include selection of the primary antibody, final

dilutions of the antibody, appropriate positive and negative controls, intra-observer discrepancies in evaluating the staining pattern, selection of scoring system, assignment of the studied populations, and so on. Standardized experiment design, procedures and statistical analysis should be applied in future studies.

1.6 Objective

Currently most types of cancer still cannot be completely cured. Early diagnosis and surgical resection of tumors is the key and most effective option of treatment. However, a considerable number of patients die because of tumor recurrence or metastasis after surgery. Thus accurate identification of the high-risk patient population and early therapeutic intervention are of essential scientific and clinical value for all cancer patients. In order to refine the risk of tumor recurrence or metastasis, specific biomarkers that correlate with tumor biological behavior and patient prognosis are needed. Wwhile these biomarkers often enhance the malignant transformation and tumor cell development, suppression of the activity of these biomarkers is an efficient strategy of developing targeted cancer drugs.

The quest for a **new generation of cancer biomarkers and therapeutic targets based on human melanoma models is the purpose of this thesis**. We collected 707 biopsies from melanoma patients across all stages, and constructed a big tissue microarray (TMA), as well as the related follow-up database. Working from this robust technological platform, we investigated the expression profile and prognostic significance of more than ten biomarkers, including Fbw7, p27, BRAF, KAI1, Dicer, Runx3, Tip60, and MMP2. For a particular biomarker, like Fbw7, we observed the impact on melanoma cell migration by overexpression and knockdown Fbw7 in melanoma cell line. By so doing, **the molecular mechanisms underlying Fbw7-mediated cell migration were investigated**. Based on the

identified biomarkers in melanoma, we also **investigated the potential value of multiple-biomarker signatures in certain subgroups of melanoma patients**, so as to identify the best markers or marker combinations are identified topermit more sensitive or robust algorithms and parameters to be put forward for clinicians to use as guides for future patient management.

Chapter 2: Material and Methods

2.1 Cell Lines and Cell Culture

Melanoma cell lines MMRU, MMAN, MMLH, PMWK, RPEP and RPM-MC were kindly provided by Dr. H. R. Byers (Boston University School of Medicine, Boston, MA, USA). Among them, PMWK is a primary cutaneous melanoma cell line; RPEP and RPM-MC are both recurrent primary melanoma cell lines; MMRU, MMAN, MMLH are metastatic melanoma cell lines. The MEWO, Sk-mel-3, Sk-mel-110 cell lines were kind gifts from Dr. A. P. Albino (Memorial Sloan-Kettering Cancer center, New York, USA.). All the melanoma cell lines were cultured in Dulbecco's Modified Eagle Media (DMEM) (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS. Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) in 5% CO_2 humidified atmosphere at 37^{o}C. Immortalized melanocytes (a gift from Dr. Meenhard Herlyn (The Wistar Institute, PA, USA)) were cultured in melanocyte growth media supplemented with 5 μg/ml bovine pituitary extract, 1 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10 ng/ml phorbol myristate acetate and 4% FBS (PromoCell, Heidelberg, Germany).

2.2 Antibodies

Mouse anti-Fbw7 (ab74054) was purchased from Abcam (Cambridge, MA, USA); Anti-actin and rabbit anti-Flag antibodies from Sigma-Aldrich (St. Louis, MO, USA); mouse monoclonal anti-NQO1, Cul1, Skp2, p27 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit polyclonal anti-Tip60 antibody from Millipore (Billerica, MA, USA); rabbit anti-phospho-p44/42 (Erk1/2), rabbit anti-p44/p42 MAPK (Erk1/2), rabbit anti-p38 MAPK antibody, rabbit anti-phospho-Akt (Thr308), rabbit anti-Akt (C67E7) were

purchased from Cell Signaling (Beverly, MA, USA); and mouse anti-Flag from Applied Biological Materials Inc. (Richmond, BC, Canada).

2.3 Expression Plasmids, siRNA and Transfection

The pFLAG-Fbw7-alpha, -beta, -gamma plasmids were kind gifts from Dr. Bruce E. Clurman from Fred Hutchinson Cancer Research Center, Seattle, USA (Grim et al, 2008) and Dr. Ceshi Chen from Albany Medical College, New York, USA (Zhao et al, 2010). The p3xFLAG-Myc-CMV-24 vector plasmid was purchased from Sigma-Aldrich (St. Louis, MO, USA). HA-tagged pcDNA-Ubiquitin plasmid was a kind gift of Dr. R. Zhang from the University of Alabama at Birmingham (Zhang & Zhang, 2008). Expression plasmids were transfected into melanoma cells by Effectene Transfection Reagent (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instruction.

Cells were grown to 50% confluence before transfection of small interfering (si)RNAs. For siRNA transfection, cells were incubated with either non-specific control siRNA, Fbw7α siRNA (siFbw7α, Qiagen SI03089240), or second Fbw7α specific siRNA (siFbw7α', Abnova H00055294-R01) using Silenfect transfection reagent (Bio-Rad, Mississauga, Ontario, Canada). Assays were performed 48 h after transfection.

2.4 Immunohistochemistry

TMA slides were de-waxed at 55°C for 30 min followed by three 5-minite washes in xylene. Tissues were rehydrated by a series of washes in 100%, 95% and 80% ethanol and distilled water. Antigen retrieval was performed by heating the tissues at 95°C for 30 minutes in 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 20 min. Nonspecific antigens were blocked by universal blocking serum (DAKO Diagnostics, Mississauga, Ontario, Canada) for 30 minutes. The TMA slides

were incubated overnight with specific antibodies at 4°C. Next day, the slides were incubated with biotin-labeled secondary antibody and streptavidin-peroxidase (DAKO Diagnostics, Mississauga, Ontario, Canada) for 30 minutes each at room temperature with light protection. The samples were developed using 3,3'-diaminobenzidine substrate (DAB, Vector Laboratories, Burlington, Ontario, Canada) and counterstained with hematoxylin. The slides were finally dehydrated and sealed with coverslips. Negative controls were performed by omitting the primary antibody for overnight incubation.

2.5 Evaluation of TMA Immunostaining

The evaluation of each biomarker staining was blindly and independently examined by three observers, including one dermatopathologist, and a consensus score was reached for each one. Immunostaining of specific protein is defined as either cytoplasmic or nuclear, or whole staining, and graded according to both intensity and percentage of cells with positive staining. The staining intensity was scored as 0, 1+, 2+ and 3+; the percentage of stain-positive cells was also scored into 4 categories: 1 (0-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). In the cases with a discrepancy between duplicated cores, the higher score from the two tissue cores was taken as the final score. The expression levels of a biomarker was evaluated by immunoreactive score (IRS) (Remmele & Stegner, 1987), calculated by multiplying the scores of staining intensity and the percentage of positive cells. χ^2 tests were applied to evaluate the statistical differences in demographic and clinical parameters, as well as in the expression levels of the biomarker. Survival time of each patient was calculated from the date of melanoma diagnosis to the death date. The correlation between protein biomarker expression and patient survival was examined by Kaplan-Meier survival curve and log-rank test. Adjusted hazard ratios and their 95% confidential intervals (CIs) were

estimated by multivariate Cox proportional hazards regression models. We used SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) software for all analyses.

2.6 Protein Extraction and Western Blot

Cultured cells were washed with cold PBS, harvested by scraping on ice and pelleted by centrifugation at 3,000 g for 5 minutes. For whole cell lysate, cell pellets were lysed in 80-100 µl modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 150mM NaCl, 1 mM EDTA, 0.1% NP-40, 0.25% sodium dodecyl sulfate) containing fresh added protease inhibitors (100 µg/ml phenylmethylsulfonyl fluoride, 1µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A). The samples were then sonicated, incubated on ice for 20 minutes, and centrifuged at 12,000 × g for 20 minutes at 4˚C before the supernatants were collected. For sub -cellular fractionation, cytoplasmic and nucleoplasmic proteins were isolated by Cytoskeletal Buffer (CSK) (10 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, and 0.5% NP-40) with protease inhibitors for 5 minutes on ice. After centrifugation at 1000 × g for 14 minutes at 4˚C, the supernatants were collected as cytoplasmic lysates, and nuclei were pelleted. The nuclei pellet was then re-suspended in modified RIPA buffer, sonicated and incubated on ice for 20 minutes. Samples were centrifuged at 12,000 g for 20 minutes at 4˚C and the supernatants were collected as nuclear lysate.

Protein concentrations were determined by performing Bradford assay (Bio-Rad, Mississauga, ON, Canada), according to manufacturer's instructions. Proteins (50 µg/lane) were separated on 8, 10, or 12% SDS-polyacrylamide gels (SDS-PAGE) and blotted onto polyvinylide difluoride (PVDF) membranes (Bio-Rad). The PVDF membranes were blocked with 5% Bovine serum albumin (BSA) in TBST (TBS containing 0.05% Tween-20) for 1 hour at room temperature before being incubated with primary antibodies prepared in 5%

BSA in TBST for 1 hour at room temperature. Blots were washed in TBST three times, for 5 minutes each and incubated with secondary antibodies labelled with the near-infrared fluorescent dyes IRDye 800 or IRDye 680 (LI-COR Biosciences, Lincoln, NE, USA) at room temperature for 1 hour before washing in TBST three times, for 5 minutes each. The blots were scanned on the Odyssey Infrared Imaging System to visualize proteins (LI-COR Biosciences) equipped with Odyssey 2.1 software. The fold change of protein expression was corrected by the differences of β-actin loading control.

2.7 Reverse Transcription and Real-time Quantitative Polymerase Chain Reaction

Total RNA of melanoma cells and normal human melanocytes was extracted by Qiazol reagent (Qiagen) according to manufacturer's protocol. RNA concentrations were measured with a spectrometer at 260 nm. 2 μg of total RNA was reverse-transcribed into cDNA with the SuperScript First-Strand Synthesis System (Invitrogen) or Transcriptor cDNA Synthesis System (Roche, Indianapolis, IN, USA) according to the manufacturer's protocols. qPCR was performed with SYBR Green Master mix system (Applied Biosystem, Carlsbad, CA, USA) or SYBR Green Master mix system (Roche, Indianapolis, IN, USA). A model of 7900HT qPCR system thermal cycler (Applied Biosystems) was used according to the manufacturer's instructions. Expression of human glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. All primer sequences that were used in this study are listed in Table 2.1.

Table 2.1 Sequences of all the primers used in this study

	Forward	Reverse
Fbw7	5'-CCACTGGGCTTGTACCATGTT-3'	5'-CAGATGTAATTCGGCGTCGTT-3'
Fbw7α	5'-TAACCCTTCCTCAAGCCA-3'	5'-GGAGTCCTCATCTACCGAAA-3'
Fbw7β	5'-GCATTTGCCTTTACTGTGG-3'	5'-TGTGCCGTAGAAACCCAT-3'
Fbw7γ	5'-TGGCTTGGTTCCTGTTGA-3'	5'-GGGCAATGATGCTAATGC-3'

2.8 Sulphorhodamine B (SRB) Assay

Cells transfected with siControl and siFbw7α were seeded into 24-well plates. At various time points after transfection, the cells were fixed with 10% trichloroacetic acid for at least 1 h at 4°C. The plates were stained with 0.057% sulphorhodamine B for 20 minutes at room temperature followed by washing in 1% acetic acid four times. The dye was dissolved in 10 mM Tris base (pH 10.5) and cellular density was measured by optical density at 490 nm.

2.9 Wound Healing Assay

Twenty-four hours after transfection, cells were washed with PBS and a standard 20 µl pipette tip was used to draw across the well to produce a wound at the centre of each well. The monolayers were then washed three times with PBS to remove floating cells and incubated in fresh complete medium for another 24 hours. Photographs of the wounds were taken from the same position at 0 and 24 hour time points. The starting lines based on the scratch at 0 hour and the numbers of migrating cells across these lines were counted to quantify the rate of cell migration.

2.10 Transwell Migration Assay

Cells were seeded in six-well plates the day before transfection to reach about 40% confluency. The cells were transfected with siControl and siFbw7α, and 5×10^{4} cells in 100 µl serum-free medium were reseeded into Permeable Support transwell (Costar, WA, USA) with 8.0 µm polycarbonate membrane in 24-well plates. 600 µl complete medium was added to each well and the cells were incubated in Transwell plate at 37°C for 4 hours to allow the cells to migrate towards the underside of the insert filter. The cells remaining on the upper side of the filter membrane were removed with a cotton swab. The cells on the lower side of the filter were fixed with 4% formaldehyde for 20 minutes at room temperature, and stained

with 600 µl of 1 µg/ml DAPI for 5 minutes. The cells were washed with water and photos were taken using an inverted fluorescence microscope at 200× magnification.

2.11 Immunofluorescent Staining

Cells were seeded on coverslips in six-well plates in complete medium, and forty-eight hours after plasmid or siRNA transfection, cells were washed with PBS and new serum-free medium applied for overnight. In the case of MEK inhibitor PD98059 (80 µM) treatment, the inhibitor was added to the serum free medium overnight. Then cells were collected after stimulation with complete medium and PD98059 for 30 minutes. Cells were fixed with 2ml of fixation solution (2% paraformaldehyde and 0.5% Triton X-100 in PBS) for 30 minutes at 4 ˚C. The coverslips were washed with PBS and then blocked in 1% BSA for 1 hour at room temperature. The cells on coverslips were stained with phalloidin-rhodamine (1 unit) for 30 minutes, followed by incubation with rabbit anti-Fbw7 antibody for 2 hours and Cy2-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) at a dilution of 1:2000 for 1 hour. DNA was stained Hoechst 33258 (20mM) for 5 minutes, and the cells were washed three times and mounted with Permount mounting medium (Fischer Scientific). Pictures were taken with a cooled mono 12-bit Retiga-Ex camera equipped with Northern Eclipse Imaging software. Intensity of F-actin staining were analyzed using ImageJ software (NIH, Bethesda, MD).

2.12 RhoA Pull-down Assay

MMRU cells were seeded in six-well plate to reach 40-50% confluency. 24 hours after transfection, cells were serum starved for 24 hours and then stimulated with complete medium for 30 minutes. The cells were lysed and cell lysate was incubated with rhotekin-RBD beads (Cytoskeleton, Inc. Denver, USA) at 4˚C for 1 hour. The beads were pelleted by

centrifugation at 5000 × g for 1 minute. The beads were washed and boiled with 2× loading buffer and subjected to western blot. 10% of protein lysate was used to examine the total RhoA expression.

2.13 Statistical Analysis for *in Vitro* Studies

The data were presented as the mean ± SD. Statistical analyses were performed using student-*t* test and p value < 0.05 was considered significant. The optimal cut-off points of TMA score were calculated using the MedCalc software, version 12.5 (MedCalc Software, Ostend, Belgium). Univariate and multivariate Cox proportional hazards regression models were performed to estimate the hazard ratios (HRs) and their 95% confidence intervals (CI) for the confirmation analysis in validation patient sets. All statistical analyses were carried out using the SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA).

Chapter 3: Establishing Melanoma Tissue Microarray and Comprehensive Clinical Database for Multiple Biomarker Studies

3.1 Background and Rationale

Tissue microarray (TMA) is a high throughput technology enabling analysis of hundreds of tissue samples on a single microscope slide. Since this methodology was developed in 1998 by Kononen et al, it has been commonly implemented in screening of biomarkers, especially in the field of oncology (Kononen et al, 1998). Traditionally, stained histologic sections from tissues affected by tumor or other diseases would be examined by a pathologist. Given the magnitude of the workload and limited human capability, this process is time-consuming, tedious and inaccurate. By contrast, TMAs provide high throughput and uniform reaction conditions, making the tissue biomarker study far more efficient and precise. This technology allows a needle to lift tissue from a formalin-fixed paraffin-embedded (FFPE) sample and place it into a predrilled hole in the prepared recipient paraffin block. Distinct tissue samples from different tissue blocks are present as cores (0.6 to 2 mm diameter), arrayed in a grid. A single slide usually contains 40 to 800 samples, depending on the core size (Rimm et al, 2001).

Immunohistochemistry (IHC) technology is used in most TMA-based biomarker study, although other methods have also been developed, such as fluorescent in situ hybridization. IHC is based on binding of antibody to its target epitope or antigen in tissue. By using a secondary reagent (either immunoperoxidase or alkaline phophatase), the stained immunohistochemical reaction makes the binding of the antibody to the antigen visible. The diaminobenzidine (DAB) generating a brown product, and amino-9-ethylcarbozole (AEC)

that giving a red reaction, are the two most commonly used reagents for visualization in IHC (Ferringer, 2012).

Combined TMA and immunohistochemistry technology represent a useful tool in discovering and validating biomarkers. Moreover, TMA and cell line combination have attractive value in identifying the role of biomarkers in certain types of cancer and validating antibodies for IHC. Therefore, to investigate the potential diagnostic and prognostic biomarkers in human cutaneous melanoma, and to study the functions of the biomarkers of interests, we established the TMA using 707 patients with different stages of melanocytic lesions and a corresponding database for future biomarker study.

3.2 TMA and Clinical Database Construction

3.2.1 Procedures for Melanoma TMA Construction

The procedures for constructing a our TMA are described here in brief. First of all, archived paraffin blocks and corresponding histological sections from potentially hundreds to thousands of patients were collected. Routine hematoxylin and eosin (H & E) stained sections of tissue from patients were examined, and areas of tumor or control normal melanocytes were cycled by a pathologist. Secondly, according to pre-designed number of samples in each stage of melanoma for TMA construction, selected patients were sorted and grouped. Corresponding clinicopathological reports were also collected for the followed database construction. Thirdly, all the patients were mixed and assigned to different blocks (in our case, we have five blocks) with one unique case number given to each individual. A small array including 40 cases of different stages of melanoma was also constructed for antibody dilution testing. Finally, all the tissue blocks were subjected to semi-automated machine (Beecher Instruments, Silver Spring, MD) for TMA construction. Taking into

account the limitation of the representative areas of the tumor, we used duplicate 0.6-mm-thick tissue cores from each donor block (Figure 3.1). Multiple 4-µm sections were cut with a Leica microtome (Leica Microsystems Inc, Bannockburn, IL) and then transferred to adhesive-coated slides using routine histology procedures. Thirty TMA sections were cut each time to avoid section loss caused by microtome. The cut sections were placed in a holder and stored in a -80 freezer to reduce the tissue oxidation and antigen loss.

Figure 3.1 Constructed 5-slides TMA and testing small array.

3.2.2 Source of Biopsies

All the biopsies we used were provided by the derma-pathologist Dr. Magdalena, Martinka, from Department of Pathology, Vancouver General Hospital (VGH), BC, Canada. The use of human skin tissues in this study was approved by the Clinical Ethics Board of the University of British Columbia and was performed in accordance with the Declaration of Helsinki guidelines. Together with Dr. Martinka, we obtained the biopsies from VGH archives from 1995 to 2009; reviewed and marked the selected samples having clear diagnosis and intact tissue. Considering limited time and labor, we originally designed to have 50 normal nevi, 100 dysplastic nevi, 100 melanomas in situ, 300 invasive primary melanomas and 150 metastatic melanomas. Eventually we collected 707 samples and stored them in room temperature before subjecting them to TMA construction (Table 3.1).

Since mucosal melanomas, uveal melanomas and ocular melanomas display distinct clinical courses and molecular phenotypes, and account for a relatively small proportion of melanoma patients, we selected only more prevalent cutaneous melanomas. We also excluded non-Caucasian patients due to the different genomics, as well as the different protein expression pattern. The information of patient survival and treatment received were retrieved from British Columbia Cancer Agency, and were updated in September, 2013.

