ROLES OF TUMOUR SUPPRESSOR APAF-1, ONCOGENES ILK AND AKT

EXPRESSION IN HUMAN MELANOMA PROGRESSION

By

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ABSTRACT

The incidence of cutaneous malignant melanoma is increasing more rapidly than any other tumor. Malignant melanoma is a life-threatening skin cancer due to its highly metastatic characteristics and resistance to radio- and chemo-therapy. It is believed that the ability to evade apoptosis is the key mechanism for the rapid growth of cancer cells. However, the exact mechanism for failure in the apoptotic pathway in melanoma cells is unclear. In the current study, we used tissue microarray (TMA) and immunohistochemistry to evaluate the expression patterns of three apoptosis-related genes in melanocytic lesions, including the tumour suppressor Apaf-1, oncogenes integrin-linked kinase (ILK) and the activated form of Akt (phospho-Akt Ser-473). Our data showed that Apaf-1 expression is significantly reduced in melanoma cells compared with normal nevi. We also found that in melanomas, strong ILK expression is significantly associated with tumour thickness. Strikingly, our TMA study on p-Akt indicated that strong p-Akt expression was associated with melanoma progression and invasion. Furthermore, strong p-Akt expression is inversely correlated with disease-specific 5-year survival of patients with primary melanoma. These data, coupled with a number of functional studies demonstrating an essential role of Akt activity in melanomagenesis, implicate that Akt signaling may serve as a promising therapeutic target for malignant melanoma.
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<tr>
<td>Apaf-1</td>
<td>apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
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<tr>
<td>CART</td>
<td>classification and regression tree</td>
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<tr>
<td>DTIC</td>
<td>alkylating agent dacarbazine</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase-3</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis protein</td>
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<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
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<tr>
<td>p-Akt</td>
<td>phospho-Akt</td>
</tr>
<tr>
<td>PDK-1</td>
<td>phosphoinositide-dependent protein kinase-1</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol (3,4,5) trisphosphate</td>
</tr>
<tr>
<td>PI-3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>TMA</td>
<td>tissue microarray</td>
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<td>UV</td>
<td>ultraviolet</td>
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This thesis is based on following papers:


   **Contribution:** I conducted immunohistochemistry, collection of patients clinicopathological information, statistical analysis and writing of the manuscript. M. Martinka helped to construct TMA, collection of patients clinicopathological information and Apaf-1 staining evaluation. J.A. Bush did the in vitro chemosensitivity assays. This project was supervised by Dr. Gang Li.


   **Contribution:** I conducted immunohistochemistry, collection of patients clinicopathological information, statistical analysis and writing of the manuscript. N. Makretsov, E. I. Campos, M. Martinka and D. Huntsman helped in TMA construction and evaluation of ILK staining. C. Huang and Y. Zhou helped in collection of patients clinicopathological information. This project was supervised by Dr. Gang Li.

Contribution: I conducted TMA construction, immunohistochemistry, collection of patients clinicopathological information, statistical analysis and writing of the manuscript. M. Martinka helped in TMA construction, collection of patients clinicopathological information and evaluation of p-Akt staining. This project was supervised by Dr. Gang Li.

Signature of Student

Signature of Research Supervisor
1.1 Cutaneous Malignant Melanoma

1.1.1 Melanoma Incidence

Cutaneous malignant melanoma is a most dangerous form of skin cancer deriving from melanocytes through malignant transformation. The incidence of malignant melanoma is increasing more rapidly than any other tumours in Caucasian population (Rigel et al., 1996). It is estimated that the life-time risk for melanoma has increased by 15-fold in the past 60 years, reaching one in 68 in America (Glass Hoover, 1989; Jemal et al., 2003; Koh and Geller, 1995, Rigel et al., 2003). In 2003, there were approximately about 54,200 and 4,250 new cases of melanoma to be diagnosed in USA and Canada, respectively (Jemal et al., 2003; National Cancer Institute of Canada: Canadian Cancer Statistics 2004, Toronto, Canada, 2004).

1.1.2 Biology of Melanocytes

Melanoma develops as the result of uncontrolled proliferation of transformed melanocytes, which are derived from the neural crest during development. Melanocytes are key components of the skin’s pigmentary system through their ability to produce melanin. These cells are located in the basal layer of the epidermis and form long dendritic processes that contact primarily with the surrounding keratinocytes, with a ratio of 30-40 keratinocytes to one melanocytes. Under the control of locally produced peptide hormones α-melanocyte-stimulating hormone or adrenocorticotropic hormone (Lerner and Mcguire, 1961; Lerner and McGuire, 1964), melanocytes produce pigment melanin in elongated, membrane-bound organelles known as melanosomes. Melanin is then
packaged into granules which are moved down dendritic processes and delivered to neighboring keratinocytes (Hearing, 1999). In the inner layers of the epidermis, melanin granules form a protective cap over the outer part of keratinocyte nuclei. In the stratum corneum, melanin granules are uniformly distributed to form a ultraviolet (UV)-absorbing blanket which reduces the amount of radiation penetrating the skin (Kawada et al., 1994; Khlgatian et al., 2002; Ortonne, 2002). Interestingly, recent evidence suggested that melanocytes may also serve as important local regulators of skin cells in addition to their ability to produce melanin. Upon UV irradiation and other stimuli, they are able to secrete a wide range of signal molecules, including cytokines, pro-opiomelanocortin peptides, catecholamines, and nitric oxide. Potential targets of these secretory products are keratinocytes, lymphocytes, fibroblasts, mast cells, and endothelial cells, all of which express receptors for these signal molecules (Slominski et al., 1993). Melanocytes may therefore act as important local regulators of skin homeostasis (Tsatmali et al., 2002).

1.1.3 Biology of Normal Nevi and Dysplastic Nevi

A melanocytic nevus (mole) is a benign proliferation of melanocytes. There is a consistent rise in the risk of malignant melanoma of the skin with an increasing number of nevi. It is estimated that 50% of melanomas have a melanocytic nevus as a precursor lesion. Clinically, normal nevi are small, usually less than 6 mm. They are usually flat or slightly raised and have even pigmentation or flesh coloration with sharply circumscribed borders. Nevi usually show three distinct morphologies following their development: junctional nevi, compound nevi and dermal nevi. Junctional nevus is the most primitive
form of nevus, characterized by a benign proliferation of nevus cells along the dermoepidermal junction. These cells have all the features of melanocytes, including the production of melanin and the presence of dendritic processes. A more developed nevus is compound nevus, showing both junctional proliferation and the presence of nevus cells in the dermis. Compound nevus begins to show evidence of hyperplasia with cords and nests of cells extending into the dermis. Dermal nevus is a more advanced nevus which has lost connection to the epidermis and is present in dermis only. It exists primarily as proliferating masses, nests and cords of cells. As the cells mature, they become less dendritic and lose the ability to generate pigment. Many purely dermal nevi may have relatively little pigment; instead, the cells become more elongated and resemble nerve cells and express nerve specific enzymes, such as cholineesterase.

Dysplastic nevus, also referred to as atypical nevus, is a mole that has begun to grow abnormally. They are acquired nevi, most developing between the age of 12 and 30, which are seen in about 4 percent of the Caucasian population in the United States. The appearance of a dysplastic nevus is different from that of a normal nevus: A dysplastic nevus is generally larger than an ordinary mole and has irregular and indistinct borders; its color frequently is not uniform and ranges from pink to dark brown; it is usually flat, but parts may be raised above the skin surface. Some of the dysplastic nevi even have the ability for spreading to adjacent parts of the skin, or through the blood and lymph circulation to other organs. Dysplastic nevi are considered potential melanoma precursors and patients with dysplastic nevi are considered to have an increased lifetime risk for melanoma. In sporadic cases of dysplastic nevi, a Caucasian living in the USA
has a 10% life-time risk of developing melanoma. In patients with familiar melanoma, the risk exceeds 50%.

1.1.4 UV Radiation and Melanogenesis

Sun and UV radiation exposures are the major environmental risk factors for melanoma. It is estimated that more than 80% of melanoma cases in Australia are due to sun exposure (Armstrong and Kricker, 1993). Intensive studies have implied that sun exposure is important in all stages of melanoma development from the initiation of nevi through invasive melanoma. Sunlight is a continuous spectrum of electromagnetic radiation that can be divided into three major regions of wavelength: the infrared, visible and UV. UV radiation, which is the most significant region that contributes to melanomagenesis, can be further subdivided into UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm) wavebands. Since the atmospheric ozone layer of the earth can block the UV light below about 300 nm, the UVC radiation cannot reach the surface of the earth, thus contributing little to the development of melanoma. Unlike UVC, UVA and UVB both reach the earth's surface in large enough amounts to cause harmful biological effects on the skin. UVB is considered to represent the most carcinogenic waveband related to melanoma risk. Nucleic acids and proteins both absorb light within the UVB range, peaking at 260 and 280 nm, respectively. Absorption of UVB by DNA causes damage that, if not repaired, can become initiating mutations in skin. UVB causes two types of DNA lesions: the cyclobutane pyrimidine dimmers and the pyrimidine (6-4) pyrimidone photoproducts (Tornaletti et al., 1996; Matsumura and Ananthaswamy, 2002). Cyclobutane pyrimidine dimers are considered to be more carcinogenic than the 6–4
photoproducts, forming almost three times as often and being repaired less efficiently (Rosenstein and Mitchell, 1987; You et al., 2001). Both types of lesions can lead to genetic mutations such as the C→T or CC→TT transitions; the latter mutation represents the hallmark of UV-induced mutagenesis. Besides these signature transitions, UVB can also induce C→A and G→T transversions and DNA strand breaks (Rosenstein and Mitchell, 1987; Linge, 1996; Cleaver and Crowley, 2002; Matsumura and Ananthaswamy, 2002). In comparison with UVB, unique UVA signature mutations have not been described. UVA is poorly absorbed by DNA, and produces predominantly oxidative lesions by photosensitization mechanisms (Cadet et al., 1997), which can be repaired by base excision repair (BER). UVB and UVA also elicit different transcriptional (Tyrrell, 1996) and inflammatory responses (Krutmann and Earnshaw, 2000), and have different effects on the immune response (De Fabo and Noonan, 1983).

Epidemiologic evidence has related the development of melanoma to intermittent over-exposure to UV (sunburns), rather than the total accumulated UV dose in a life-time (Holman and Armstrong, 1984). It has been hypothesized that childhood may be a particularly susceptible time for sun exposure (Armstrong and Kricker, 2001; Whiteman et al., 2001), but recent studies indicated that there is no difference in risk of melanoma between sunburns in childhood and older ages (Pfahlberg et al., 2001, Elwood and Jopson, 1997). Melanin, a brown-black pigment, is synthesized in melanosomes within epidermal melanocytes, and then distributed to surrounding keratinocytes through melanocytic dendritic processes (Hearing, 1999). Melanin can absorb UV photons before they interact with other cellular components (Kawada et al., 1994; Ortonne, 2002). Under normal conditions, prolonged sun exposure allows melanocytes to increase
melanin production as well as the transportation of melanin to form protective caps above the nuclei of suprabasal keratinocytes. However, during intense intermittent exposure, melanocytes apparently do not have time to synthesize melanin to protect surrounding keratinocytes as well as themselves. Under this circumstance, melanocytes receive large doses of UV radiation without protection from increased melanin synthesis. In addition, melanocytes contain several prosurvival or antiapoptotic proteins, which may inhibit cell death following intense UV exposure (Klein-Parker et al., 1994; Plettenberg et al., 1995; Morales-Ducret et al., 1995) and allow the heavily damaged melanocytes to survive. In addition, UV irradiation may also increase the chances of melanocytes transformation by stimulate melanoma genesis by modulating growth factors, inhibiting the endogenous antioxidant system, or inhibiting cell-mediated immunity (Berking et al., 2001).