Table 3.1 Summary of constructed TMA

Category	No	%
Normal Nevi	48	6.8
Dysplastic Nevi	100	14.1
Melanoma in situ	90	12.7
Primary invasive melanoma	310	43.8
Metastatic melanoma	159	22.5
With 5-y follow up	520	73.6
With 10-y follow up	347	49.1
Total	707	

3.2.3 Clinicopathological Characteristics of Patients

We collected the clinicopathological information and follow up survival data of each selected patient, and established a comprehensive clinical database. The clinicopathological parameters included age, sex, thickness, Clark's level, AJCC stage, subtype, location, mitotic rate, ulceration, lympho-vascular invasion, lymphocyte response, regression, histological satelitosis, diagnose date, collection date, survival status, death date, death cause and time of follow (Table 3.2, 3.3, 3.4). The clinical database was established by myself and double checked by two other researchers, Drs. G Chen, and Z Zhang.

Table 3.2 Clinicopathologic characteristics of all melanoma patients in constructed TMA

Variable	No. (%)	Sub-variable	No. (%)
Age-yr			
Median (SD)	59.7 (16.7)		
Range	7-95		
≤ 30	24 (4.4)		
30-60	252 (45.0)		
≥ 60	283 (50.6)		
Sex			
Female	233 (41.7)		
Male	326 (58.3)		
AJCC			
0	90 (16.1)		
I	176 (31.5)	IA	95 (17)
		IB	79 (14.1)
II	134 (24.0)	IIA	46 (8.2)
		IIB	56 (10.0)
		IIC	32 (5.7)
III	109 (19.5)	IIIA	8 (1.4)
		IIIB	33 (5.9)
		IIIC	67 (12.0)
IV	50 (8.9)		

Table 3.3 Clinicopathologic characteristics of primary melanomas in constructed TMA

Variable	No. (%)
Age-yr	
Median (SD)	60.8 (17.17)
Range	7-95
≤ 30	17 (4.2)
30-60	180 (45.0)
≥ 60	203 (50.8)
Sex	
Female	182 (45.5)
male	218 (54.5)
Tumor thickness (mm)	
T1 (< 1.00)	194 (48.5)
T2 (1.01 - 2.00)	81 (20.2)
T3 (2.01 - 4.00)	58 (14.5)
T4 (> 4.00)	66 (16.5)
Unspecified	1 (0.2)
Ulceration	
Present	67 (16.8)
Absent	333 (83.2)
Mitotic rate (no./ mm^2)	
0	239 (59.8)
< 1.00	57 (14.2)
1.00 – 10.00	78 (19.5)
> 10.00	26 (6.5)
Location	
Head and neck	109 (27.2)
Trunk	151 (37.8)
Extremity	140 (35.0)
Subtype	
Superficial spreading	160 (40.0)
Nodular	49 (12.2)
Lentigo maligna	98 (24.5)
Others	30 (7.5)
Unspecified	63 (15.8)
Lymphocyte response	
Non-brisk	151 (37.8)
Brisk	36 (9.0)
Unspecified	213 (53.2)
Histological satelitosis	
Positive	10 (2.5)
Negative	300 (75.0)
Unspecified	90 (22.5)
Regression	
Positive	32 (8.0)
Negative	368 (92.0)
Total	400 (100)

Table 3.4 Clinicopathologic characteristics of metastatic melanomas in constructed TMA

Variable	No. (%)
Age-yr	
Median (SD)	57.2 (15.1)
Range	23-94
≤ 30	7 (4.4)
30-60	90 (56.6)
≥ 60	62 (39.0)
Sex	
Female	51 (32.1)
male	108 (67.9)
Stage at diagnosis	
Regional spread	65 (40.9)
Distant metastasis	88 (55.3)
Unspecified	6 (3.8)
Metastatic Nodes	
N1	28 (17.6)
N2	14 (8.8)
N3	67 (42.1)
Metastatic site	
M1a	10 (6.2)
M1b	7 (4.4)
M1c	33 (20.8)
Total	159 (100)

3.2.4 Multiple Biomarker Studies

Based on the freshly constructed TMA, I have cooperated with several lab colleagues to investigate biomarkers of interest in human cutaneous melanoma, including Skp2, Runx3, p27, Tip60, JWA, p300 and KAI1 (Table 3.5). Most of the results have been published in cancer related journals (Appendix A) (Chen et al, 2012; Chen et al, 2011a; Chen et al, 2011b; Lu et al, 2013; Rotte et al, 2013; Zhang et al, 2011). The prognostic values of identified

biomarkers require further independent validation studies, so that these data can be translated from bench to clinical settings.

Table 3.5 Multiple biomarkers identified by the constructed TMA

Marker	Sample number	Antibody	Expression	Prognosis
Skp2	527 (436 melanomas and 91 nevi)	Mouse monoclonal (Santa Cruz, US)	Cytoplasmic Skp2 was increased in melanomas, and correlated with AJCC stage, tumor thickness and ulceration.	Cytoplasmic Skp2 was associated with worse DSS of PM, but not MM
RUNX3	528 (440 melanomas and 88 nevi)	Mouse monoclonal (Medical and Biological Lab, Japan)	Nuclear RUNX3 expression was lost in melanomas, compared with dysplastic nevi	Loss of RUNX3 was associated with worse DDS in both PM and MM
P27	499 (428 melanomas and 91 nevi)	Mouse monoclonal (Santa Cruz, US)	Nuclear p27 was reduced in melanoma, whereas cytoplasmic p27 was increased	Cytoplasmic p27 was associated with worse OS and DS in MM
Tip60	553 (448 melanomas and 105 nevi)	Rabbit polyclonal (Millipore, US)	Tip60 expression was reduced in both PM and MM	Reduced Tip60 expression was associated with worse DS in both PM and MM
JWA	505 (383 melanomas and 122 nevi)	Mouse monoclonal (Abmax, China)	JWA expression was reduced in both PM and MM	Reduced JWA expression was associated with worse DS and OS
p300	474 (392 melanomas and 82 nevi)	Mouse monoclonal (Millipore, US)	Nuclear p300 was lost, whereas cytoplasmic compartment was increased, and correlated with AJCC stage II and III	Nuclear p300 loss was associated with worse OS and DS

PM, primary melanoma; MM, metastatic melanoma; DS, disease-specific survival; OS, overall survival.

3.3 Discussion

Biomarker development can be divided into five phases: candidate biomarker discovery, specific antibody validation, investigation in training retrospective cohort, validation of the results in independent cohort, and finally, further confirmation by randomized, prospective clinical trials (Pepe, 2005). TMA technology can serve as a useful tool, and dramatically speed the process of each of these phases. For example, biomarker screening using TMAs

can triage hundreds or thousands of potential candidates, and only a few likely candidates need be studied in detail. TMAs provide many benefits. First of all, the workflow is far more efficient with TMAs, than that of conventional histological evaluation of tissue samples. By arraying hundreds of tissue samples on a single slide, TMAs allow minimized effort and time for a pathologist to read the slides. After the up-front effort of constructing the TMAs for the first time, each subsequent analysis using the TMA sections takes only a few days, compared with a couple of months previously. Secondly, TMAs save the tissue and minimize the damage to donor block by taking a small core of it from blocks. So that the blocks can beused repeatly after contributing to the TMA. Thirdly, TMAs save the reagents, and provide a uniform condition for the staining procedures. This consistency and uniformity are significant improvements over traditional methods where batch to batch variability often influenced the staining. Finally, because of the efficiency in processing hundreds of samples at the same time, TMAs dramatically increase the number of tumors that can be analyzed in a cohort study. And a larger population base increases the significance and reliability of the biomarkers studies

However, limitations of this technology do exist. As mentioned before, a major shortcoming of TMA technology is related with the small size of the tissue cores used for TMA construction. Due to the heterogeneity of tumor, the small TMA cores may not accurately reflect the staining pattern observed from the whole tissue sections. Usually two 0.6 mm cores are used for one biopsy in construction of TMA, and this size is believed to adequately represent the staining for the whole tissue (Torhorst et al, 2001). Larger core size or numbers may have to be considered as an improvement for the evaluation of very heterogeneous biomarkers, such as markers of hypoxia (Iakovlev et al, 2007). Several

technical difficulties also contribute to limitations of TMA, such as tissue core loss and sampling error during tissue extraction and the storage process. The problem of tissue loss is of greater concern when analyzing early stage skin lesions, such as dysplastic nevi or melanomas in situ. These lesions are usually small and admixed with normal stroma or other type of cells. Although the initial tissue blocks have been examined by a pathologist, there is no guarantee that sections cut from deeper levels of a TMA block might still contain tumor. In our case, we found up to 200 tissue core loss after the first 20 sections cut.

Because of the nature of IHC staining, antibody specificity is essential for getting trustworthy data from TMA study. IHC staining and true antigen expressions are not necessarily synonymous. Multiple factors may result in false positive immunostaining without true expression, including inappropriate high antibody concentration or excess antigen retrieval (Ferringer, 2012). Therefore, independent and internal control should be appropriately adjusted, and several factors should be considered in TMA/IHC experiments, including antibody source, antigen retrieval methods, antibody concentration testing and so on. In addition, staining of melanin, collagen, keratinocytes, penetrated lymphocytes, or some other cells may also be disturbing to scoring and analyzing results. A system called Automated Quantitative Analysis (AQUA) has been developed by Dr. Rimm and his group to distinguish tumor between stromal elements using multicolor immunofluorescent histochemistry (McCabe et al, 2005). Some other systems using feature extraction based methods have been used for the same purpose. In our case, we have performed H & E and S100 staining in parallel with TMA sections to address this issue.

Despite the controversy over the power of identifying and validating biomarkers, TMA has been accepted as a powerful tool in screening tumor tissue biomarkers. Through

the collaborative effort of the pathologist and lab members, we developed a TMA consisting of 707 patients, with 559 melanomas and 148 nevi. This TMA serves as a template for the examination of candidate biomarkers of melanoma and up to 15 biomarkers with prognostic value have been identified and studied, confirming the efficiency and usefulness of TMA.

Chapter 4: Prognostic Significance of Fbw7 Expression in Human Melanoma and Its Role in Cell Migration

4.1 Background and Rationale

Tumor metastasis is the major cause of cancer-related mortality. While melanoma that is diagnosed early can be completely cured by surgical excision, patients with metastatic melanoma are faced with very poor prognosis, with an average survival of 6-8 months (Cummins et al, 2006). Therefore, finding the factors driving melanoma metastasis is crucial for designing novel therapeutic regimens for melanoma. Fbw7 is a member of the F-box protein family, which function as a substrate recognition component in SCF E3 ubiquitin ligase. As a result of alternative splicing of their first exon, *Fbw7* encodes three transcripts (Fbw7α, β and γ), which have distinct subcellular localizations: Fbw7α localizes to the nucleus, while Fbw7β and γ to cytoplasm and nucleolus, respectively (Spruck et al, 2002). Most of currently identified Fbw7 substrates have been shown as oncogenes in multiple types of human cancer, including c-Myc, cyclin E, Notch, c-Jun, mTOR and Mcl-1 (Cheng & Li, 2012). Loss of Fbw7 function leads to chromosomal instability and contributes to oncogenesis (Minella et al, 2002). Moreover, reduced expression or loss-of-function mutations of *Fbw7* have been frequently found in diverse human cancers and the overall mutation rate is about 6% as shown in a comprehensive genetic study on primary tumors (Akhoondi et al, 2007). Recent studies suggest that Fbw7 may have functions in cell migration and metastasis. Gastric cancer patients with lymph node metastasis show significantly reduced Fbw7 protein expression levels compared with normal gastric mucosa patients (Li et al, 2012). However, as a general tumor suppressor in human cancers, the

expression of Fbw7 and its exact function in melanoma cell migration have been unknown previously.

To investigate the role of Fbw7α in melanoma progression, we used tissue microarray (TMA) and immunohistochemistry to examine the expression of Fbw7 protein in different stages of human melanocytic lesions. In-vitro functional and mechanism studies of Fbw7 in melanoma cell lines were also conducted.

4.2 Results

4.2.1 Reduced Nuclear Fbw7 Expression is Correlated with Melanoma Progression

To investigate the expression level of Fbw7 in the biopsies of pigmented lesions, immunohistochemical staining of normal nevi, dysplastic nevi, primary melanoma, and metastatic melanoma were performed on TMA slides (Figure 4.1). The staining was predominantly in the nucleus as the arrows indicate (Figure 4.1c), so only nuclear staining was measured. The specificity of the Fbw7 monoclonal antibody was examined by immunofluorescence and western blotting analysis (Figure 4.2). Notably, Fbw7 staining was significantly reduced in primary melanoma compared to dysplastic nevi ($P = 0.020$, χ^2 test) and further reduced in metastatic melanoma compared to primary melanoma ($P = 0.011$, χ^2 test). The most significant difference of Fbw7 expression was shown between dysplastic nevi and metastatic melanoma ($P = 0.001$, χ^2 test; Figure 4.1e). This result indicates that Fbw7 may affect melanoma metastasis through its regulation of metastasis-related substrates. However, there is no significant difference in Fbw7 staining between normal nevi and dysplastic nevi ($P = 0.670$, χ^2 test, Figure 4.1e).

We also examined the correlation between Fbw7 nuclear staining and the clinicophathologic characteristics. Our data showed significant correlation between nuclear Fbw7 expression

and tumor thickness and AJCC stages (P = 0.029 and 0.013, respectively, Table 4.1). These differences suggest that Fbw7 may play a role in melanoma progression. We did not observe any significant correlation between nuclear Fbw7 staining and sex, tumor ulceration or location. Surprisingly, we found significant correlation between Fbw7 staining in different age groups and tumor subtypes (P = 0.038 and 0.049, respectively, χ^2 test, Table 4.1). Fbw7 expression is higher in younger groups and in superficial spreading melanomas; however, the underlying mechanisms remain unknown.

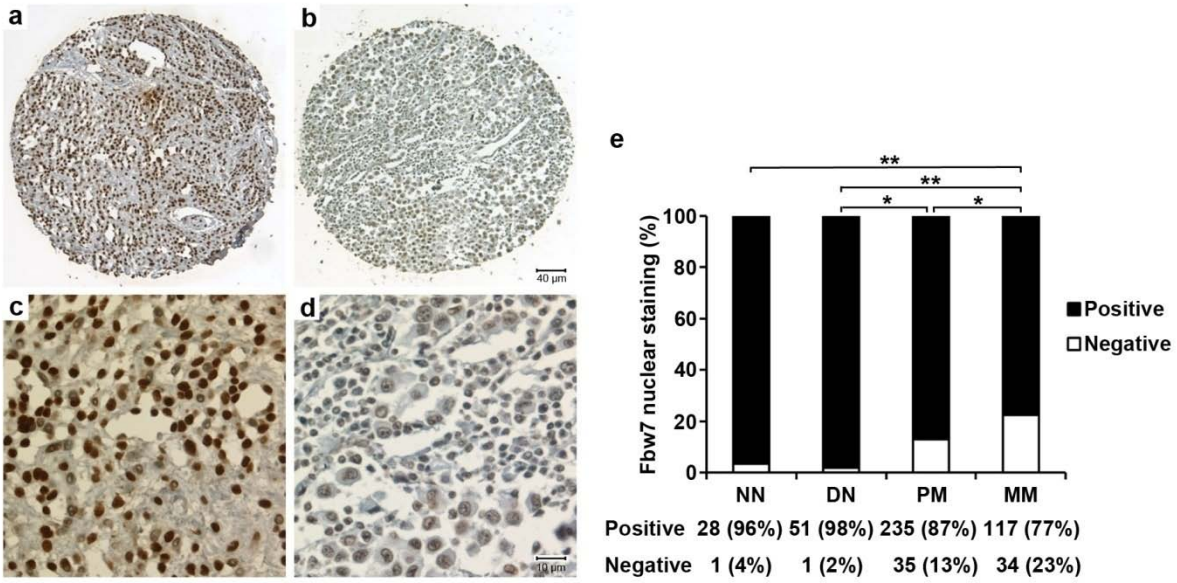

Figure 4.1 Fbw7 expression is reduced in human advanced melanoma.

(**a-d**) Representative images of Fbw7 immunohistochemical staining in human melanoma TMA. (**a,c**) Normal nevus with positive staining. (**b,d**) Metastatic melanoma with negative staining. Bar = 40 μm for **a** and **b**. Bar = 10 μm for **c** and **d**. (**e**) Reduced Fbw7 expression correlates with melanoma progression. NN, normal nevi; DN, dysplastic nevi; PM, primary melanoma; MM, metastatic melanoma.

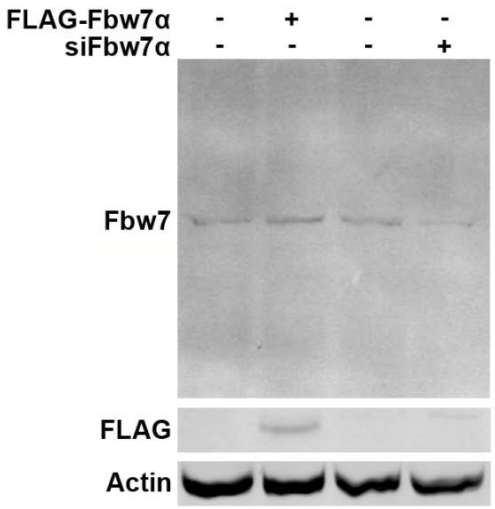

Figure 4.2 Specificity of Fbw7 monoclonal antibody.

FLAG-Fbw7α plasmid or siRNA targeting Fbw7 was transfected into MMRU cell line, and subjected to western blot analysis. Beta-actin expression was used as loading control.

Table 4.1 Fbw7 staining and clinicopathologic characteristics of 420 cases of melanoma

Variables	Fbw7 Staining			
	Negative	Positive	Total	*P* Value[a]
Primary melanoma (n=270)				
Age				
≤ 60	11 (8.5%)	118 (91.5%)	129	0.038
> 60	24 (17.0%)	117 (83.0%)	141	
Gender				
Male	24 (16.6%)	121 (83.4%)	145	0.059
Female	11 (8.8%)	114 (91.2%)	125	
Tumor thickness				
≤ 2.0 mm	14 (9.1%)	140 (90.9%)	154	0.029
> 2.0 mm	21 (18.1%)	95 (81.9%)	116	
Ulceration				
Absent	25 (11.5%)	192 (88.5%)	217	0.153
Present	10 (18.9%)	43 (81.1%)	53	
Tumor subtype				
Superficial spreading melanoma	7 (7.2%)	90 (92.8%)	97	0.049
Lentigo maligna melanoma	6 (11.3%)	47 (88.7%)	53	
Nodular melanoma	7 (15.2%)	39 (84.8%)	46	
Other	16 (21.6%)	58 (78.4%)	74	
Site[b]				
Sun-exposed	10 (14.9%)	57 (85.1%)	67	0.581
Sun-protected	25 (12.3%)	178 (87.7%)	203	
Metastatic melanoma (n=150)				
Age				
≤ 60	23 (26.1%)	65 (73.9%)	88	0.145
> 60	10 (16.1%)	52 (83.9%)	62	
Gender				
Male	24 (23.3%)	79 (76.7%)	103	0.569
Female	9 (19.1%)	38 (80.9%)	47	
American Joint Committee on Cancer stage				
I	12 (8.3%)	133 (91.7%)	145	0.013
II	23 (18.4%)	102 (81.6%)	125	
III	15 (24.2%)	47 (75.8%)	62	
IV	17 (19.3%)	71 (80.7%)	88	

[a] χ^2 test.

[b] Sun-protected sites: trunk, arm, leg and foot; Sun-exposed sites: head and neck.