1.1.5. Melanoma Progression, Treatment and Prognosis

The clinical and histological features of melanoma development and progression have been well described, and a sequence of steps has been proposed: step 1, common acquired and congenital nevi with structurally normal melanocytes; step 2, dysplastic nevi with structural and architectural atypia; step 3, radial growth phase primary melanoma without metastatic tendency; step 4, vertical growth phase of primary melanoma with tendency for metastasis; and step 5, metastatic melanoma (Clark, 1991). The progression from each stage to the next is associated with specific biologic changes, which are based on experimental models and clinical and histopathologic observations. In the stage of common nevi, the growth of nevus cells begins with limited and controlled proliferation of cells in the epidermis. After penetrating into the dermis, the nevus cells withdraw from the cell cycle and undergo a final terminal differentiation (Prieto et al.,
However, in the stage of dysplastic nevi, it appears that nevus cells grow following a completely different pathway and some dysplastic nevi appear to be precursor lesions of malignant melanoma (Clark et al., 1978, Clark et al., 1984). In the radial growth phase, melanoma cells show radial spread, usually confined to the intraepidermal compartment, or the cells may invade the dermis, but do not form a nodule. In this stage, the disease is still localized and restricted to the skin, and surgical excision can result in clinical cure (Clark et al., 1989; Rossi et al. 1997). In the vertical growth phase, the lesion develops vertically, forming a true tumour. The melanoma in this stage has an important negative impact on patient survival. In general, when melanoma cells reach the vertical growth phase, they have also acquired metastatic potential.

To date, the success of systemic therapy for melanoma remains unsatisfactory. Melanoma is associated with one of the highest mortality rates, particularly for advanced disease. Melanoma can rapidly metastasize to other organs such as lung, liver, brain, bone and small intestine and is highly resistant to radiation and chemotherapy (Schadendorf et al., 1994; Serrone and Hersey, 1999; Garbe, 1993; Helmbach et al., 2001). Although early melanomas are curable with surgical excision (Balch et al., 2001), up to 20% of patients will develop metastatic tumours (Houghton and Polsky, 2002). Patients with metastatic melanoma have a poor prognosis, with a median survival of only 6-10 months (Jemal et al., 2002). To date, the melanoma prognosis relies largely on clinical and pathological parameters. The clinical melanoma stages at diagnosis is still the most important prognostic factor; the 10-year overall survival is 71% for patients with AJCC stages I and II disease, 30% for stage III disease, and about 10% for stage IV disease (Balch, 1992; Balch et al., 2001). For primary melanomas, the most important
prognostic factors were ulceration, Breslow thickness, age, primary site, and sex. Breslow thickness is usually used as the indicator of the degree of melanoma invasion in primary tumours. It is measured in millimetres from the overlying granular layer of the epidermis to the deepest easily identifiable tumour cells with an ocular micrometer. It can be divided into four categories: ≤0.75 mm, low risk; 0.75-1.50 mm, intermediate risk; 1.5-4 mm, high risk; >4 mm, very high risk. So far, the Breslow thickness is the best indicator of prognosis in primary malignant melanoma (Balch et al., 2001).

1.2 Apoptosis and Melanoma

1.2.1 Melanoma: A Chemo-resistant Tumour

The main obstacle in treating melanoma especially for advanced diseases is its resistant characteristic to chemotherapy (Gilchrest et al., 1999; Li et al., 2002). The alkylating agent dacarbazine (DTIC) was the only FDA-approved drug for the treatment of malignant melanoma as a single agent, only deliver an overall response rate ranging from 10% to 20% and complete remissions in only 5% of patients (Anderson et al., 1995; Serrone et al., 2000). Temozolomide, a DTIC derivative, has been shown the improved response to brain metastasis, but it does not significantly increase patients overall survival (Atkins et al., 2002). Other chemotherapeutic drugs that have been tried for melanoma treatment include nitrosoureas (carmustine, lomustine), vinca alkaloids (vincristine, vinblastine), platinum compounds (cisplatin, carboplatin) and taxanes (Taxol, docetaxel), but none of these single agents showed an improvement over DTIC (Becker, et al. 2000). Although various combination chemotherapies showed increased response
rates in some studies, none of these succeeded in prolonging survival (Lakhani et al., 1990; Middleton et al., 2000).

Experiments both in vitro and in vivo further confirmed that the chemo-resistant characteristic of melanoma cells is either intrinsic or develops during application of chemo-drugs. To predict tumour cell sensitivity in well-established human melanoma cell lines, Schadendorf et al. (1994) used a soft agar culture system and demonstrated that there was a high degree of resistance in melanomas against all cytostatic drugs studied, suggesting the presence of intrinsic cellular mechanisms confer drug resistance. Another in vitro study by Kern et al. (1997) showed that exposure of human melanoma cells increases drug resistance in a dose-dependent manner. In vivo experiments using melanoma xenografts derived from patient tumours have confirmed the resistance of melanoma cells to chemotherapeutic drugs (Osieka, 1984). The reasons for the chemoresistant phenotypes in melanomas are still largely unknown and the underlying cellular resistance mechanisms need to be urgently clarified.

1.2.2 Dysregualtion of Apoptosis and Chemo-resistance

Most chemotherapy drugs ultimately act through induction of apoptosis (programmed cell death) to kill susceptible cells (Fisher, 1994; Houghton, 1999; Kaufmann et al., 1999). Although the molecular mechanism for drug resistance in melanoma is still poorly understood, it appears that the low therapeutic efficacy in this disease is likely due to a relative inability to induce apoptosis (Soengas and Lowe, 2003, Li et al., 1998). Apoptosis is a type of cell death involving characteristic morphological
and biochemical changes which proceeds in part via aggregation and multimerization of upstream death effector molecules that sequentially activate a cascade of caspases (Rathmell, 2002). It is characterized by cellular shrinking, condensation and margination of the chromatin and ruffling of the plasma membrane. Eventually, the cell becomes divided into the apoptotic bodies which consist of cell organelles and nuclear material surrounded by an intact plasma membrane (Van Cruchten et al., 2002). Apoptosis plays an important role in development, homeostasis and anticancer protection of multicellular organisms. In melanoma, tumour cells within lesions demonstrate an inherently low level of spontaneous apoptosis (Mooney et al., 1995; Staunton and Gaffney, 1995), and resistance to apoptosis has been correlated with increased metastatic potential in animal models of melanoma (Glinsky et al., 1997). There is also evidence for increased resistance to apoptosis in melanocytic nevi compared to isolated melanocytes (Alanko et al., 1999), suggesting that the acquisition of apoptosis resistance may be an early step in the malignant transformation.

\(P53\) was the first tumour suppressor gene linked to apoptosis. Mutations in \(p53\) were found to be associated with advanced tumour stage and poor patient survival in a broad spectrum of human malignancies (Schmitt and Lowe, 1999; Lowe and Lin, 2000; Wallace-Brodeur and Lowe, 1999). In the skin, excessive exposure to UV radiation induces apoptosis, which presumably serves to eliminate heavily damaged cells. Thus, tumour suppressors like \(p53\) engage apoptotic pathways to prevent the accumulation of mutated or oncogenically transformed cells (Lowe et al., 1994; Soengas et al., 1999). Furthermore, Ziegler et al. showed that loss of \(p53\) function in UV-damaged skin leads to
the survival of damaged cells thereby initiating tumour development. Hence, loss of apoptotic function can impact tumour initiation, progression and metastasis.

However, contrary to other malignancies, p53 mutations are very rare in melanoma (Albino et al., 1994; Montano et al., 1994; Ragnarsson-Olding et al., 2002), suggesting that dysregulation of other apoptotic regulators rather than p53 itself may contribute to distorted apoptosis of melanoma cells and thus the disease progression. In recent years, application of molecular techniques has revealed the involvement of several genes in this process. These molecules can be categorized into two main groups based on their functions: (1) Pro-apoptotic effectors, i.e. molecules that induce apoptosis; and (2) Pro-survival effectors, i.e. molecules that prevent apoptosis (Table 1.1). The fate of a cell depends on a complex net balance of these positive and negative regulators which will determine whether or not apoptosis is ultimately engaged.

1.2.3 Tumour Suppressor Apaf-1

Apoptotic protease activating factor-1 (Apaf-1) gene was first identified in 1997 from HeLa cell extracts as a cytochrome-c-dependent activator of caspases (Zou et al., 1997). Apaf-1 is a 130 kDa protein, which comprises an N-terminal caspase recruitment domain (CARD), followed by a region with high homology to CED-4 and a C-terminal domain containing multiple WD-40 repeats (also known as beta-transducin repeats), which are involved in protein–protein interactions. The CARD is a protein fold consisting of α-helical bundle, which act as a docking region to recruits caspase-9 (Hofmann et al., 1997). The central CED-4 domain of Apaf-1 includes a DNA binding domain which
dATP/ATP binds to. In addition, the COOH-terminal portion of Apaf-1 has multiple WD-40 repeats in which can binds to cytochrome c. Apaf-1 is a crucial component of the mitochondrial mediated cell death pathway. Upon pro-apoptotic stimulation, cytochrome c is released from the mitochondria into the cytosol and binds Apaf-1 and in the presence of ATP, forming a ring-like complex with a sevenfold symmetry referred to as the 'apoptosome' (Acehan et al., 2002). The apoptosome binds and activates caspase-9, which in turn recruits and activates caspas-3 and/or -6 and -7, leading to apoptosis. In addition, Apaf-1 knockout mice exhibit embryonic lethality and defective brain development. Embryonic stem cells from these animals are resistant to several mitochondrially mediated apoptotic stimuli (Yoshida et al., 1998; Cecconi et al., 1998).

1.2.4 Oncogene ILK

The interactions between cell and extracellular matrix (ECM) regulate many physiological and pathological processes. Integrin-linked kinase (ILK), a key component of cell-ECM structure, was first identified using a yeast two-hybrid genetic screen in 1996. ILK interacts with the cytoplasmic domain of both the β1 and β3 integrins and is widely expressed in tissues throughout the body. ILK is a ~59KD protein kinase with four ankyrin repeats in its N-terminus, which are responsible for protein-protein interactions, followed by a phosphoinositide-binding motif normally present in pleckstrin-homology domains. The integrin-binding site is located in the extreme C-terminus, formed by the last two subdomains of the kinase catalytic domain (Hannigan et al., 1996).
ILK is believed to function as the effector of phosphatidylinositol 3-kinase (PI3-K) signaling, which positively regulates protein kinase B (PKB)/Akt activity and negatively regulates glycogen synthase kinase-3 (GSK-3) activity (Delcommenne et al., 1998). As a proto-oncogene, ILK is involved in several oncogenesis-related events, including suppression of apoptosis and promotion of cell survival, as well as cell migration and invasion (Yoganathan et al., 2002). It has been shown that over-expression of ILK in epithelial cells leads to anchorage-independent cell growth and cell cycle progression (Novak et al., 1998). ILK overexpression in mammary epithelial cells showed a highly invasive phenotype, which ultimately can lead to tumour formation in nude mice (Wu et al., 1998). So far, increased expression and activity of ILK have been correlated with malignancy in several human tumour types, including breast, prostate, brain, and colon carcinomas (Marotta et al., 2001; Graff et al., 2001; Troussard et al., 2001; Attwell et al., 2000).