4.2.2 Strong Fbw7 Staining Correlates with Better 5-year Survival of Melanoma Patients

A total of 328 patients had complete clinical information. To evaluate the correlation between nuclear Fbw7 expression and 5-year patient survival, we constructed Kaplan-Meier survival curves using overall or disease-specific 5-year patient survival data to analyze the cases with positive versus negative Fbw7 staining. Our analysis revealed that overall survival in the positive Fbw7 staining group was 56.96% compared to 44.07% in negative group. The log-rank analysis showed that the differences are significant (overall survival, $P = 0.037$; disease-specific survival, $P = 0.015$; Figure 4.3). These data implicate Fbw7 as a potential prognostic marker in clinical practice. However, multivariate Cox regression analysis showed that nuclear Fbw7 expression is not an independent factor for predicting both overall and disease-specific patient survival ($P = 0.195$ and 0.118, respectively, Table 4.2). This result is not surprising because that Fbw7 nuclear expression reduces during melanoma progression (as AJCC stage increases) dilutes the effects of Fbw7 in predicting patient survival, when AJCC is incorporated into the multivariate cox regression analysis.

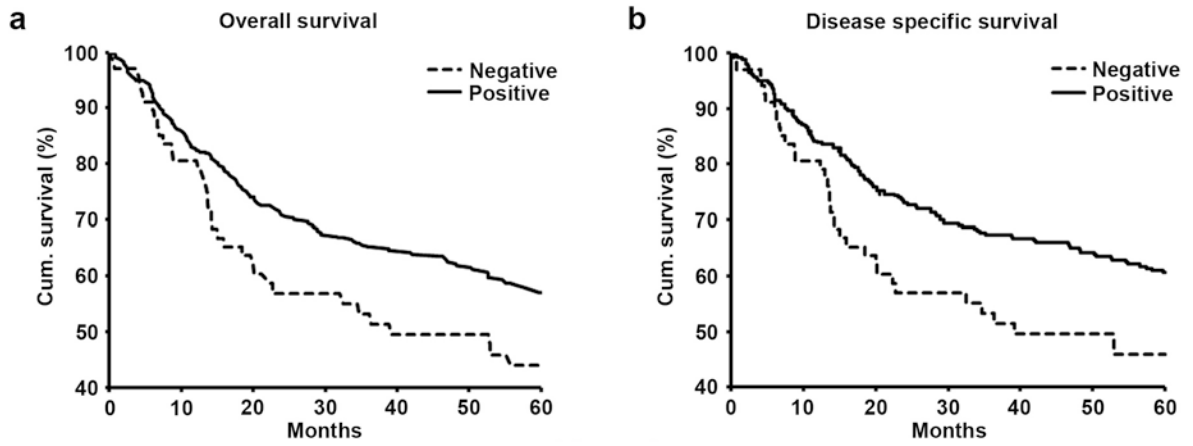

Figure 4.3 Kaplan-Meier survival analyses of melanoma patients.

Patients with negative Fbw7 expression have a significantly worse (**a**) overall and (**b**) disease-specific 5-year survival than those with positive Fbw7 expression (*P* = 0.037 and 0.015, respectively, log-rank test).

Table 4.2 Multivariate Cox regression analysis on 5-year overall and disease-specific survival of 420 melanoma patients

Variables *	Overall survival					Disease-specific survival				
	β †	SE	HR	95% CI	*P*	β †	SE	HR	95% CI	*P*
Age	-0.305	0.154	3.912	0.54-1.00	0.048	-0.256	0.161	2.522	0.56-1.06	0.112
Sex	-0.191	0.159	1.447	0.61-1.13	0.229	0.240	0.166	2.097	0.57-1.09	0.148
AJCC	-1.474	0.161	83.641	0.17-0.31	0.000	-1.610	0.172	87.673	0.14-0.28	0.000
Fbw7	0.248	0.191	1.679	0.88-1.87	0.195	0.307	0.196	2.447	0.93-2.00	0.118

*Coding of variables: Age was coded as 1 (≤60 years), and 2 (>60 years). Sex was coded as 1 (male) and 2 (female). AJCC was coded as 1 (Stages I and II), and 2 (Stages III and IV). Fbw7 was coded as 1 (negative) and 2 (positive). † β, regression coefficient. Abbreviations: SE, standard error of β; HR, hazard ratio; CI, confidence interval.

4.2.3 The Expression of Fbw7α Isoform in Melanoma Cell Lines

Since we observed reduced expression of nuclear Fbw7 in our patient tissue samples, we examined Fbw7 expression level in both different melanoma cell lines and normal melanocytes by western blot. Our data showed that 6 of 9 melanoma cell lines expressed less Fbw7 protein, while only one melanoma cell line (RPM-MC) showed more Fbw7 and other two (Sk-mel-3 and Sk-mel-110) had similar level of Fbw7 as compared to normal melanocytes (Figure 4.4a). We also investigated the mRNA expression in different melanoma cell lines by RT-PCR. We used primers for all Fbw7 isoforms and specific primers targeting each isoform (Table 2.1). As shown in Figure 4.4b, the total Fbw7 mRNA expression is significantly reduced in melanoma cell lines (7.7% on average) as compared to normal melanocytes. Moreover, our data indicated that α isoform is the predominant form of Fbw7 protein expressed in melanoma cell lines (Figure 4.4c), which is consistent with previous report (Grim et al, 2008). Reduced Fbw7 protein and mRNA expression in melanoma cell lines further confirmed our TMA results, indicating Fbw7 might play a tumor suppressive role in melanoma progression.

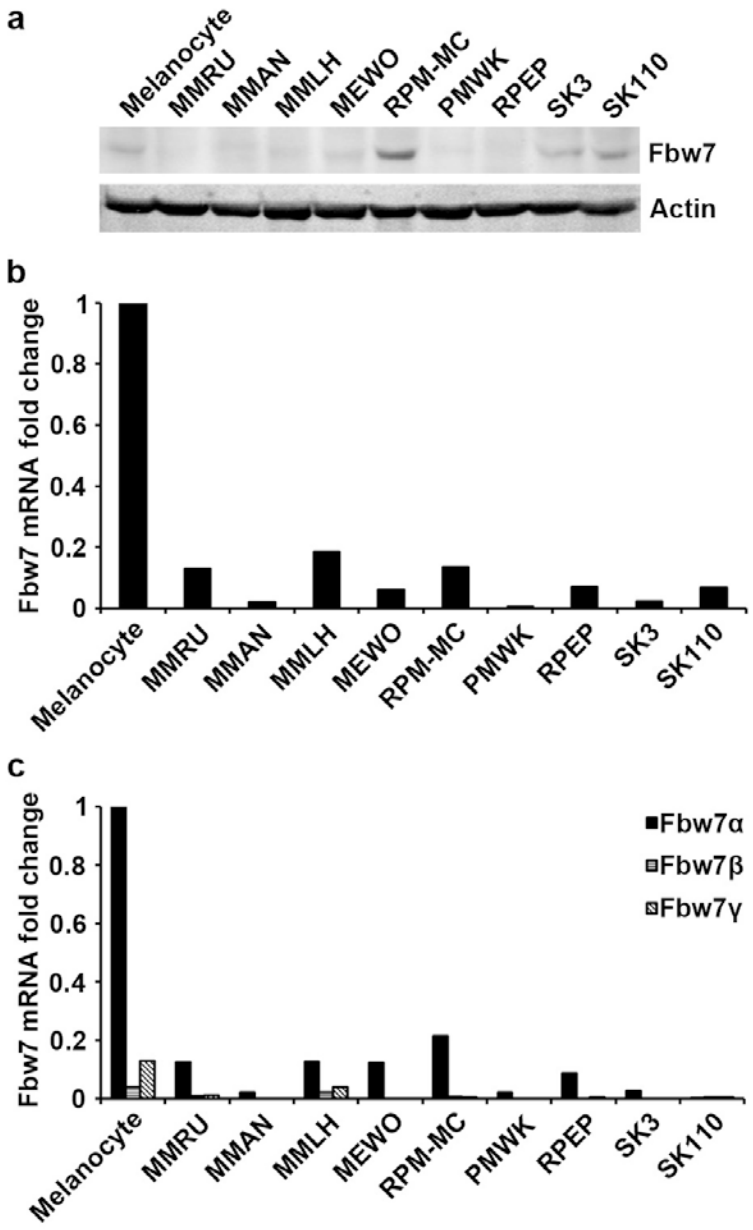

Figure 4.4 Fbw7 mRNA expression in normal melanocytes and melanoma cell lines.

(**a**) Fbw7α protein expression level was reduced in melanoma cell lines compared with normal melanocytes. (**b**) Total Fbw7 mRNA level was reduced in melanoma cell lines compared with melanocytes. (**c**) Comparison of mRNA expression of three Fbw7 isoforms in melanocytes and melanoma cell lines.

4.2.4 *In-vitro* Functional Study of Fbw7

Previous reports have focused on the function of Fbw7 in cell proliferation (Ekholm-Reed et al, 2004; Welcker et al, 2004), because a majority of its substrates are cell cycle regulation and cell proliferation related proteins, such as c-Myc and cyclin E. However, our data indicate that ectopic Fbw7a expression does not change the cell proliferation of melanoma cell line MMRU by SRB assay (Figure 4.5). Examining some known Fbw7 substrates, such as cyclin E and c-Myc by western blot (Figure 4.5), we observed only subtle decrease of c-Myc protein expression upon Fbw7α overexpression. Transwell invasion assay was also performed, but no significant difference was observed between the Fbw7 knock-down group and the control group (data not shown).

To investigate whether the reduction of Fbw7α affects the migration of melanoma cells, we examined the rate of cell migration by wound healing assay after both overexpression and knockdown of Fbw7. As we found the Fbw7α isoform to be the predominant form expressed in melanoma cell lines, we overexpressed the 3×FLAG-Fbw7α plasmid in MMRU and RPEP melanoma cell lines. Our results showed that overexpression of Fbw7α caused about 50% reduction in melanoma cell migration as compared to the control group in MMRU cell line (Figures 4.6a and b). Similarly, we found that Fbw7α overexpression inhibited phosphorylated ERK1/2 expression by western blot (Figure 4.6c), suggesting that Fbw7α may affect metastatic melanoma cell mobility through the MAPK-ERK signalling pathway. We also checked other signalling pathways related to cell migration, such as MAPK-p38 and PI3K-Akt pathways. The data showed similar expression level of p38 and p-Akt in Fbw7α overexpression and in the vector control group, suggesting that those signalling pathway are not involved in Fbw7α (Figure 4.5). Conversely, we

observed significant increase of cell migration after Fbw7α knockdown in two melanoma cell lines (Figure 4.7), confirming the effects of endogenous Fbw7α on melanoma cell migration.

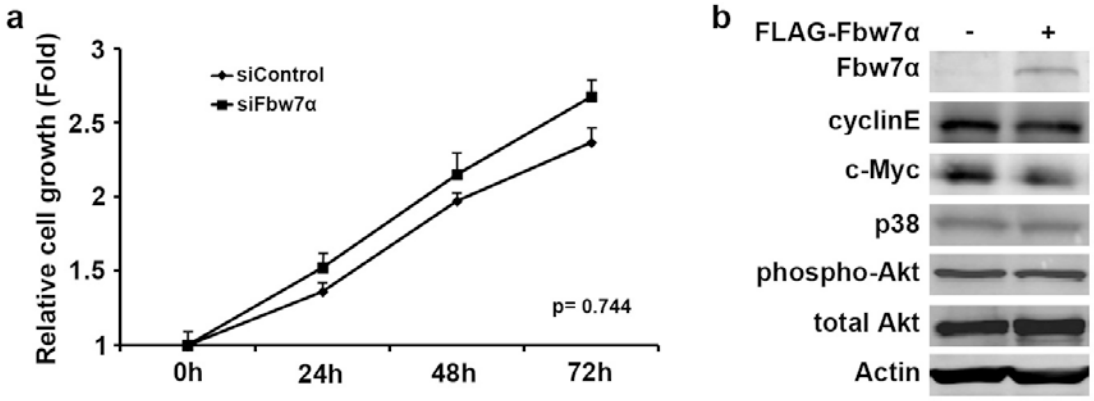

Figure 4.5 Effect of Fbw7α knockdown on melanoma cell proliferation and the expression of cyclin E, c-Myc, p38 and Akt.

(**a**) MMRU cells were transfected with siControl and siFbw7α siRNAs. After 48 h, the cells were counted and 5×10^4 cells were reseeded into 96-well plates. Cells were fixed at 0, 24, 48 and 72 h, and subjected to SRB assay. The results are expressed as the mean value of triplicate samples ± SD (error bars). (**b**) MMRU cells were transfected with FLAG-vector and FLAG-Fbw7α plasmids. 24 h after transfection, cells were lysed and subjected to western blot analysis. Specific antibodies were used to detect the downstream molecules.

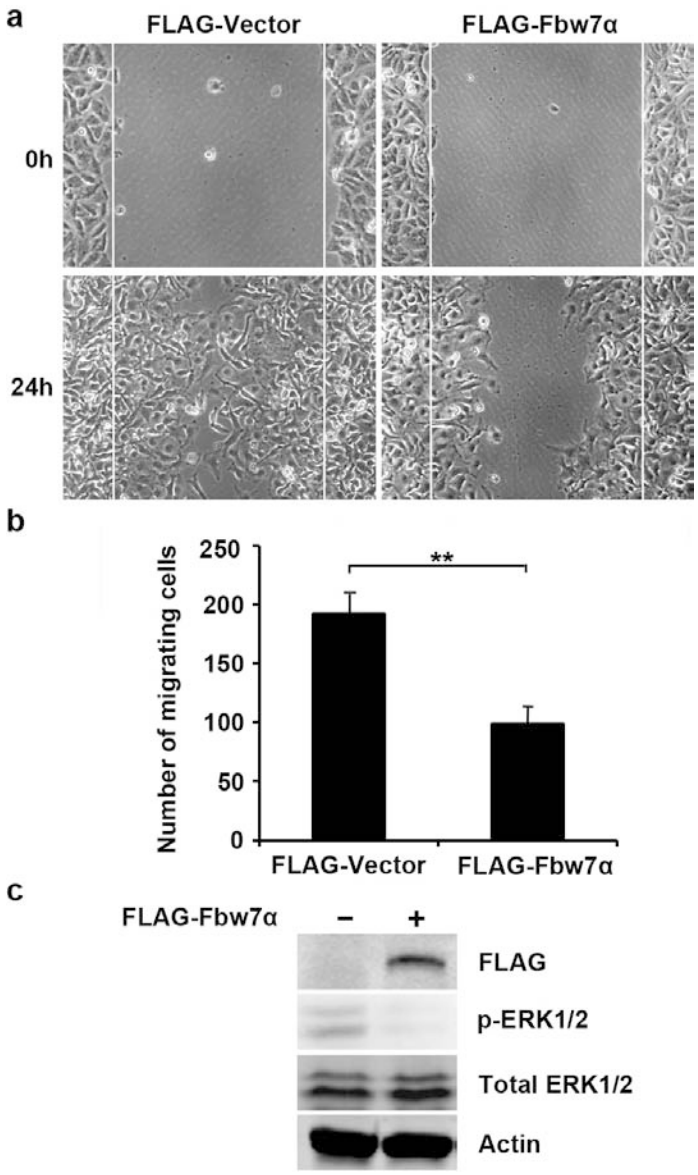

Figure 4.6 Enforced Fbw7α expression inhibits melanoma cell migration.

(**a**) Representative images of the effects of Fbw7α overexpression on melanoma cell migration. (**b**) The inhibition of Fbw7α on melanoma cell migration was quantified by counting the migrated cells in five random fields of each well, error bars equal to means ± SD. The data were obtained from three independent experiments. $^{**}P < 0.01$. (**c**) Western blotting analysis of Fbw7α expression, total ERK1/2 and phosphor ERK1/2.

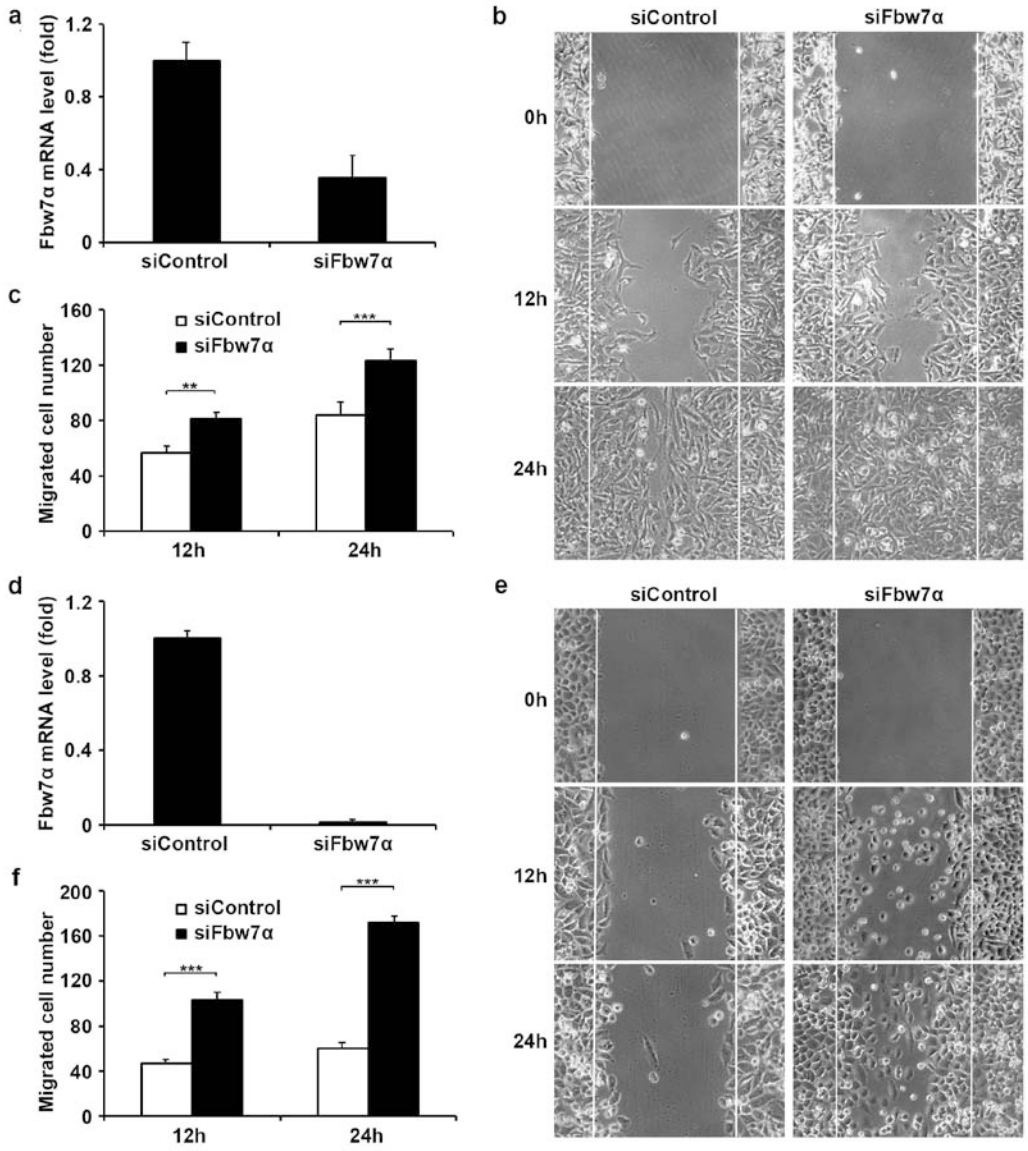

Figure 4.7 Fbw7α knockdown enhanced melanoma cell migration.