1.2.5 Oncogene Akt

The oncogene PKB/Akt was originally characterized as the human homologues for the viral oncogene v-Akt (Staal, 1987). To date, three members of the Akt family have been identified, named Akt1, Akt2 and Akt3 (PKBα,β,γ) (Staal, 1987; Murthy et al., 2000). Although being products of different genes, they are all closely related to each other, sharing up to 80% of amino acid homology. Each isoform exhibits a pleckstrin homology (PH) domain in the N-terminal region and a kinase domain with two phosphorylation sites: Thr308 and Ser473. Thr308 and Ser473 phosphorylation occurs in response to growth factors and other extracellular stimuli, and is essential for maximal
Akt activation (Alessi et al., 1996). Typically, growth factors bind to receptors and activate the receptor associated PI3-K, leading to the production of the second messenger phosphatidylinositol (3,4,5) trisphosphate (PIP3), which in turn recruits Akt via its PH domain to the plasma membrane (Testa et al., 2001). Upon membrane localization, Akt is phosphorylated at Thr-308 by phosphoinositide-dependent protein kinase-1 (PDK-1), a kinase which is thought to be constitutively active. Thr308 is necessary for the kinase activation, but for full kinase activation, phosphorylation of Ser-473 is also required. The kinase phosphorylating this residue has not been identified yet, however, several findings suggested that ILK plays an important role in this process (Lynch et al., 1999; Persad et al., 2001).

Once activated, Akt modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression and cellular growth. As a key effector of PI3-K signaling pathway, Akt has been shown to play a critical role in controlling the balance between cell survival and apoptosis (Franke et al., 1997). Upon activation, Akt delivers anti-apoptotic signals by phosphorylating Bad and procaspase-9 (Datta et al., 1997), as well as the forkhead family of transcription factors such as AFX, FKHR, and FKHRL1, which in turn induce the expression of proapoptotic factors (Rena et al., 1999; Biggs et al., 1999; Wolfrum et al., 2003; Brunet et al., 1999; Kops et al., 1999). In addition, Akt can promote cell survival by indirectly activating the pro-survival transcription factor NF-κB through the phosphorylation of I-κB kinase (Romashkova and Makarov, 1999; Ozes et al., 1999). Besides its anti-apoptotic function, Akt also acts to promote cell proliferation and growth (Zhou et al. 2001; Liang et al. 2002; van Weeren et
al. 1998; Diehl et al., 1998; Ghosh-Choudhury et al. 1999) and angiogenesis (Sodhi et al., 2001; Kosmidou et al., 2001).

1.3 Objective

The objective of this study is to gain information on the relationships between tumour suppressor Apaf-1, oncogene ILK and Akt expression and melanoma progression. We, thus, used tissue microarray (TMA) technology and immunohistochemistry to evaluate the expression of Apaf-1, ILK and phospho-Akt (Ser-473) in different stages of human melanocytic lesions and correlates the expression pattern with the clinicopathological parameters. Furthermore, we also examined the prognostic value of these molecules to predict patient 5-year survival.
Table 1.1 Summary of pro-survival and pro-apoptotic molecules involved in melanoma and their probable mechanisms of action

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Action</th>
<th>Mechanisms of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>Pro-apoptotic</td>
<td>Interacts with Bax, cytochrome c, Apaf-1, Noxa and PUMA, leading to caspase activation</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Pro-apoptotic</td>
<td>Interacts with p53 pathway</td>
</tr>
<tr>
<td>Bax</td>
<td>Pro-apoptotic</td>
<td>p53/Bcl-2/Bcl-X1/c-Myc interactions</td>
</tr>
<tr>
<td>Bid</td>
<td>Pro-apoptotic</td>
<td>Activation of Bax and Bak</td>
</tr>
<tr>
<td>Noxa</td>
<td>Pro-apoptotic</td>
<td>Induction of mitochondrial release of cytochrome c, activation of Bax and Bak</td>
</tr>
<tr>
<td>Puma</td>
<td>Pro-apoptotic</td>
<td>Mitochondrial release of cytochrome c, activation of procaspase 9</td>
</tr>
<tr>
<td>TNF</td>
<td>Pro-apoptotic</td>
<td>TNF/TNF-R1/intracellular signalling transducers/caspase interactions</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Pro-apoptotic</td>
<td>Assembly of adaptor components, activation of caspases and release of cytochrome c into the cytoplasm</td>
</tr>
<tr>
<td>Fas and FasL</td>
<td>Pro-apoptotic</td>
<td>Cysteine proteases/ICE/caspase 8 proenzyme interactions, PITSLRE kinases</td>
</tr>
<tr>
<td>IFN</td>
<td>Pro-apoptotic</td>
<td>Activation of proteins involved in the apoptotic pathways, downregulation of antiapoptotic molecules such as Bcl-2</td>
</tr>
<tr>
<td>αvβ3</td>
<td>Variable</td>
<td>Altered expression of pro-apoptotic p27kip1 and its sequestering protein</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Variable</td>
<td>Downregulation of Bcl-2 and alteration of adhesion conditions</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Pro-survival</td>
<td>Apaf-1/caspases/Bax interactions, blocking c-Myc-induced apoptosis</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Pro-survival</td>
<td>Alteration of transcription of certain genes, inhibition of TNF-mediated apoptosis</td>
</tr>
<tr>
<td>Survivin</td>
<td>Pro-survival</td>
<td>Inhibition of caspases, association with the mitotic spindle, association with Cdk4</td>
</tr>
<tr>
<td>Livin</td>
<td>Pro-survival</td>
<td>Binds to caspases, inhibits processing of procaspase 9</td>
</tr>
<tr>
<td>Akt</td>
<td>Pro-survival</td>
<td>Activation of NF-κB and its downstream effectors</td>
</tr>
<tr>
<td>ML-IAP</td>
<td>Pro-survival</td>
<td>Inhibition of downstream caspases, interaction with SMAC</td>
</tr>
</tbody>
</table>
CHAPTER 2. MATERIAL AND METHODS

2.1 TMA Construction

Formalin-fixed, paraffin-embedded tissues from 16 human normal nevi, 66 dysplastic nevi, 204 primary melanomas, and 58 metastatic melanomas were used for our study. All specimens were obtained from the 1990-1998 archives of the Department of Pathology, Vancouver General Hospital. The use of human skin tissues in this study was approved by the medical ethical committee of the University of British Columbia and was performed in accordance with the Declaration of Helsinki Guidelines. The most representative tumour area was carefully selected and marked on the H&E-stained slide. The TMAs were assembled using a tissue-array instrument (Beecher Instruments, Silver Spring, MD). Duplicate 0.6-mm-diameter tissue cores were taken from each biopsy. Three composite high-density TMA blocks containing 107, 126, and 111 cases from a total of 344 patients were designed. Multiple 4-μm sections were cut with a Leica microtome and transferred to adhesive-coated slides. One section from each TMA was routinely stained with H&E. The remaining sections were stored at room temperature for immunohistochemical staining. For Apaf-1 and ILK studies, only the TMA slide containing 107 patients was used for immunohistochemistry. For p-Akt study, three TMA slides with a total of 344 cases were used for immunohistochemistry.

2.2 Antibodies

Rabbit polyclonal antibody against Apaf-1, ILK and p-Akt (Ser-473) were purchased from Pharmingen (Pharmingen, BD Biosciences, Ontario, Canada), Stressgen
(Stressgen, Victoria, British Columbia, Canada) and Cell Signaling (Cell Signaling Technology, Beverly, MA), respectively.

2.3 Immunohistochemistry of TMAs

The TMA slides were dewaxed by heating at 55°C for 30 min and by three washes, 5 min each, with xylene. Tissues were rehydrated by a series of 5-min washes in 100%, 95%, 80% ethanol and distilled water. Antigen retrieval was performed by heating the samples at 95°C for 30 min in 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. After blocking with universal blocking serum (DAKO Diagnostics, Mississauga, ON, Canada) for 30 min, the primary antibody against Apaf-1, ILK and p-Akt (Ser 473) were used at dilutions of 1:300, 1:100 and 1:100, respectively, 4°C overnight. The sections were then incubated with biotin-labeled secondary antibody and streptavidin-peroxidase for 30 min each (DAKO Diagnostics, Mississauga, ON, Canada). The samples were developed with 3,3'-diaminobenzidine substrate (Vector Laboratories, Burlington, ON, Canada) and counterstained with hematoxylin. Then, the slides were dehydrated following a standard procedure and sealed with coverslips. Negative controls were performed by omitting primary antibodies during the primary antibody incubation.

2.4 Evaluation of Immunostaining

The Apaf-1, ILK and p-Akt staining in TMAs was examined blinded by two to four independent observers (including one dermatopathologist) simultaneously, and a consensus score was reached for each core. For p-Akt staining, the positive reaction of p-
Akt was scored into 4 grades according to the intensity of the staining: 0, 1+, 2+, and 3+. The percentages of p-Akt positive cells were also scored into 4 categories: 0 (0%), 1 (1-33%), 2 (34-66%), and 3 (67-100%). The sum of the intensity and percentage scores is used as the final staining score. The staining pattern of the biopsies was defined as follows: 0, negative; 1-2, weak; 3-4, moderate; 5-6, strong. For Apaf-1 and ILK staining, because most of the biopsies showed homogenous staining, only staining intensity was evaluated: 0, negative; 1, weak; 2, moderate; and 3, strong. In the cases with discrepancy between duplicated cores, the higher score from the 2 tissue cores was taken as the final score.

2.5 Statistical Analysis of TMA

Statistical analysis was performed with the SPSS 11.5 software (SPSS, Chicago, IL). The $\chi^2$ test was used to compare the quantitative differences of staining in different stages of melanoma progression. The association between p-Akt staining and the clinicopathological parameters of the primary melanoma patients, including age, gender, tumour thickness, ulceration, histological subtype, and location, was also evaluated by $\chi^2$ test. The classification and regression tree (CART) (Gordon and Olshen, 1985; Averbook et al., 2002), Kaplan-Meier method and log-rank test were used to evaluate the correlations between Apaf-1, ILK and p-Akt expression and patient survival. Cox regression model was used for multivariate analysis. A $P$ value of <0.05 was considered significant.
CHAPTER 3*. REDUCED APAF-1 EXPRESSION IN HUMAN CUTANEOUS MELANOMAS

3.1 Rationale and Hypothesis

The main obstacle in treating melanoma is its resistant characteristic to conventional chemotherapy, with an overall response rate of only 20% to 40% (Gilchrest et al., 1999; Mc Clay and Mc Clay, 1996; Green and Schuchter, 1998; Li et al., 2002). Although the molecular mechanism for drug resistance in melanoma is still poorly understood, it appears that the low therapeutic efficacy in this disease likely relates to a relative inability to induce apoptosis (Li et al., 1998, Soengas and Lowe, 2003). In addition, resistance to apoptosis has been correlated with increased metastatic potential in melanoma (Glinsky et al., 1997). Recently, Apaf-1 gene (Zou et al., 1997), a downstream effector of p53, which links release of cytochrome c to activation of caspase-9 in mitochondrion-mediated apoptosis pathway (Li et al., 1997), is found to be inactivated in melanoma presumably by methylation (Soengas et al., 2001). Specifically, LOH of Apaf-1 alleles was detected in 42% of the metastatic melanoma specimens and more than 50% of cell lines that derived from metastatic melanoma showed negative Apaf-1 expression. Strikingly, restoring physiological levels of Apaf-1 through gene transfer or treatment with methylation inhibitor can dramatically enhances chemosensitivity in Apaf-1-deficient cell lines, which raises the possibility that restoring Apaf-1 regulation to some melanomas would have therapeutic benefit.