(**a-c**) MMRU cells; (**d-f**) RPEP cells. (**a,d**) Fbw7α mRNA expression in siControl and siFbw7α cells by real-time PCR. (**b,e**) Representative images show Fbw7α knockdown induced melanoma cell migration. (**c,f**) The induction of Fbw7α knockdown on melanoma cell migration was quantified by counting the migrated cells in five random fields of each well, error bars equal to means ± SD. The data were obtained from three independent experiments. $^{**}P < 0.01$. $^{***}P < 0.001$.

4.2.5 Selective MEK Inhibitor Treatment Abolished Fbw7α Knockdown Induced Melanoma Cell Migration

Previous studies have shown that ERK1/2 plays a central role in the migration of numerous cell types, and that the MAPK-ERK pathway inhibitor PD98059 and U0126 could be used to inhibit the migration of multiple cell types in response to cell matrix, such as growth factors and other stimuli (Favata et al, 1998; Klemke et al, 1997). PD98059 and U0126 are both highly selective inhibitors of MEK1 activation and the MAP kinase cascade (Crews et al, 1992; Favata et al, 1998). They bind to the inactive forms of MEK1 and prevent activation by upstream activators, such as b-raf and c-raf (Rosen et al, 1994). Two different siRNAs against Fbw7α were used to knockdown the protein, western blot analysis showed better efficacy with first siRNA (Figure 4.8c). After Fbw7α knockdown, we treated the cells with PD98059 and measured cell migration by wound healing assay. Fbw7α knockdown enhanced cell migration by 3-fold, while PD98059 inhibited Fbw7α knockdown-induced cell migration by 50% (Figure 4.8). We performed both wound healing and transwell migration assays with and without U0126 treatment. U0126 treatment also inhibited Fbw7α KD-induced cell migration (Figure 4.9), confirming that the MAPK-ERK1/2 signal pathway is an important mechanism responsible for the increased cell migration in Fbw7α-KD melanoma cells.

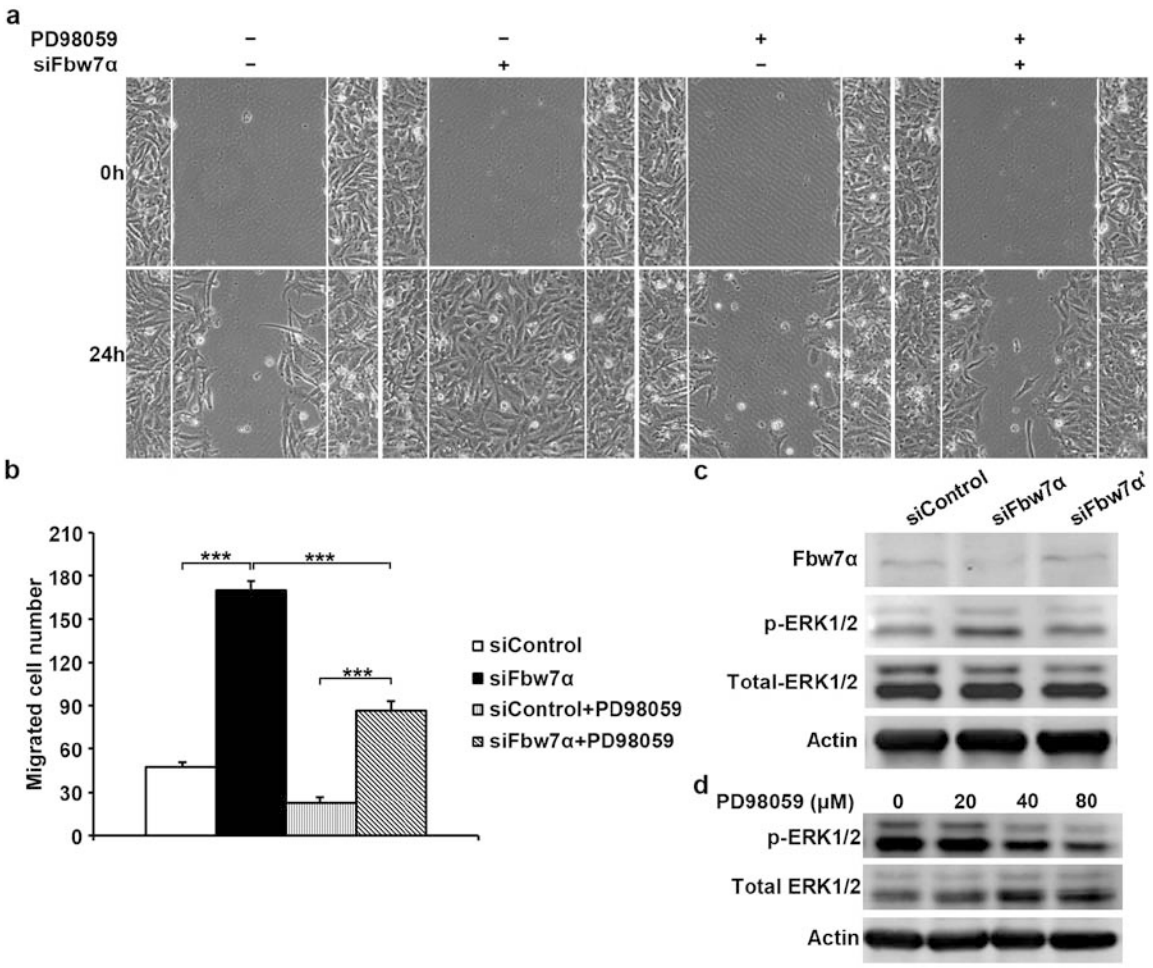

Figure 4.8 Treatment of selective MEK inhibitor PD98059 abolished Fbw7α knockdown induced melanoma cell migration.

(**a**) Representative images show the effect of PD98059 on melanoma cell migration. After Fbw7α knockdown, MMRU cells were treated with 80 μM PD98059 for 24 h in serum free medium, then the wound healing assay was performed. (**b**) Quantification of (**a**). The induction of Fbw7α knockdown on melanoma cell migration was quantified by counting the migrated cells in five random fields of each well, error bars equal to means ± SD. Data were obtained from three independent experiments. ***$P < 0.001$. (**c**) Western blotting analysis showed efficient Fbw7α knockdown using two siRNAs. (**d**) Western blotting analysis of ERK1/2 phosphorylation by MEK inhibitor PD98059.

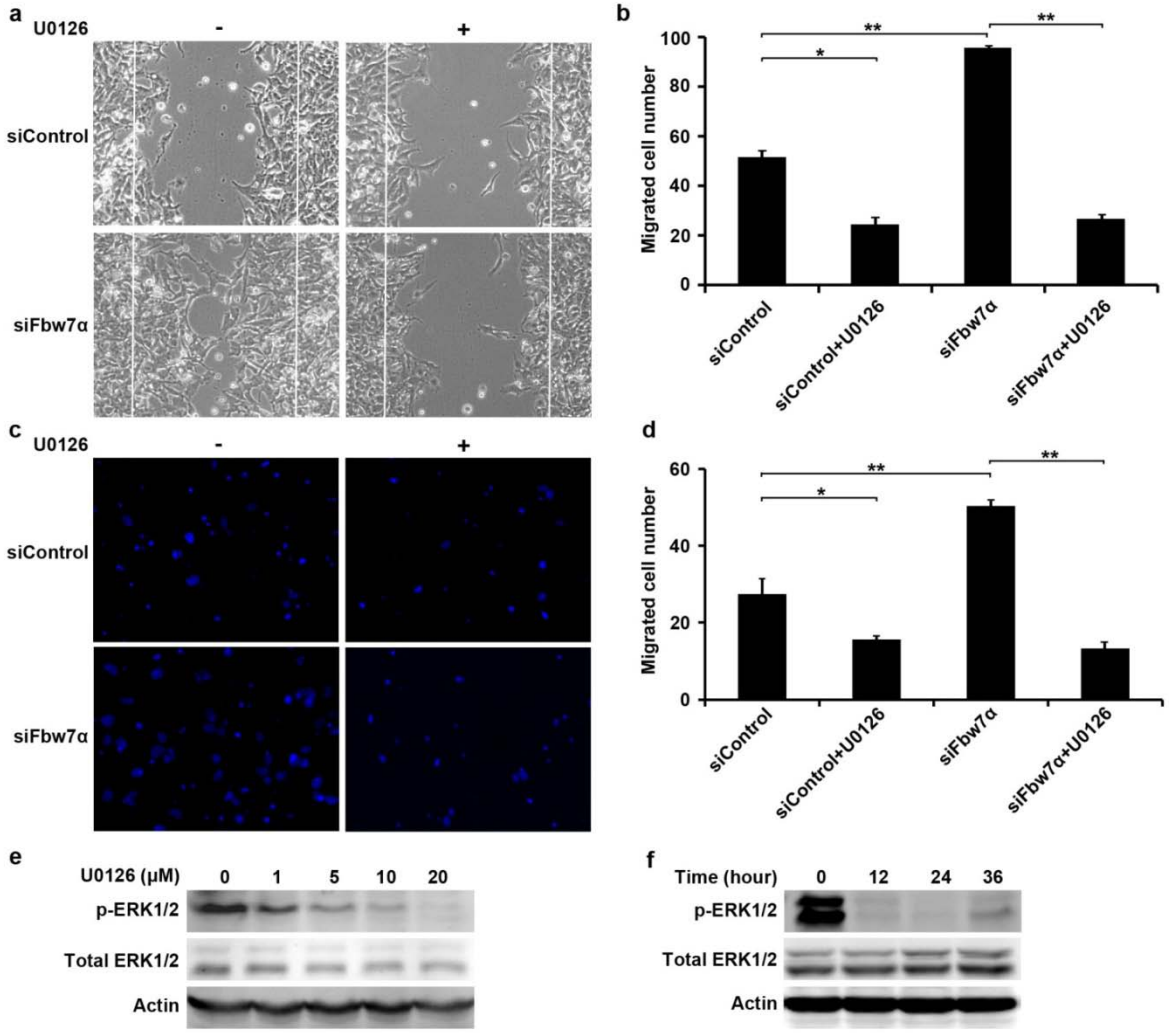

Figure 4.9 Fbw7α knockdown enhanced melanoma cell migration which was abolished by U0126 treatment.

(a,c) Representative images of wound healing assay (a) and transwell migration assay (c) show that Fbw7α knockdown induced melanoma cell migration which was abolished by U0126 treatment (20 μM for 24 h). (b,d) Migrated cells were quantified by counting the migrated cells in five random fields of each well, error bars equal to means ± SD. The data were obtained from three independent experiments. **P < 0.01. ***P < 0.001. (e,f) The dose response and time course of U0126 on p-ERK expression were examined by western blot analysis.

4.2.6 ERK/MAPK Activity is Required for Increased Stress Fiber Formation in Fbw7α-KD Melanoma Cells

Previous data showed that RhoA activity and F-actin stress fiber formation is important for cell migration (Gao et al, 2006). RhoA is a small G-protein acting as a molecular switch that transit cellular signals through an array of effector proteins, thereby mediating a diverse number of cellular responses including cytoskeletal reorganization (Nobes & Hall, 1995; Ridley et al, 1992). The RhoA protein switches between an active GTP-bound state and an inactive GDP-bound state (Hall, 1998). To further investigate the mechanism underlying Fbw7α-KD induced cell migration, RhoA pull-down assay was done to measure RhoA activity by pull-down of GTP-bound RhoA. MMRU cells transfected with siControl and siFbw7α were serum starved for 24 h and RhoA activity was induced by serum stimulation for 30 min, and subjected to protein extraction and pull-down steps. Figure 10a shows that RhoA activity is very low in siControl cells, and markedly increased in siFbw7α group, which was abolished by MEK inhibitor PD98059 (Figure 4.10a). After serum stimulation, we stained the cells with rhodamine-conjugated phalloidin. The intensity of F-actin staining was measured by Image J software. We observed reduced stress fibre formation in the Fbw7α overexpression group (Figure 4.10b). Our results also indicated that serum-induced stress fiber formation is more excessive in Fbw7α-KD MMRU cells than in those transfected with control siRNA (Figure 4.10c), and that PD98059 treatment abrogated the Fbw7α knockdown induced stress formation in Fbw7α-KD cells (Figure 4.10d). These data confirmed that MAPK-ERK signal is required for Fbw7α-KD induced RhoA activity and stress fiber formation.

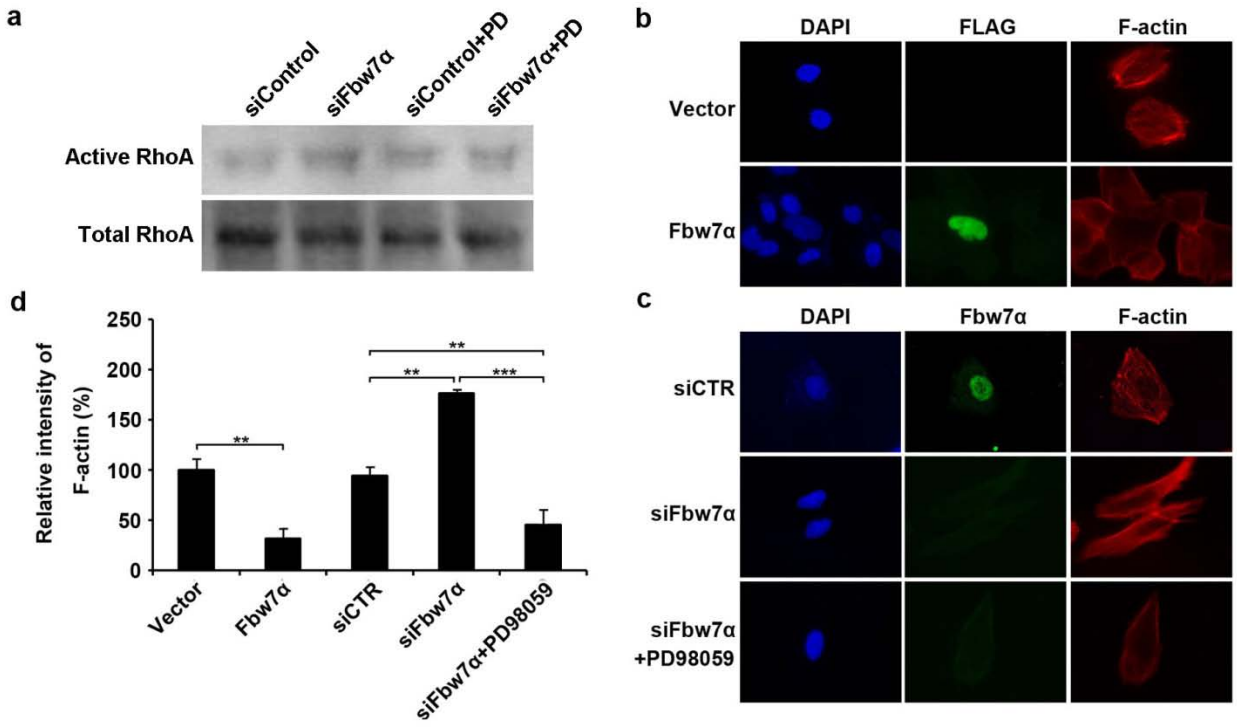

Figure 4.10 Fbw7α affects RhoA activity and stress fiber formation.

(**a**) Fbw7α KD-induced RhoA activity was abolished by PD98059. MMRU cells transfected with siControl and siFbw7α siRNAs were serum starved for 24 h, and serum stimulation for 30 min. (**b**) Decreased stress fiber formation in Fbw7α overexpression MMRU cells. MMRU cells were transfectd with FLAG-Fbw7α or vector control, followed by serum starvation for 24 h and serum stimulation for 30 min. (**c**) Increased stress fiber formation after Fbw7α KD. MMRU cells were transfected with siControl and siFbw7α siRNAs, followed by serum starvation for 24 h and serum stimulation for 30 min. For MEK inhibitor treatment, MMRU cells were incubated with serum free medium containing 80 μM PD98059 for 24 h. Magnification, 630×. (**d**) Quantification of (**b** and **c**). Relative intensity of F-actin was examined by Image J software, $^{**}P < 0.01$, $^{***}P < 0.001$.

4.3 Discussion

Well-known as a tumor suppressor, Fbw7 expression was significantly reduced in gastric cancer, colorectal cancer and glioma at both mRNA and protein levels (Hagedorn et al, 2007; Iwatsuki et al, 2010; Yokobori et al, 2009). Meanwhile, loss-of-function mutations of *Fbw7* were frequently found in various cancers. *Fbw7* mutation rates in cholangiocarcinoma, T-cell acute lymphocytic leukemia, endometrial carcinoma and colorectal cancer were reported as 35%, 31%, 9% and 9%, respectively (Akhoondi et al, 2007; Nakayama & Nakayama, 2005; Welcker & Clurman, 2008; Yan et al, 2006). However, no study yet has been conducted on the expression and biological functions of Fbw7 in melanoma. In the present study, we sought to determine the role of Fbw7 in melanoma progression. Initially, we used TMA technology and immunohistochemistry to investigate the expression level of Fbw7 in various stages of melanocytic lesions. Based on 420 patients at different stages of melanoma and 81 cases of nevi, our data showed that the expression of Fbw7 was significantly reduced in primary melanoma and metastatic melanoma as compared todysplastic nevi. We found that most staining is in the nucleus and we confirmed that the nuclear Fbw7α isoform is the predominant form of Fbw7 protein expressed in melanoma. We have for the first time found that reduced nuclear staining of Fbw7 correlated with the melanoma progression.

We also found that Fbw7 expression is significantly correlated with melanoma thickness and AJCC stages (Table 4.1), indicating that Fbw7 may function in the vertical growth phase of melanoma. Interestingly, our analysis showed that reduced Fbw7 expression is significantly correlated with poor 5-year patient survival for all melanomas (Figure 4.3), which is consistent with the previous reports on gastric cancer, colorectal cancer and glioma (Hagedorn et al, 2007; Iwatsuki et al, 2010; Yokobori et al, 2009). Moreover, multivariate

Cox repression analysis revealed that Fbw7 is an independent factor for predicting both overall and disease-specific 5-year patient survival (P = 0.047 and 0.018, respectively; Table 4.2). Additionally, our analysis revealed the correlation between Fbw7 expression and patient age, as well as the subtype of melanoma (P = 0.038 and 0.049, respectively). More studies are required to establish the relationship between melanoma subtype and related genetic changes. Previous studies have shown that the progression of melanoma requires multiple genetic alterations. For example, BRAF is mutated in ~60% to 70% of superficial spreading melanomas (Haluska et al, 2006). A comprehensive screening of the *Fbw7* mutation in different subtypes of melanomas may address this question.

Although the Fbw7 ubiquitin ligase mediated protein degradation pathway has been extensively studied, and a number of Fbw7 substrates ehas been identified in the past decade, the mechanisms responsible for the regulation of Fbw7 expression are poorly understood. Recent studies indicated that several regulators can directly modulate Fbw7 mRNA expression, such as microRNAs. Xu et al. have found that the endogenous protein and activity levels of Fbw7 key substrate cyclin E could be upregulated by overexpression of miR-223 through reducing Fbw7 mRNA level, and that increased genomic instability was observed as a consequence (Xu et al, 2010). Lerner et al. also have shown that miR-27a inhibits Fbw7-mediated ubiquitylation and degradation of the cyclin E (Lerner et al, 2011). These data indicate that the degradation of Fbw7 substrate could be affected by the upstream regulators which transcriptionally regulate Fbw7 expression. However, the mechanisms of Fbw7 reduction in melanoma require further investigations.

Previous studies have shown that Fbw7α mRNA expression is much higher than that of the other two isoforms, Fbw7β and Fbw7γ, in most cell lines and primary cultured cells

(Spruck et al, 2002). Furthermore, it has been reported that the Fbw7α is the isoform responsible for the degradation of key Fbw7 substrates, such as cyclin E, c-Myc and SREBP1 (Grim et al, 2008). As we found that Fbw7α is the predominant form expressed in melanoma cell lines, we overexpressed Fbw7α by introducing FLAG tagged plasmid. We found that Fbw7α significantly suppresses melanoma migration (Figure 4.6), while Fbw7α-KD cells showed enhanced ability of migration by both wound healing and transwell migration assays, which was abolished by both selective MEK inhibitor PD98059 and U0126 (Figures 4.8 and 4.9). Previous studies indicated that MAPK family proteins play crucial roles in cell migration (Huang et al, 2004). Since it has been reported that ERK1/2 governs cell movement by phosphorylating myosin light chain kinase (MLCK), caplain or FAK (Deak et al, 1998), we therefore examined the expression level and phosphorylation status of ERK1/2. Interestingly, we observed reduction of phospho-ERK1/2 expression, while observing no change in total ERK1/2 expression after transfected melanoma cell line with Fbw7α plasmid (Figure 4.6). Consistently, phospho-ERK1/2 expression was increased after Fbw7α knockdown, while total ERK1/2 expression was comparable with that in the siControl group (Figure 4.8). These data suggests that the upstream regulators of ERK1/2 might be affected by Fbw7α. Notably, a recent report showed that SEL-10/Fbw7 regulated LIN45/Braf stability in *C. elegans*, implicating another possible mechanism responsible for Fbw7-mediated MAPK-ERK pathway regulation (de la Cova & Greenwald, 2012). The fact that two well-known MEK inhibitor PD98059 and U0126 treatment abolished Fbw7α KD-induced melanoma cell migration confirmed that Fbw7α regulates cell migration through ERK1/2. To further investigate the role of Fbw7α in melanoma cell migration, we performed RhoA pull-down assay and stress fiber formation assay. We showed that active GTP-bound RhoA

expression level was significantly increased by Fbw7α knockdown and was brought back to control level by PD98059 and U0126 treatment. Similar effects were observed in stress fiber formation assay, suggesting that the MAPK-ERK pathway was indeed involved in Fbw7α-mediated melanoma cell migration (Figures 4.8, 4.9, and 4.10).

Another possible pathway that may be involved in Fbw7α KD-induced melanoma cell migration is the KLF5-FGF-BP-pERK signalling pathway. Because the Krüpple-like transcription factor 5 (KLF5) has been shown to promote cell proliferation, migration and tumorigenesis, it is broadly considered as an oncogene (Liu et al, 2009). Previous study has shown that KLF5 promotes kerotinocyte migration through integrin-linked kinase (Yang et al, 2008), and KLF5 has been found to be targeted by Fbw7 for ubiquitin-mediated degradation (Zhao et al, 2010). Moreover, KLF5 enhances ERK phosphorylation in bladder cancer cells (Liu et al, 2009). Fbw7 may suppress melanoma cell migration through increase KLF5 degradation, but on the other hand, KLF5 down-regulation weakened p-ERK level. The role of this KLF5-mediated pathway in melanoma cell migration requires more investigation.

In conclusion, we found the α isoform of Fbw7 protein is the major form expressed in human melanoma cell lines and tumor biopsies, and reduced Fbw7 expression predicts worse 5-year patient survival. Consistent with previous studies on other types of cancer, Fbw7 functions as a tumor suppressor in melanoma. Fbw7 protein regulates melanoma cell migration through the MAPK-ERK signalling pathway, and it may serve as a prognostic marker and potential therapeutic target for the treatment of human melanoma.

Chapter 5: Prognostic Value of KAI1/p27 Combination in Primary Melanomas

5.1 Background and Rationale

About 50% of primary melanoma patients developed metastases within 15 years after diagnosis, and the occurrence rate of metastasis was 15% of patients with a thin (thickness < 1 mm) melanoma after their initial diagnosis (Kalady et al, 2003). However, the currently used staging system, the AJCC criteria, lacks the ability to assign precisely risk of metastasis in specific patients. Biomarkers that could stratify melanoma patients based on risk of progression or metastasis may help clinicians to evaluate individual patients' metastasis risks better and thus plan treatments accordingly.

Our previous studies have identified several important biomarkers in melanoma, including BRAF, Dicer, Fbw7, p27, KAI1, MMP2 and Tip60. These studies have also shown that these markers have prognostic value (Chen et al, 2012; Chen et al, 2011b; Cheng et al, 2013; Jafarnejad et al, 2013; Safaee Ardekani et al, 2013). The purpose of this phase of our project is to evaluate the usability of these markers in combination to more precisely predict risks of mortality than is possible with the markers used individually.

5.2 Results

5.2.1 KAI1 and p27 Best Differentiate Metastatic Melanomas from Primary Disease

First of all, logistic regression analysis was performed to identify the signature genes that most efficiently discriminated metastatic melanomas from primary melanomas. A representative staining of BRAF, Dicer, Fbw7, KAI1, MMP2, P27 and Tip60 is shown in Figure 5.1. As shown in the images, cytoplasmic staining was observed for BRAF, MMP2, Dicer, KAI1, and Tip60, whereas nuclear staining is observed for Fbw7 and P27.

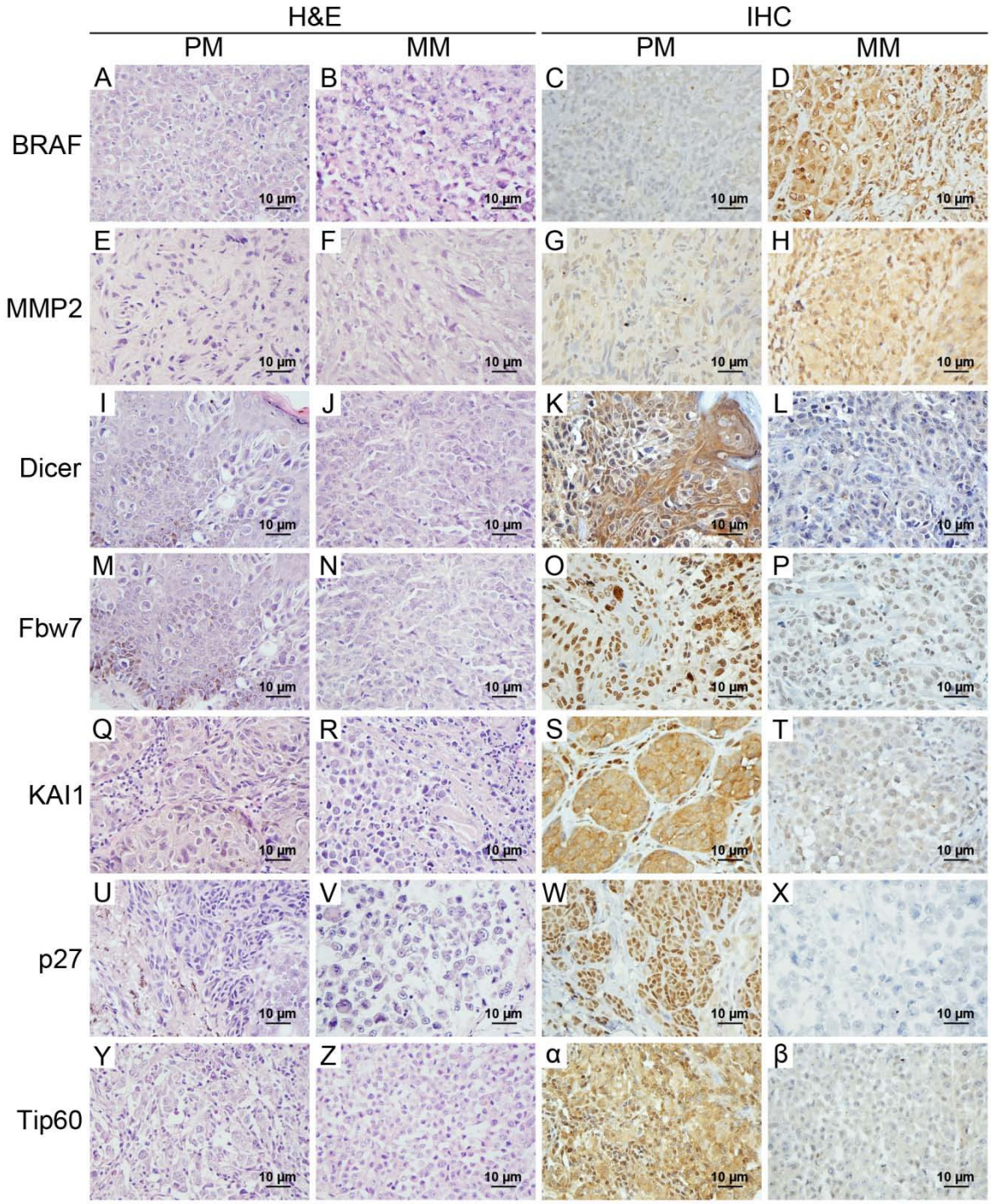

Figure 5.1 Representative H&E and immunohistochemical staining of 7 candidate biomarkers in primary melanomas and metastatic melanomas.

Magnification, × 400.

Metastatic melanomas had elevated BRAF and MMP2 expression, and showed low levels of Dicer, Fbw7, KAI1, p27 and Tip60, as compared to primary melanomas. The optimal cut-off values of IRS scores for the seven markers were calculated by MedCalc software (version 12.5, Ostend, Belgium). Univariate analysis showed that BRAF, Dicer, KAI1, P27 and Tip60 were differentially expressed in metastatic melanoma as compared to primary melanoma. Yet multiple logistic regression analysis indicated that the KAI1 ($P = 6.321\times10^{-5}$) and p27 ($P = 9.677\times10^{-5}$) were the two most significant biomarkers compared with the other 5 (Table 5.1). KAI1 and p27 were expressed in 95.24% and 78.10% of metastatic tumors, and in 66.21% and 50.34% of primary melanomas, respectively. This difference between primary melanoma and metastatic melanoma is highly significant ($P < 0.001$), indicating that loss of KAI1 or p27 may represent a relatively robust feature of more advanced melanomas. Furthermore, we ran the same analysis on the combination of KAI1 and p27, and found loss of both proteins better differentiate metastatic melanoma (Table 5.2).

Table 5.1 Comparison of expression of seven biomarkers in primary versus metastatic melanomas

Biomarker	Primary		Metastatic		*P* value	
	-	+	-	+	Univariate	Multivariate
BRAF	79 (54.48)	66 (45.52)	36 (34.29)	69 (65.71)	0.002	0.037
Dicer	10 (6.90)	135 (93.10)	23 (21.90)	82 (78.10)	0.001	0.053
Fbw7	22 (15.17)	123 (84.83)	21 (20.00)	84 (80.00)	0.318	0.666
KAI1	96 (66.21)	49 (33.79)	100 (95.24)	5 (4.76)	0.000	0.000
MMP2	102 (70.34)	43 (29.66)	63 (60.00)	42 (40.00)	0.089	0.166
P27	73(50.34)	72(49.66)	82(78.10)	23(21.90)	0.000	0.000
Tip60	30(20.69)	115(79.31)	46(43.81)	59(56.19)	0.000	0.029

Table 5.2 Definition of metastasis-like subgroup in primer melanoma

Group	Expression	PM (%)	MM (%)	Chi-square	*P* value
ML	KAI1- P27-	48(33.10)	78(74.29)	41.32	0.000
NML	KAI1+/P27-&KAI1-P27+	97(66.90)	27(25.71)		

5.2.2 Loss of KAI1 and p27 in a Subgroup of Primary Melanoma

The combination of loss of KAI1 and p27 was observed in a majority of metastatic melanomas, and this tissue biomarker may serve as a particular signature. To evaluate the ability of this biomarker combination to distinguish metastatic melanoma from primary tumor, we further classified a metastasis-like group (ML) of 48 patients with negative expression of both KAI1 and p27 (33.10%), and a non-metastasis-like group (NML) of 97 patients with positive expression of either KAI1 or p27 (66.90%) or both. Representative images of the expression pattern were shown in Figure 5.2.

To reduce potential classification-bias, we established a classification tree based on the identified tissue biomarker signature. Losses of KAI1 and p27 were plotted in a classification tree (Figure 5.3). The initial split in the tree occurred at KAI1, and two major groups then emerged, KAI1 positive and KAI1 negative. Those negative with KAI1 staining were further subdivided by p27 expression status. The classification tree shows a considerable separation for KAI1-p27- subgroup versus KAI1+ and p27+ subgroups. Finally, the 48 metastasis-like cases in primary melanomas and 78 metastatic cases were identified in the KAI1-p27- branch. KAI1+p27- and KAI1+p27+ cannot be distinguished by this classification tree because the ability of these two markers to discriminate metastatic tumor was primarily due to loss of either KAI1 or p27. The classification tree results indicated KAI1-p27- can be treated as a unit biomarker signature for metastatic melanoma and may enhance accuracy in identifying patients with high-risk primary melanoma shareing the biological feature with those of advanced melanomas.

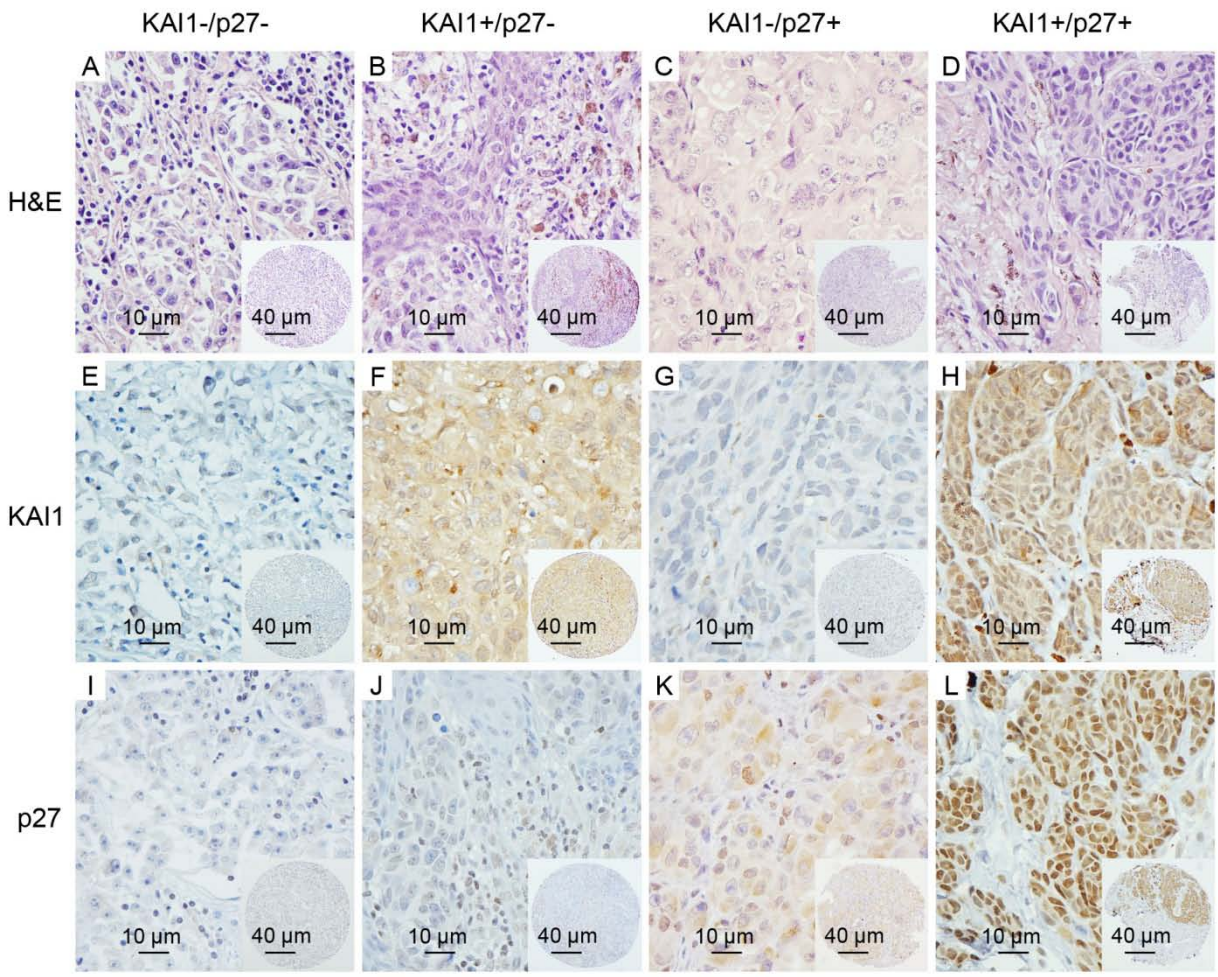

Figure 5.2 Representative images of expression of KAI1 and p27 in primary melanomas.

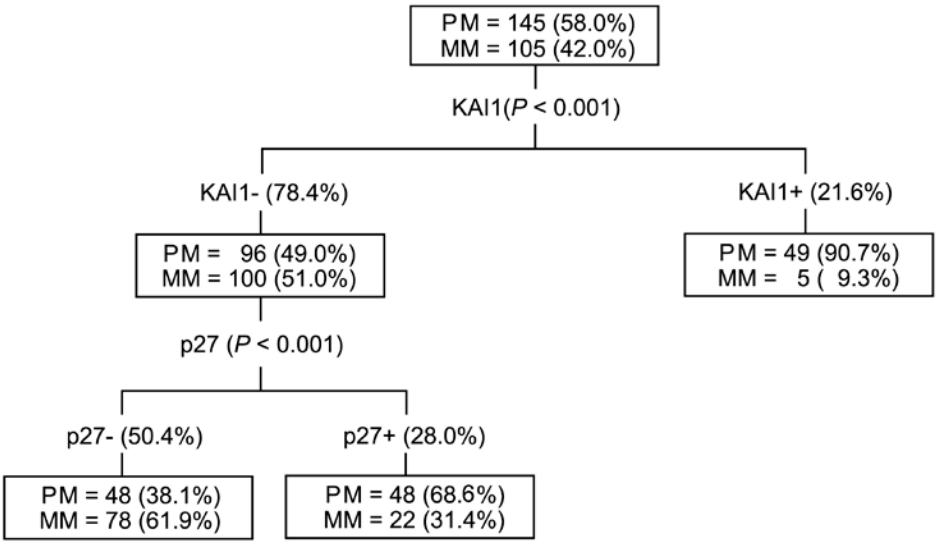

Figure 5.3 Classification tree for identification of metastasis-like subgroup with KAI-P27-.

Optimal tree chosen by cross-validation after preliminary step classifying by the negative (KAI1-) or positive (KAI1+) of KAI1 ($P < 0.001$), the KAI- branch then was further divided by p27, and a branch was identified with KAI-p27-, which was significant separated with KAI-p27+ branch ($P < 0.001$). In the KAI-p27- branch includes 38.1% of primary melanomas and 61.9% of metastatic melanomas.

5.2.3 Loss of KAI1 and p27 Predicts Poorer Survival

To examine the prognostic value of this combination of biomarkers, we performed survival analysis in a training cohort of 145 patients (48 in ML group, 97 in NML group) with primary melanoma patients. Kaplan-Meier survival curves showed that disease-specific survival is significantly reduced for patients with metastasis-like group (52.1%) compared to the non-metastasis-like subgroup (76.3%, $P = 0.002$, Figure 5.4A). To validate the difference

of clinical outcomes between metastasis-like and non-metastasis-like groups in the training cohort, survival analysis was performed in an independent validation cohort of 92 patients with primary melanoma. The results showed that the NML group had significantly improved disease-specific survival (87.9% in NML versus 70.6% in ML, *P* = 0.03, Figure 5.4B). In addition, the same trend was observed in the combined patients cohort (*P* = 3.00E-4, Figure 5.4C).