* A version of this chapter has been published. Dai DL, Martinka M, Bush JA, Li G. Reduced Apaf-1 expression in human cutaneous melanomas. Br J Cancer. 2004 Sep 13;91(6):1089-95.
To further investigate the role of Apaf-1 in melanoma progression, we, in the present study, used tissue microarray technology and immunohistochemistry to evaluate the Apaf-1 expression level in primary human melanoma at different stages.
3.2 Results

3.2.1 Clinicopathological Features of TMA

Initially, there are 87 human primary melanomas and 16 normal nevi were used for our TMA study. Due to loss of biopsy cores or insufficient tumour cells present in the cores, 70 cases of primary melanoma and 13 cases of nevi could be evaluated for Apaf-1 staining. For the 70 primary melanoma cases in which Apaf-1 staining was available, there were 38 male and 32 female, with ages ranging from 21 to 93 years (mean=58). For melanoma staging, we used Breslow thickness as our criteria for evaluating Apaf-1 expression: 53 tumours were ≤1.5 mm thick (low-risk melanoma) and 17 were >1.5 mm (high-risk melanoma) (Marghoob et al., 2000). Among the 70 cases, 32 were superficial spreading melanoma, 12 were lentigo maligna melanoma, and other 26 cases consisted of desmoplastic melanoma, acrolentigous melanoma, and nodular melanoma. In total, 14 melanomas were located in sun-exposed sites (head and neck), and 56 were located in sun-protected sites (trunk, arm, leg, and feet). Tumour ulceration was observed in nine cases (Table 3.1).

3.2.2 Apaf-1 Expression in Human Melanoma

We examined Apaf-1 expression in primary melanomas and normal nevi by immunohistochemistry. Various levels of Apaf-1 expression were observed in the cytoplasm of the biopsies (Figure 3.1). Among the 70 melanoma primaries, six cases (8.6%) showed negative (0), 36 cases (51.4%) weak (1+), 21 cases (30%) moderate (2+), and seven cases (10%) strong (3+) Apaf-1 staining. In contrast to melanoma primaries,
majority (76.9%) of the nevi had moderate or strong Apaf-1 expression (eight and two cases, respectively), while only one case stained negative and two cases showed weak Apaf-1 staining (Table 3.2). A significant difference in the staining pattern between tumours and nevi was observed ($\chi^2 = 6.02$, $P=0.014$).

3.2.3 Apaf-1 Expression and Clinicopathological Parameters or 5-year Survival

To assess whether reduced Apaf-1 expression is associated with melanoma progression, we examined Apaf-1 expression in 70 melanoma primaries at various stages of invasion. As shown in Table 3.1, Apaf-1 expression was distributed fairly evenly in the two Breslow thickness categories ($P>0.05$, $\chi^2$ test). Tumour ulceration is often considered an indicator for melanoma prognosis (Vihinen et al., 2003), but in our study, we did not find any correlation between Apaf-1 expression and tumour ulceration status ($P>0.05$, $\chi^2$ test). In addition, we did not find correlation between Apaf-1 expression with age, sex, tumour subtype, or location of tumours (sun-protected vs sun-exposed) (Table 3.1). To evaluate whether Apaf-1 staining might be related to patient survival, a Kaplan-Meier survival curve was constructed using overall 5-year disease-specific survival to evaluate the biopsies stained negative or moderate (0, 1+, 2+) vs those stained strong (3+) for Apaf-1 expression (Fig. 3.2). Our data did not show a correlation between Apaf-1 staining and 5-year patients survival ($P>0.05$, log-rank test).
Table 3.1 Apaf-1 expression and clinicopathological characteristics of 70 primary melanomas.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Neg. to Weak</th>
<th>Moderate</th>
<th>Strong</th>
<th>Total</th>
<th>P Value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apaf-1 Staining</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Variable</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤57</td>
<td>23 (64%)</td>
<td>9 (25%)</td>
<td>4 (11%)</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>&gt;57</td>
<td>19 (56%)</td>
<td>12 (35%)</td>
<td>3 (9%)</td>
<td>34</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20 (52%)</td>
<td>12 (32%)</td>
<td>6 (16%)</td>
<td>38</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>Female</td>
<td>32 (69%)</td>
<td>9 (28%)</td>
<td>1 (3%)</td>
<td>32</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>Tumour thickness (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤1.5</td>
<td>32 (60%)</td>
<td>14 (26%)</td>
<td>7 (13%)</td>
<td>53</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>&gt;1.5</td>
<td>10 (59%)</td>
<td>7 (41%)</td>
<td>0 (0%)</td>
<td>17</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>Ulceration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>36 (59%)</td>
<td>18 (30%)</td>
<td>7 (11%)</td>
<td>61</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>Present</td>
<td>6 (67%)</td>
<td>3 (33%)</td>
<td>0 (0%)</td>
<td>9</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>Tumour subtype(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSM</td>
<td>17 (54%)</td>
<td>12 (38%)</td>
<td>3 (9%)</td>
<td>32</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>LMM</td>
<td>7 (59%)</td>
<td>4 (33%)</td>
<td>1 (8%)</td>
<td>12</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>Other</td>
<td>18 (69%)</td>
<td>5 (19%)</td>
<td>3 (12%)</td>
<td>26</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>Site(^c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sun-protected</td>
<td>32 (57%)</td>
<td>18 (32%)</td>
<td>6 (11%)</td>
<td>56</td>
<td>(P&gt;0.05)</td>
</tr>
<tr>
<td>Sun-exposed</td>
<td>10 (71%)</td>
<td>3 (21%)</td>
<td>1 (7%)</td>
<td>14</td>
<td>(P&gt;0.05)</td>
</tr>
</tbody>
</table>

\(^a\) Chi-square test for negative to moderate versus strong Apaf-1 expression.