The univariate Cox proportional hazards regression analysis showed that ML subgroup had significantly worsened disease-specific survival, with a hazard ratio of 2.44 (95% CI: 1.47-4.00; P < .001), indicating that the risk of dying in the ML subgroup was 2.44 times greater compared to that of the NML subgroup. Multivariate Cox proportional hazards regression model adjusted for age, sex, ulceration, thickness were assessed (Table 5.3). In the multivariable Cox model, the KAI1-/p27- signature was found to be of independent prognostic significance for disease- specific survival (*P* = 0.004). More important, compared to KAI1 and p27 as an individual prognostic marker, the KAI1-/p27- signature is more closely associated with melanoma patient survival: the *P* value is 0.004 for KAI1-/p27-, and 0.044, 0.181 for KAI1 and p27, respectively. It is noteworthy that ML subgroup exhibited higher hazard ratio for survival (HR = 2.08; 95% CI = 1.27-3.45), as compared to the thickness (HR = 1.07; 95% CI = 1.03-1.11).

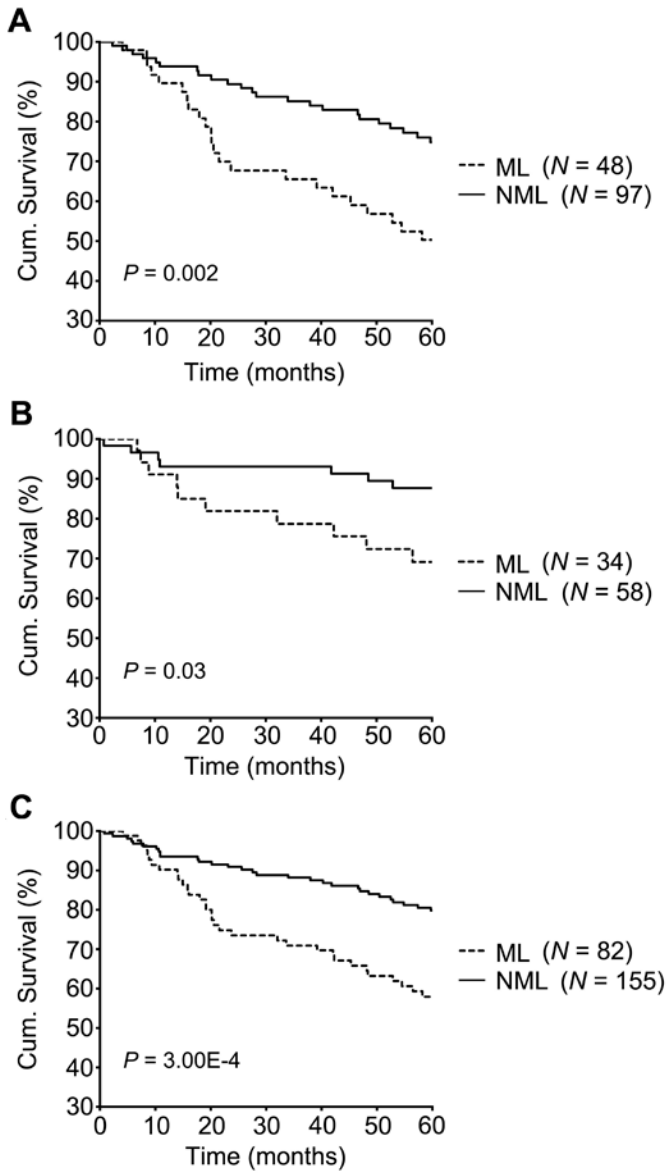

Figure 5.4 Disease-specific survival analysis in NML and ML subgroups.

Disease-specific survival in different subgroups of primary melanomas was analyzed by the Kaplan–Meier survival method. Patients in metastasis-like subgroup showed poorer prognosis than patients in non-metastasis-like subgroup in the training cohort (A), in the validation cohort (B), and in the combination cohort (C). Log-rank test *P* value was indicated in the graphs.

Table 5.3 Cox proportional hazard regression analysis in primary melanomas with combination cohort

	Univariate Cox Proportional		Multivariate Cox Proportional	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age	1.03(1.01-1.04)	0.001	1.01(0.99-1.03)	0.268
Sex	1.02(0.62-1.67)	0.937	0.91(0.55-1.51)	0.716
Thickness	1.09(1.06-1.12)	9.989E-8	1.07(1.03-1.11)	2.374E-4
Ulceration	4.35(2.65-7.16)	6.510E-9	3.56(2.09-6.07)	3.105E-6
KAI1	2.42(1.43-4.10)	0.001	1.74(1.02-2.97)	0.044
p27	1.56(1.00-2.44)	0.051	1.37(0.87-2.12)	0.181
Subgroup	2.44(1.47-4.00)	4.649E-4	2.08(1.27-3.45)	0.004

HR, hazard ratio; 95% CI, 95% confidence interval

5.2.4 KAI1 and p27 Expression was Associated with Tumor Thickness

Our data showed that the metastasis-like subgroup displaying a more aggressive clinical phenotype, and we further found that metastasis-like subgroup melanomas had greater tumor thickness as compared to those of non-metastasis-like subgroup melanomas (4.94 vs. 2.66 mm; $P = 0.001$, Table 5.4). This finding may explain why melanoma patients in the ML subgroup suffer from worse prognosis. The other clinicopathological characteristics and associations with subgroups are shown in Table 5.4. These parameters include age, gender, subtype, thickness, and the presence of ulceration. No significant correlation was found in the proportion of age, gender, clinical subtype and ulceration between metastasis-like and non-metastasis-like subgroups.

Table 5.4 Clinicopathological characteristics of NML and ML subgroups in combination cohort of patients with primary melanoma

Variable	ML (N = 82)	NML (N = 155)	P value
Age	63.42 ± 1.97	59.48 ± 1.53	0.123
Sex			0.194
Male	48(58.54)	77(49.68)	
Female	34(41.46)	78(50.32)	
Thickness	4.94 ± 0.73	2.66 ± 0.24	**0.000**
Ulceration (%)			0.117
Absent	57(69.51)	122(78.71)	
Present	25(30.49)	33(21.29)	
Subtype (%)			0.830
AL	4(4.88)	6(3.87)	0.714
LM	8(9.76)	18(11.61)	0.663
N	20(24.39)	28(18.06)	0.249
SS	27(32.93)	62(40.00)	0.285
Other	7(8.54)	13(8.39)	0.969

5.3 Discussion

In present study, we performed statistical analysis on a patient cohort with large sample size, and identified the metastasis-like subgroup of primary melanomas in order to develop clinically effective classification model. We demonstrated that simultaneous loss of both KAI1 and p27 was a novel molecular feature associated with metastasis, discriminating between primary melanoma and metastatic melanoma, identifying a metastasis like subgroup (ML subgroup) within primary melanoma patients, and constituting a strong prognostic marker for poor survival in patients with primary melanoma.

Initially, our data demonstrated that KAI1- and p27- were the two markers among 7 previously identified independent markers that showed the most significant difference in expression level between metastatic melanomas (95.24% vs. 78.10%) and primary melanomas (66.21% vs. 50.34%, $P < 10E\text{-}4$). This result was consistent with previous finding that highly aggressive tumors showed the lowest KAI1 and p27 expression levels (Lijovic et al, 2002; Wander et al, 2011). Our study extends previous work in that the combination of KAI1 and p27 loss is a metastatic feature and significantly differentiates metastatic melanomas from primary melanomas ($P < 0.001$). Tumor metastasis suppressor KAI1 has previously been shown to interfere with multiple steps of the metastatic cascade, including proliferation, invasion, and migration (Maloney et al, 2013). The p27 regulated metastasis of human melanoma (Hoshino et al, 2011).

Using the metastatic signature (loss of both KAI1- and p27-), a metastasis-like subgroup was identified accounting for 33.10% of primary melanomas. Tumors identified within the same subgroup were more likely to present with similar clinical features. Using the CART tree analysis, the 48 metastasis-like primary melanoma cases and 78 metastatic

melanoma cases without both KAI1 and p27 expression were separated on the KAI1- or p27- branch. Kaplan-Meier survival analysis showed that the patients in ML subgroup have a worse 5-year survival rate compared with the patients in NML subgroup (52.1% vs 76.3%). More important, multivariate Cox regression analysis revealed that KAI1-/p27- is an independent prognostic factor in primary melanomas, showing a stronger correlation with patient survival than when used as individual markers. Our results suggest that loss of KAI1 and p27 leads to enhanced metastatic potential for primary melanomas, and that restoring expression or function of KAI1 and p27 can be a potential strategy for melanoma therapy. KAI-p27- signature could potentially identify a distinct subgroup in primary melanoma patients that need to be monitored more closely and treated more aggressively.

Chapter 6: Identification of Stage-specific Biomarkers of Melanoma

6.1 Background and Rationale

Numerous methods have been developed to aid clinicians to refine the risk of disease progression and assess the prognosis of melanoma patients, so that individualized treatment can be designed accordingly. The current widely adopted system is the American Joint Committee on Cancer (AJCC) staging system that divides patients into four stages: Stage I (Breslow thickness < 2mm) and Stage II (Breslow thickness > 2 mm) define primary invasive melanoma, whereas Stage III and Stage IV define local regional and distant metastases, respectively (Balch et al, 2009). For Stage I and II, the treatment of choice is surgery. For Stage III and IV patients, surgery is often not possible, and additional treatments such as chemotherapy, targeted therapy or immunotherapy are needed.

Several pathological parameters such as thickness, ulceration and mitotic rate and serum biomarkers such as LDH levels have been taken into account to subdivide patients into AJCC Stages. Subgroups assignments according to this system behave differently in clinical outcome, strongly supporting the biological validity of the system. However, at present, there has been no systematic analysis of molecular biomarkers with ability to further refine risk prediction for AJCC individual stages. In this study, we attempt to identify histological markers that are prognostically significant for each individual AJCC Stage. Six previously reported independent melanoma biomarkers, including BRAF, MMP2. P27, Dicer, Fbw7 and Tip60 (Chen et al, 2012; Chen et al, 2011b; Cheng et al, 2013; Jafarnejad et al, 2013; Rotte et al, 2012; Safaee Ardekani et al, 2013) were chosen for this purpose.

6.2 Results

6.2.1 Study Populations Used for Biomarker Discovery and Confirmation Studies

Two patient populations with a total of 439 patients were used in our study. The first biomarker discovery population consisted of 254 patients (148 primary melanomas and 106 metastatic melanomas) for whom the expression levels of all 6 markers are available, permitting inter-biomarker comparisons. For the validation purposes, 185 additional cases (133 primary melanomas and 52 metastatic melanomas) were added for whom the expression levels are available for individual markers. These cases were added to the discovery population to form the validation population. The distribution and selected demographic characteristics of melanoma patients are listed in Table 6.1. As shown in Figure 6.1, the 5 year survival for Stages I, II, III, and IV are 91.3%, 62.1%, 42.6% and 8.0%, respectively, in a total of 439 patients. The prognosis of each of the AJCC Stages in both the discovery population and in the confirmation population is similar and consistent with that reported in the literature, suggesting that our study patient population is similar to previously reported melanoma patient populations (Balch et al, 2009).

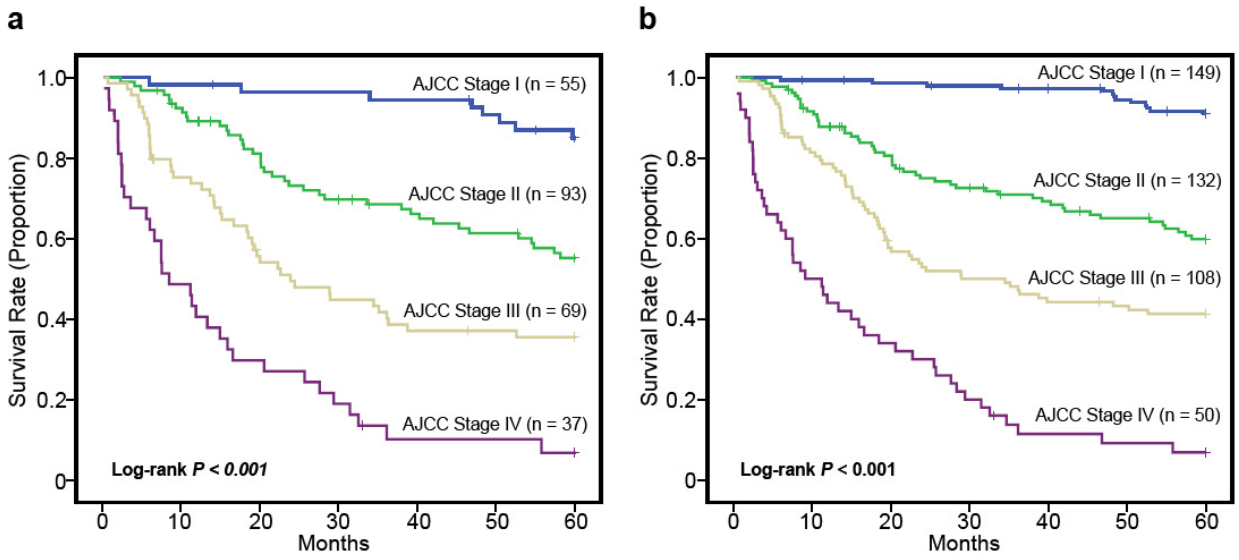

Figure 6.1 5-year Kaplan-Meier survival analysis of AJCC staging in discovering population.

(**a**) and expanded validation population (**b)** of melanoma patients. **a.** Discovering population with 254 patients: proportions of survival are 85.5% for stage I, 58.1% for stage II, 37.7% for stage III, 8.1% for stage IV (Log-rank $P < 0.001$); **b**. Validation population with 439 patients: proportions of survival are 91.3% for stage I, 62.1% for stage II, 42.6% for stage III, 8.0% for stage IV (Log-rank $P < 0.001$).

Table 6.1 Clinicopathologic characteristics of study population of melanoma patients

Variable	Discovery population (N = 254)	Validation population (N = 439)
Age − yr		
Median	61	60
Range	7-95	7-95
Sex – no. (%)		
Male	148 (58)	260 (59)
Female	106 (42)	179 (41)
Breslow thickness of primary tumor– no. (%)		
≤ 2 mm	61 (41)	158 (56)
> 2 mm	86 (58)	122 (43)
Unspecified	1 (1)	1 (1)
Ulceration of primary tumor– no. (%)		
Positive	48 (32)	64 (23)
Negative	100 (68)	217 (77)
Subtype of primary tumor – no. (%)		
Superficial spreading melanoma	52 (35)	115 (41)
Lentigo maligna melanoma	15 (10)	40 (14)
Acrolentigous melanoma	6 (4)	10 (4)
Nodular melanoma	35 (24)	48 (17)
Unspecified	40 (27)	68 (24)
AJCC stage		
I	55 (22)	149 (34)
II	93 (37)	132 (30)
III	69 (27)	108 (25)
IV	37 (14)	50 (11)

6.2.2 Identification of Best Biomarker Candidates for AJCC Stage I to IV

Our lab had previously identified six prognostic biomarkers based on the established tissue microarray (TMA). To further understand the role of these markers in melanoma progression and to select the best stage-specific prognostic markers, we compared the expression patterns of these six biomarkers in primary and metastatic melanomas. As shown in Figure 6.2, BRAF and MMP2 proteins show a progressive increase from Stage I to Stage IV, whereas Tip60 loss is most pronounced in metastatic melanoma (Stage III and IV) as compared to primary melanomas (Stages I and II). In contrast, p27, Dicer, and Fbw7 show similar expression changes across different AJCC Stages. Interestingly, alteration of BRAF protein was found to be most dramatic between AJCC Stages I and II ($P < 0.001$, χ^2 test), and strong BRAF expression accounts for only 23.6% in Stage I, as compared to 57.0% in Stage II (Figure 1A). This phenomenon may be explained by the fact that activation of the MAPK signalling pathway caused by BRAF increase promotes tumor cell growth and proliferation (Cohen et al, 2002; Satyamoorthy et al, 2003), thereby influencing the melanoma progression from AJCC Stage I to Stage II. Expression of the cell cycle inhibitor $p27^{Kip1}$ is also negatively regulated by Ras/Raf cascades (Zhang & Liu, 2002), and we found $p27^{Kip1}$ to be down-regulated in AJCC Stage II, as compared to Stage I (Figure 1C). This change, however, is not statistically significant ($P = 0.103$, χ^2 test), perhaps due to the limited case number.

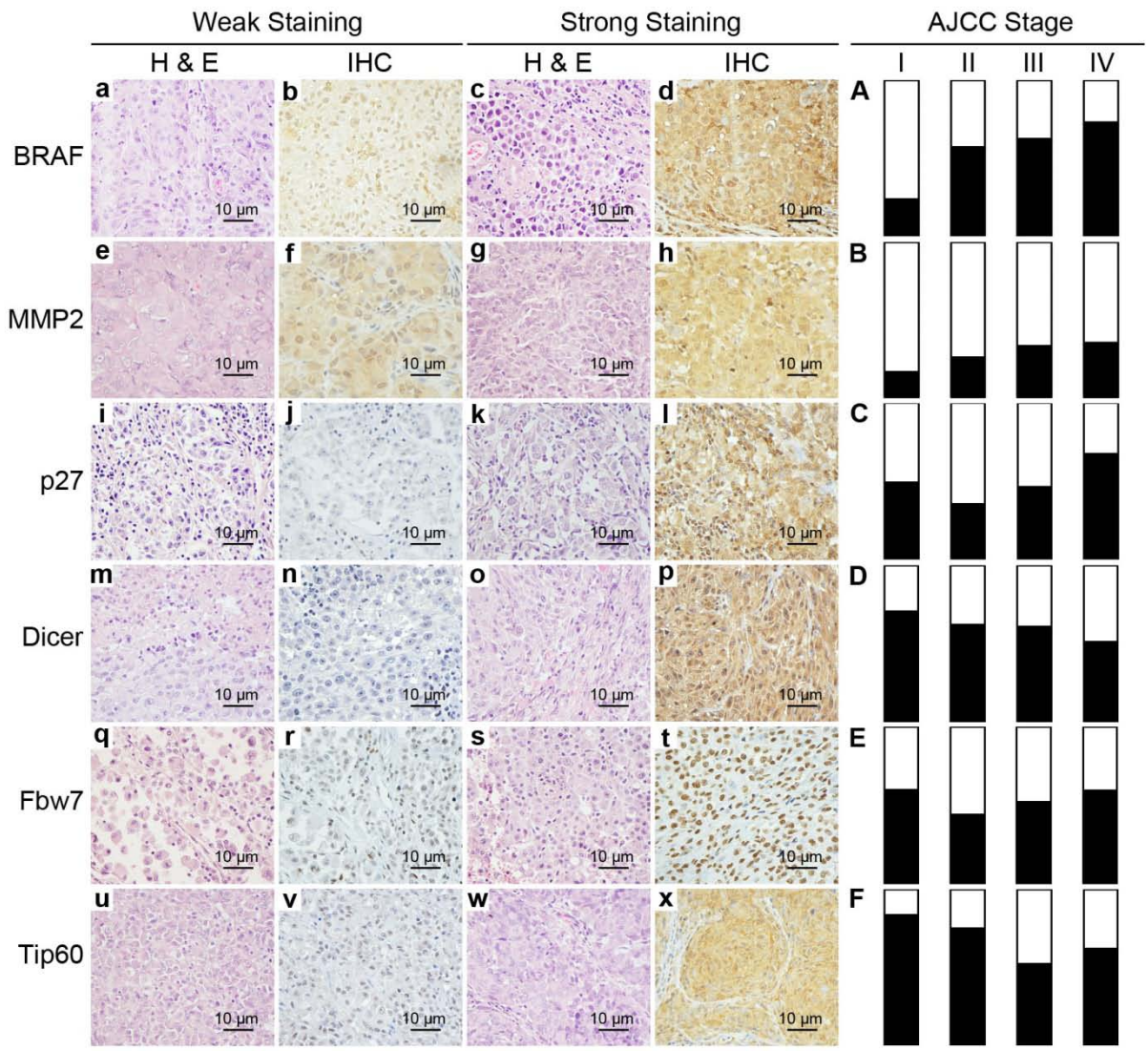

Figure 6.2 Expression levels of 6 biomarkers are changed across melanoma AJCC stages.

(a-x) Representative weak and strong immunohistochesistry stain and related haematoxylin and eosin stain images for BRAF (**a-d**), MMP2 (**e-h**), p27 (**i-l**), Dicer (**m-q**), Fbw7 (**r-u**) and Tip60 (**v-x**); (**A-E)** Percentage of weak and strong staining in AJCC Stage I, II, III and IV of 6 biomarkers.

6.2.3 Expression of Six Chosen Biomarkers are Significantly Changed During Melanoma Progression

To find the best biomarker for a specific AJCC Stage, we analyzed the prognostic correlation of 6 biomarkers by Kaplan-Meier survival and multivariate Cox-regression analyses adjusting for the important clinical variables, such as age, gender, ulceration, and tumor thickness. A discovery population of 254 patients was studied, and a single candidate marker for each specific AJCC Stage was selected based on the lower *P* value of multivariate analysis.

For Stage I, of the 6 makers, only BRAF protein expression emerged as a significant prognostic marker based on Log-rank test (Figure 6.3a) and showed the most significant *P* value compared with other markers in the multivariate Cox-regression analysis (Table 6.2). For Stage II melanomas, both MMP2 and P27 showed significant prognostic values based on survival and Cox-regression analyses (Table 2, Figure 6.4d and 6.4e), MMP2, however, appeared to have the stronger *P* values in both analyses (*P* = 0.004, Log-rank test, *P* = 0.001, Cox regression analysis; as compared to p27, *P* = 0.004, Log-rank test, *P* = 0.028, Cox regression analysis). For Stage III and Stage IV patients, only p27 emerged as having, or being close to having prognostic significance based on both analyses (*P* = 0.013 and 0.100, Log-rank test, in Stage III and IV, respectively; *P* = 0.024 and 0.068, Cox regression analysis, in Stage III and IV, respectively) (Table 6.2, Figures 6.5e, and 6.6e). Moreover, p27 cytoplasm expression was significantly increased in AJCC Stage IV, as compared to Stage III (P = 0.037, $\square^2$ test) (Figure 6.1C), suggesting that p27 is an important prognostic factor in advanced melanoma. This data is consistent with our previous report that cytoplasm p27 was

significantly associated with melanoma progression and a poorer 5-year patient survival (Chen et al, 2011b).

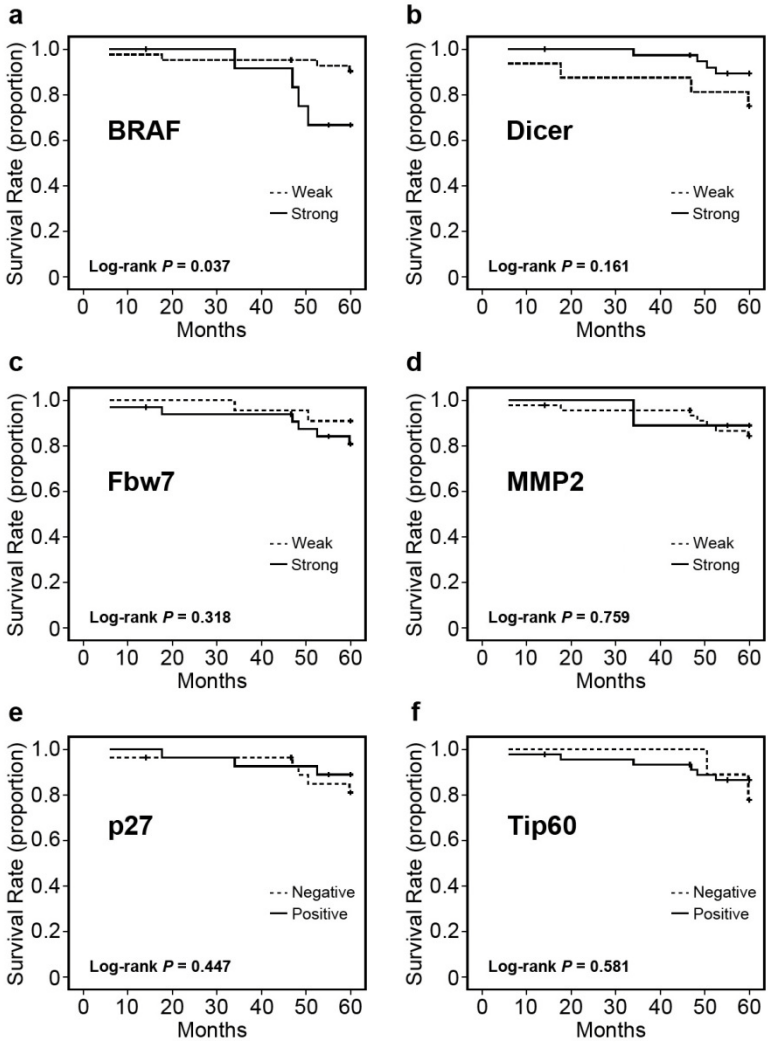

Figure 6.3 5-year survival analysis of six candidate markers in AJCC stage I melanomas in a discovering set of patients.

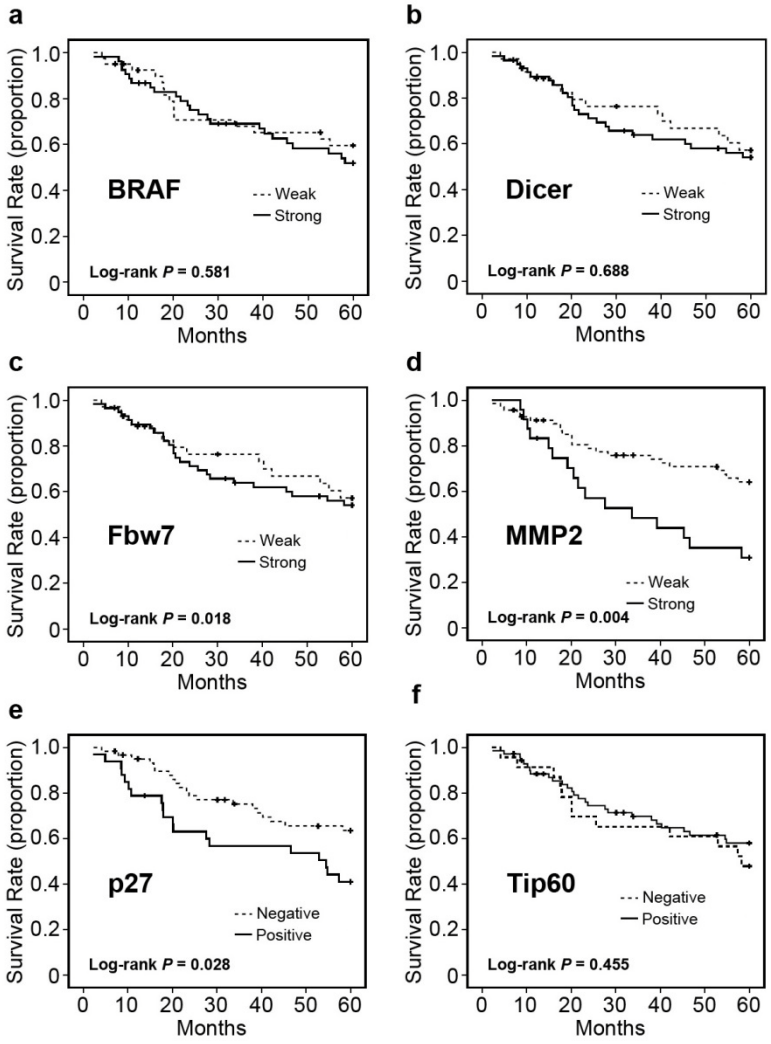

Figure 6.4 5-year survival analysis of six candidate markers in AJCC stage II melanomas in a discovering set of patients.

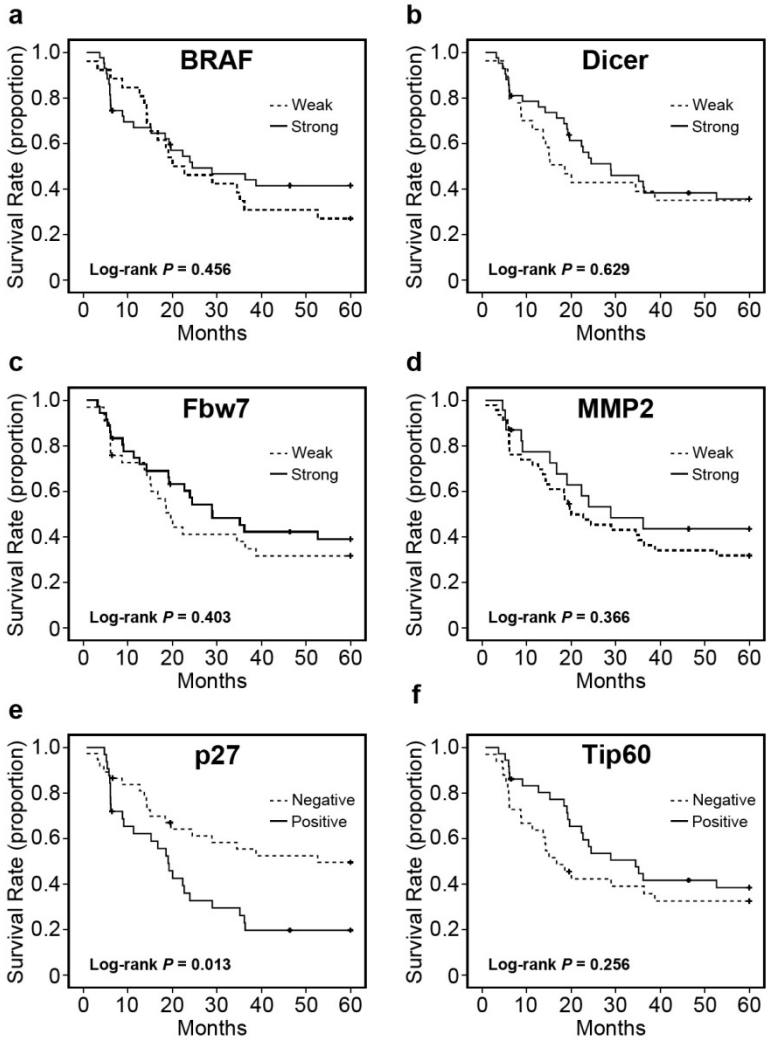

Figure 6.5 5-year survival analysis of six candidate markers in AJCC stage III melanomas in a discovering set of patients.

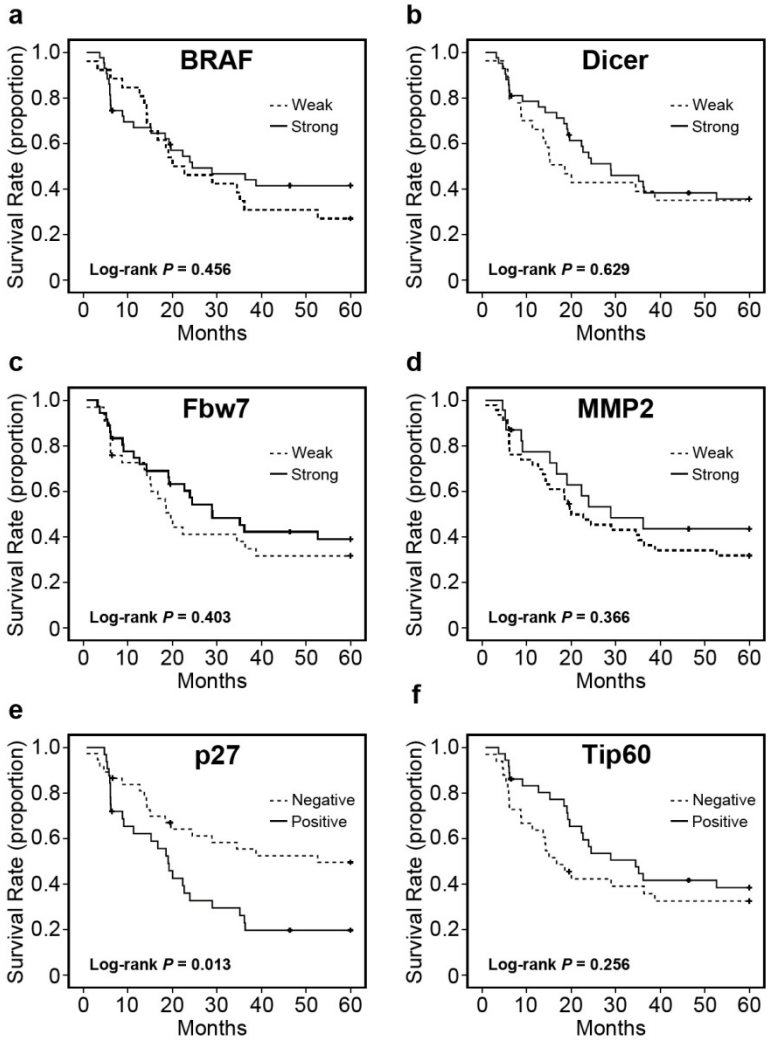

Figure 6.6 5-year survival analysis of six candidate markers in AJCC stage IV melanomas in a discovering set of patients.

Table 6.2 Comparison of prognostic value of candidate markers in each AJCC stage of melanoma in discovery patient cohort #

Factor	AJCC I (N = 55)			AJCC II (N = 93)			AJCC III (N = 69)			AJCC IV (N = 37)		
	HR	**95% CI**	**P value**	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
Age	1.01	0.96-1.06	0.784	1.01	0.99-1.03	0.406	0.99	0.97-1.02	0.504	1.02	0.99-1.06	0.225
Gender	1.19	0.23-6.26	0.834	1.04	0.50-2.20	0.915	1.81	0.93-3.53	0.080	3.05	1.11-8.39	0.031
Thickness	1.79	0.36-8.94	0.479	1.07	1.01-1.13	0.018	-	-	-	-	-	-
Ulceration	-	-	-	3.05	1.45-6.44	0.003	-	-	-	-	-	-
BRAF	3.86	0.81-18.30	**0.089***	0.81	0.38-1.70	0.575	0.87	0.46-1.65	0.673	0.91	0.38-2.17	0.824
Dicer	0.43	0.08-2.24	0.312	1.85	0.82-4.18	0.139	0.93	0.48-1.82	0.833	1.59	0.52-4.89	0.421
Fbw7	3.03	0.50-18.25	0.227	1.81	0.89-3.71	0.103	0.68	0.35-1.33	0.263	0.91	0.37-2.23	0.828
MMP2	1.55	0.14-17.75	0.726	3.85	1.69-8.77	**0.001***	0.94	0.42-2.08	0.874	0.95	0.36-2.53	0.923
P27	0.41	0.08-2.02	0.274	2.83	1.39-5.78	0.004	2.12	1.11-4.08	**0.024***	2.27	0.94-5.47	**0.068***
Tip60	0.55	0.06-4.79	0.588	0.41	0.18-0.93	0.033	0.91	0.44-1.90	0.799	0.36	0.11-1.16	0.085

*****: selected marker for confirmation analysis in expanded patient cohort

#: This patient cohort has survival data for all of the six candidate biomarkers

6.2.4 Conformation of Stage-specific Biomarkers

To confirm the prognostic value of each stage-specific marker, we performed additional analysis in additional patient, as well as combined population (discovery plus additional patients). Because BRAF expression emerged as the strongest biomarker for Stage I melanoma in the discovery phase, we performed both survival and multivariate Cox-regression analyses based on 73 Stage I patients (analysis was not executed in additional patients, because none of the 18 patients died, Table 6.3). As shown in Table 6.4, BRAF possesses a hazard ratio of 4.48 and a *P* value of 0.049 (95% CI: 1.01-19.91). Not surprisingly, 93.2% of patients with weak BRAF expression survived, as compared to 69.2% of those with strong BRAF expression survived, for at least 5 years ($P = 0.010$, Log-rank test).

Among 123 patients with Stage II melanoma, we performed confirmation analyses for MMP2 and p27, since both of them emerged to be significant biomarkers in the discovery population. MMP2 demonstrated a hazard ratio of 6.38 and 3.85 ($P = 0.002$, 95% CI: 1.39-4.45) in additional and combined cohorts, respectively (Table 6.3, Table 6.4), and significantly correlated with worse 5-year patient survival ($P < 0.001$, Log-rank test) (Figure 6.6). However, p27 did not show prognostic significance (Data not shown).

In Stage III and IV melanomas, the only marker emerging from the discovery phase was p27. Using the expanded patient population, this emergence was confirmed in Kaplan-Meier analysis for both Stage III and Stage IV population. In additional patients of Stage III and IV (27 and 12 patients in Stage III and IV, respectively), no significant influence was observed (Table 6.3), which may due to relatively small case number. However, for 95 patients had Stage III melanoma, cytoplasm p27 showed a hazard ratio of 1.78 ($P = 0.032$,

95% CI: 1.05-3.02, Table 6.4), and p27 expression dramatically affected the patient survival (*P* = 0.018, Log-rank test). For 49 Stage IV patients, the hazard ratio of p27 was 2.36 (*P* = 2.36, 95% CI: 1.14-4.87, Table 6.4), and p27 significantly correlated with poor patient survival (P = 0.046, Log-rank test). These data confirmed the observation that the best biomarkers for specific AJCC Stages I, II, III and IV, are BRAF, MMP2, p27 and p27, respectively.

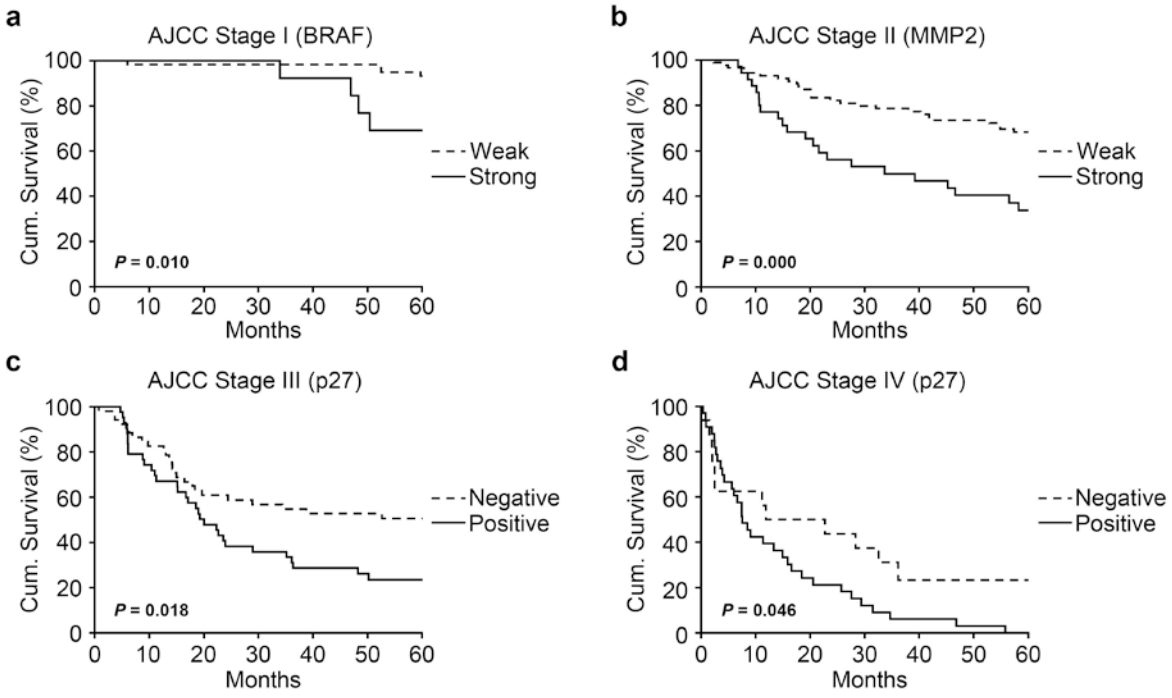

Figure 6.7 5-year Kaplan-Meier survival analyses for determined stage-specific biomarkers in expanded population of melanoma patients.

(**a**) Strong BRAF expression significantly correlates with worse 5-year survival in AJCC Stage I patients (73 patients, *P* = 0.010, log-rank test). (**b**) Strong MMP2 expression significantly correlates with worse 5-year survival in AJCC Stage II patients (123 patients, *P* = 0.000, log-rank test). (**c**) Strong cytoplasm p27 expression significantly correlates with

worse 5-year survival in AJCC Stage III patients (95 patients, $P = 0.018$, log-rank test). (**d**) Strong cytoplasm p27 expression significantly correlates with worse 5-year survival in AJCC Stage IV patients (49 patients, $P = 0.018$, log-rank test).

Table 6.3 Validation of prognostic biomarker for each AJCC stage melanoma in additional melanoma patients #

Factor	AJCC I (N = 18) *			AJCC II (N = 30)			AJCC III (N = 27)			AJCC IV (N = 12)		
	HR	**95% CI**	**P value**	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
Age	0	NA	1	1.08	0.03-3.07	0.30	0.38	0.46-4.41	0.54	0.44	0.11-2.94	0.51
Gender	0	NA	1	0.89	0.40-13.4	0.35	1.41	0.54-12.5	0.24	1.88	0.02-2.04	0.17
Thickness	0	NA	1	4.16	0.00-0.91	0.04	-	-	-	-	-	-
Ulceration	0	NA	1	2.34	0.05-1.46	0.13	-	-	-	-	-	-
BRAF	0	NA	1	-	-	-	-	-	-	-	-	-
MMP2	-	-	-	6.38	0.00-0.50	**0.012**	-	-	-	-	-	-
P27	-	-	-	-	-	-	0.91	0.19-1.79	0.34	2.14	0.03-1.64	0.14

#: In addition to the discovery cohort, which has data available for all 6 candidate biomarker; there are additional patients, for whom data is available for specific marker, but not for all 6 markers.

*****: No patient out of the 18 stage died, thus the Cox regression analysis was not able to be executed.

Table 6.4 Confirmation of prognostic biomarker for each AJCC stage melanoma in combined (discovery and additional cohorts) population

Factor	AJCC I (N = 73)			AJCC II (N = 123)			AJCC III (N = 95)			AJCC IV (N = 49)		
	HR	**95% CI**	**P value**	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
Age	1.01	0.96-1.06	0.710	1.00	0.98-1.02	0.749	0.99	0.98-1.01	0.421	1.00	0.98-1.03	0.674
Gender	1.81	0.41-7.94	0.432	0.86	0.47-1.59	0.638	1.81	0.84-2.43	0.183	3.05	0.97-4.50	0.061
Thickness	1.60	0.35-7.21	0.543	1.05	1.00-1.09	0.045	-	-	-	-	-	-
Ulceration	-	-	-	3.33	1.74-6.34	0.000	-	-	-	-	-	-
BRAF	4.48	1.01-19.91	**0.049**	-	-	-	-	-	-	-	-	-
MMP2	-	-	-	3.85	1.39-4.45	**0.002**	-	-	-	-	-	-
P27	-	-	-				1.78	1.05-3.02	**0.032**	2.36	1.14-4.87	**0.020**

6.3 Discussion

Studies focusing on melanoma biomarkers and high-throughput immunohistochemistry analysis have identified numerous biomarkers with prognostic value for melanoma. Our results show that no one individual prognostic marker is ideal for all AJCC Stages. For Stage I, the best marker is BRAF. For Stage II, the best marker if MMP2, and for Stages III and IV, the best marker is p27. It is currently unknown what biological functional properties of these markers underlie the stage-specific prognostic value. It is possible that the biological challenges to the melanoma cells are different during each step of melanoma progression and metastasis. Further studies are needed to elucidate this speculation.

BRAF is the most commonly mutated gene in melanoma (mutations Occur in 50-70%), and mutation of this gene (majority arising at codon 600) has been considered as the key somatic event in melanoma pathogenesis (Davies et al, 2002; Michaloglou et al, 2008; Yazdi et al, 2003). Targeting mutated BRAF and consequently shutting down the MAPK signaling pathway has been directly translated into therapeutic management in melanoma. This strategy has lead to the approval of two small molecule inhibitors of BRAF by FDA: vemurafenib and dabrafenib (Bollag et al, 2012; Chapman et al, 2011b; Hauschild et al, 2012). However, the protein expression profiles and the precise prognostic value of BRAF in melanoma remain largely unknown. Our group has previously reported that BRAF expression was increased in primary and metastatic melanomas, and predicted worse survival in patients with primary melanoma (Safaee Ardekani et al, 2013). Here, we have further shown that increased expression of BRAF protein (regardless of the mutation status) in Stage I melanoma is a significant prognostic marker, in that the higher the BRAF expression the more likely the patient will experience a worsened outcome and ultimate death. It has been

demonstrated that IHC analysis using anti-BRAF antibody was highly sensitive and specific for detection of BRAF V600E mutation in melanoma (Long et al, 2013). Our data suggested that IHC stain of BRAF protein could serve as a useful prognostic marker in early stage of melanoma as well. Recent studies have demonstrated BRAF to be important for tumor growth and maintenance in melanoma models (Hoeflich et al, 2006; Wellbrock et al, 2004), whereas BRAF seems to possess relatively low oncogenic activity as compared to RAS and PI3K (Stahl et al, 2004; Wu et al, 2003). These observations may have implications of the prognostic value of BRAF expression in Stage I melanomas.

As a well-known oncogene in cancer, MMP2 promotes tumor invasion and metastasis by digesting the extracellular matrix surrounding the malignant tissue (Deryugina & Quigley, 2006; Hofmann et al, 2000). Multiple studies have shown that MMP2 is among the strongest prognostic markers for cutaneous melanoma (Gould Rothberg et al, 2009; Vaisanen et al, 2008). Based on comparison of multiple markers in the same database, we further found the prognostic value of MMP2 to be specific to thick melanomas (Stage II), whereas for Stage I, its value is not apparent. MMP2 has no prognostic value for metastatic melanomas, and the mechanism underlying this phenomenon needs further investigation.

p27 encodes an inhibitor protein regulateing the cell cycle G_0-S phase transition and has been shown to be an atypical tumor suppressor when it is localized in the nucleus (Chu et al, 2008). However, in many cancers, p27 is mislocalized, and this mislocalization is associated with a poor prognosis (Chu et al, 2008; Wander et al, 2011). Multiple mechanisms control the loss of nuclear p27 and increase of cytoplasmic p27 (Wander et al, 2011). Chen G and et al. have shown that an increase of cytoplasmic p27 was associated with poor 5-year survival of metastatic melanoma patients (Chen et al, 2011b). Here we show that when it is

expressed in the cytoplasm, p27 is a predictor for worsened prognosis for AJCC Stage III and IV melanoma patients. Interestingly, its value for primary melanoma is not apparent. The fact that it is significant for both Stage III and Stage IV melanomas may indicate that these two melanoma stages face similar physiological challenges as reflected by the expression status of p27.

At present, the AJCC Stages are used to guide management of melanoma patients, especially when combined with detailed clinicopathologic characteristics. For Stage I and Stage II patients, ulceration status and individual tumor thickness as well as mitotic figures are used to guide if Sentinel-node biopsy (SLND) is needed along with localized tumor excision. If SLND is positive, the prognosis is much worsened (Balch et al, 2009). Although the 5-year survival of SLND-positive patients is widely variable from 64-91% (van der Ploeg et al, 2011), SLND is the standard procedure for stratifying primary melanoma patients. Our results showed that histological staining with BRAF (Stage I) and MMP2 (Stage 2) are strong prognostic factors. It is possible that histological stain, which is much easier and readily available, can provide similar or even better value as compared to SLND. It remains to be tested if these histological markers can replace the invasive and technically challenging SLND.

For patients at Stage III and Stage IV, there are at present few factors that can be used to guide clinical management. Our results indicate that p27 expression may identify unique patient subgroups of Stage III /IV patients who have low risks for mortality. Therefore, these subgroups might be selected for observation without going through invasive or toxic treatments. By contrast, the high risk group based on p27 expression might benefit from a more proactive treatment regimen. This information may also be a guide in selecting

appropriate patient populations to undertake future clinical trials in evaluating novel therapies.

Taken together, our study identified stage-specific biomarkers for cutaneous melanoma to further stratify patients into different risk subsets, which enables clinicians to treat selectively those patients more likely to develop distant metastatic disease.

Chapter 7: Conclusions

7.1 Summary of Findings

In Chapter 3, we analyzed the advantages and weaknesses of tissue microarray/ immunohistocheistry technologies and their applications in discovering and validating useful biomarkers in human cutaneous melanoma. In order to establish our platform for biomarker studiy, we constructed a TMA containing 707 samples (559 melanomas and 148 nevi controls) from various stages of melanocytic lesions, and a linked database with clinical follow up with a range of 5 to 20 years. Using TMA/IHC technology and the corresponding clinical database, we investigated the expression profile and prognostic significance of more than ten markers, including Skp2, Fbw7, p27, BRAF, KAI1, Dicer, Runx3, Tip60, and MMP2. Some of these prognostic markers identified have shown significant correlation with melanoma progression and clinical outcome, indicating they may be of promising value in clinical practice.

Among the biomarkers identified using our TMAs, we took special interest in Fbw7, an E3 ubiquitin ligase mediating turnover of multiple proto-oncogenes. Subsequently, we conducted studies on Fbw7. Significant correlation between Fbw7 loss and melanoma progression (shown by tumor thickness and AJCC stage) was discovered in the TMA study. The prognostic value of Fbw7 was also observed, loss of Fbw7 predicting significantly worse overall and disease-specific 5 year patient survival. To further understand the effects of Fbw7 expression on melanoma cell lines, *in vitro* functional experiments were performed. Our data demonstrated that the alpha isoform of Fbw7, located in the cell nucleus, is the dominant form expressed in melanoma. SRB assay showed no significant effects of Fbw7 expression on cell proliferation and trans-well chamber invasion assay demonstrated that the ability of

tumor cells to invade was also not affected by Fbw7α knock-down. However, knock-down of Fbw7α was found to promote melanoma cell migration and ERK phosphorylation was shown to be up-regulated. We also determined that selective MEK-inhibitor treatment (PD98059 and U0126) diminished cell migration induced by Fbw7α knock-down, indicating the MAPK signal pathway is indeed required in this process. Moreover, RhoA activity and stress fiber formation were markedly enhanced by Fbw7α knock-down, and abolished by MEK-inhibitor treatment as well, further confirming the role of MAPK pathway in Fbw7α regulated cell migration. Taken together, our findings indicate that Fbw7 is an important independent melanoma prognostic biomarker and that loss of Fbw7 is one of the events contributing to this disease. Activation of the ERK-MAPK signaling pathway by Fbw7 loss may be targeted in future treatment of metastatic melanoma.

Spurred by the numerous melanoma biomarkers identified in our laboratory, we further explored the potential value of these biomarkers, or combination of them in predicting clinical outcome. In Chapter 5, we found that loss of KAI1 and p27 was a common characteristic in higher stage melanoma (KAI1 expression drops from 95.24% in PM to 66.21% in MM, and p27 expression drops from 78.10% to 50.34%). Furthermore, loss of both KAI1 and p27 defines a high-risk subgroup of primary melanoma patients with worse survival (76.3% versus 52.1% in the KAI1-/p27- group and the control group, respectively). An independent validation group confirmed this data. In addition, we also found that loss of KAI1 and p27 in primary melanoma was significantly associated with tumor thickness, and loss of both proteins was an independent prognostic factor for melanoma patient survival.

In Chapter 6, we identified stage-specific biomarkers of melanoma by comparing previous biomarkers in the same patient cohort. Our data indicated that BRAF and MMP2

are the best prognostic biomarkers, in AJCC Stage I and II, respectively; but that in AJCC Stage III and IV, p27 emerges as the best biomarker to predict clinical outcome. Moreover, in each stage, BRAF, MMP2 and p27 correlated with worse 5-year melanoma-specific survival (P = 0.010, 0.000, 0.018, 0.046, respectively, Log-rank test). Our study for the first time identified stage-specific biomarkers in melanoma, which may assist clinicians to better stratify risk of disease progression and design personalized therapeutic modalities.

In sum, our TMA-based biomarker studies of melanoma identified a series of promising biomarkers with significant prognostic value in human cutaneous melanoma, and indicated some could serve as therapeutic targets. The study demonstrates that a combination of high-throughput tissue microarrays and the corresponding clinical database represents a powerful technological platform for clinically relevant biomarker discovery and validation.

7.2 Limitations of This Study and Future Directions

Due to the weakness of TMA/IHC technologies and lack of experiences in TMA, this study has several limitations. First of all, the clinical database we constructed is somewhat out of date to allow us to step into the targeted therapy age for melanoma treatment. Lagging behind the advanced technologies now available for melanoma, our database contains no information about the sentinel lymph node biopsy status, phenotype with key mutations, such as *BRAF* V600 or *NRAS* Q61 mutations, as well as the LDH level for metastatic melanoma patients. Limited information restrains our further investigation, (e.g. interesting biomarkers that can predict response of targeted small molecules), thus it is important to plan well ahead and collect samples and clinical information for certain purpose or questions to be addressed prior to TMA construction. Secondly, the quality of TMA study should be better controlled, according to the REMARK criteria (McShane et al, 2005a; McShane et al, 2005e). Proper

independent and internal control should have been used in IHC staining, and the specificity of antibody should have been carefully validated to ensure that the immunoreactions show true expression of biomarkers. Since alarge number of biomarkers have been identified, it is extremely important to find the right “promising” proteins up front, and avoid waste of time and effort caused by false positive data. Thirdly, except for a few biomarkers (RUNX3 and p300), most of the TMA-based biomarker studies do not have an independent validation cohort to confirm our results. Thus further confirmation using an additional set of TMA is needed to validate data from previous studies. Last but not least, we should consider other preservative conditions or methods to store these precious sections to avoid the loss of cores and antigens in cut TMA sections. For example, a nitrogen desiccator could be used to keep the activity of the antigen within the tissue.

Yet, we have discovered a novel function of Fbw7 in regulation of melanoma cell migration, through the ERK-MAPK signaling pathway. In future, it will be important to identify the direct substrate of Fbw7 in the MAPK cascades, which may represent a breakthrough in understanding the mechanism of melanoma progression and metastasis. Limited in time, we did not have a chance to examine thoroughly all the possible functions of Fbw7 in cancer progression. Therefore, more functional studies are required to understand fully the role of Fbw7 in melanoma, or other types of malignancies. In addition, a most recent study by Ellisen and colleagues has shown that Fbw7 and its substrate Mcl-1 control a dominant survival pathway in squamous cell carcinoma, and that a loss-function mutation on the *Fbw7* gene is associated with poor response to standard chemotherapy (He et al, 2013). This interesting study suggests Fbw7 could be a potential biomarker to predict response to clinical chemotherapy, through the regulation of its complicated downstream substrates.

TMA for specific purpose can be constructed to address particular questions. For example, we can compare biomarker expression in tissues from various areas of the tumor, such as radical versus vertical phases of melanoma, central versus edge of the melanoma nest. In this way, local expression of proteins can be compared, and the influence of heterogeneity of tumor can be evaluated. Another opportunity from use of TMA is discovering biomarkers that predict clinical response to novel targeted reagents. Although these targeted therapies are commonly targeting known molecules in signaling pathways, the profiles that those genes or proteins confer to drug resistance or patient response are largely unknown. Previous studies have shown that the proteins demonstrating response to targeted small molecules most likely lie downstream of the targeted molecule or in alternative signaling pathways. TMA is an ideal tool for screening possible predictive biomarkers.

TMA technology provides the possibility of investigating multiple biomarkers in up to thousands of tissue samples per patient, and dramatically improves the efficiency of biomarker studies. This thesis study demonstrates the effectiveness of TMA platform in biomarker discovery and validation: we identified a series of useful prognostic biomarkers of human cutaneous melanoma; we found the biomarker combination (KAI1 and p27) as an stronger (compared to when use as a individual biomarker) independent prognostic factor in primary melanomas; we determined the stage-specific biomarker in each individual AJCC stages of melanoma. There is no question that TMA will play an important role in defining biomarkers with prognostic and predictive value.

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Appendices

Appendix A: Publications based on the established TMA and clinical database, described in Chapter 3.

1. Chen G, **Cheng Y**, Zhang Z, Martinka M, Li G. Cytoplasmic Skp2 expression is increased in human melanoma and correlated with patient survival. PLoS One. 2011 Feb 28; 6 (2):e17578.
2. Zhang Z, Chen G, **Cheng Y**, Martinka M, Li G. Prognostic significance of RUNX3 expression in human melanoma. Cancer. 2011 Jun 15; 117 (12):2719-27.
3. Chen G, **Cheng Y**, Zhang Z, Martinka M, Li G. Prognostic significance of cytoplasmic p27 expression in human melanma. Cancer Epidemiol Biomarkers Prev. 2011 Oct;20(10):2212-21.
4. Chen G, **Cheng Y**, Zhang Z, Martinka M, Li G. Role of Tip60 in human melanoma cell migration, melanoma metastasis and patient survival. J Invest Dermatol. 2012 Nov;132(11):2632-41.
5. Lu J, Tang Y, Farshidpour M, **Cheng Y**, Zhang G, Jafarnejad S et al. JWA inhibits melanoma angiogenesis by suppressing ILK signalling and is an independent prognostic biomarker for melanoma. Carcinogenesis. 2013 Sep 24. [Epub ahead of print].
6. Lu J, Tang Y, **Cheng Y**, Zhang G, Yip A, Martinka M *et al.* ING4 regulates JWA in angiogenesis and their prognostic value in melanoma patients. Brit J Cancer. 2013 Oct 24. doi: 10.1038/bjc.2013.670. [Epub ahead of print].
7. Rotte A, Bhandaru M, **Cheng Y**, Sjoestroem C, Martinka M, Li G. Decreased expression of nuclear p300 is associated with disease pregression and worse prognosis of melanoma patients. PLoS One. 2013 Sep 30;8(9):e75405. doi: 10.1371/journal.pone.0075405.

8. Sjoestroem C, Khosravi S, **Cheng Y**, Ardekani GS, Martinka M, Li G. DLC1 expression is reduced in human cutaneous melanoma, and correlates with patient survival. Modern Pathology. Accepted.
9. Tang Y, **Cheng Y**, Martinka M, Li G. Prognostic significance of KAI1/CD82 in human melanoma and its role in cell migration and invasion through the regulation of ING4. Carcinogenesis. Accepted.