\(^b\) SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; Other includes desmoplastic melanoma, acrolentigous melanoma, and nodular melanoma.

\(^c\) Sun-protected sites: trunk, arm, leg, and feet. Sun-exposed sites: head and neck.
Table 3.2 Distribution of Apaf-1 expression in normal nevi and primary melanomas.

<table>
<thead>
<tr>
<th>Apaf-1 Staining</th>
<th>Neg. to Weak</th>
<th>Moderate</th>
<th>Strong</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal nevus</td>
<td>3 (23%)</td>
<td>8 (62%)</td>
<td>2 (15%)</td>
<td>13</td>
</tr>
<tr>
<td>Primary melanoma</td>
<td>42 (60%)</td>
<td>21 (30%)</td>
<td>7 (10%)</td>
<td>70</td>
</tr>
</tbody>
</table>
Figure 3.1 Representative images of Apaf-1 immunohistochemical staining in human melanocytic lesions. A, Normal nevus with moderate Apaf-1 expression. B, Primary melanoma with weak Apaf-1 expression. C, Primary melanoma with moderate Apaf-1 expression; D, Primary melanoma with strong Apaf-1 expression. Magnification, X400.
Figure 3.2 Correlation between Apaf-1 expression and 5-year patient disease-specific survival. Apaf-1 expression is not correlated to 5-year patient survival ($P>0.05$, log-rank test).
3.3. Discussion

Acquired resistance to apoptosis is a hallmark of cancers (Hanahan and Weinberg, 2000), which can allow cancer cells to survive and enable the establishment of metastasis or resistance to chemotherapy. Malignant melanoma is a particularly aggressive form of cancer in this regard. It is both highly metastatic and resistant to chemotherapy. Many chemotherapeutic agents, such as taxanes, vinca alkaloids and platinum-associated drugs, have failed to introduce a significant response in melanomas.

Defects in the apoptotic pathways can be the cause of tumour progression and resistance to chemotherapy. Inactivation of Apaf-1, a key effector of the intrinsic apoptosis pathway, and its effect on chemotherapy have been observed in several tumour types, including gastrointestinal cancer, leukemia, ovarian cancer and melanoma (Yamamoto et al., 2000; Jia et al., 2001; Soengas et al., 2001; Wolf et al., 2001; Liu et al., 2002). In this study, to better understand the role of Apaf-1 in melanoma development, we used TMA technology and immunohistochemistry to investigate Apaf-1 expression level in primary human melanoma biopsies. Our results demonstrated that Apaf-1 expression is significantly reduced in melanoma compared to normal nevi ($P=0.014$) (Figure 2). However, despite the Apaf-1 reduction in melanoma, our data showed that Apaf-1 expression is not related to melanoma thickness (Table 3.1) or 5-year patient survival (Figure 3.2), suggesting that Apaf-1 reduction is an early event of melanoma tumorigenesis, possibly at the initiation stage.

The reduction of Apaf-1 expression in melanoma biopsies observed in our study is in agreement with previous studies demonstrating inactivation of Apaf-1 in melanoma
The reversion of Apaf-1 expression by the methylation inhibitor 5-aza-2'-deoxycytidine in melanoma cells (Soengas et al., 2001) suggests that hypermethylation of Apaf-1 promoter or the upstream regulators may contribute to the reduction/loss of Apaf-1 expression in melanoma. Normal expression of Apaf-1 is an important component in the p53/mitochondrial intrinsic apoptosis pathway. Proapoptotic signal from p53 can result in the release of cytochrome \( c \) from the mitochondria. In the presence of cytochrome \( c \) and dATP, Apaf-1 binds to procaspase-9, leading to caspase-9 activation and initiation of a protease cascade (Li et al., 1997). Thus, reduced Apaf-1 expression in melanoma may terminate the apoptotic signal from mitochondria and disable the p53 apoptotic program.

Regardless the significant difference of Apaf-1 expression between normal nevi and primary melanomas, it is possible that the 13 cases of normal nevi in our study may not represent the whole normal nevi population due to the relative small case number. Apaf-1 expression in normal nevi is worth further investigating by including more normal nevi cases.

Our data that Apaf-1 reduction did not correlate with tumour thickness is in agreement with the findings by Fujimoto et al (2004), who showed that the pattern of Apaf-1 LOH did not correlate with tumour Breslow thickness, while Apaf-1 mRNA expression level was significantly lower in Apaf-1 LOH positive tumours. Contrarily, Baldi et al (2004) reported that reduced Apaf-1 expression correlated with melanoma thickness. The following reasons may result in the discrepancy between our results and
those by Baldi et al: (1) different antibodies used for immunohistochemical staining; and (2) different technical approaches for immunohistochemistry. We used TMA technique for our study and all the nevi and tumour biopsies were assembled in one slide. Therefore, all the samples had exactly the same treatment for each step during immunohistochemical staining. On the other hand, Baldi et al. used regular immunohistochemistry for their study, so there may exist inconsistency between different slides during the staining procedure for 106 biopsies. Future TMA studies of additional tumour biopsies will validate the role of Apaf-1 in melanoma progression.

Our finding that no significant correlation between reduced Apaf-1 expression and tumour thickness favours a multiple-event model that a number of important molecular changes occur sequentially during melanoma progression. Dysfunction of other components in the apoptotic pathway as well as the survival pathway may contribute to melanoma progression. For example, survivin, a member of the inhibitor of apoptosis protein (IAP) family, was strongly expressed in human melanomas but not in normal melanocytes, and overexpression of survivin in the sentinel lymph nodes from melanoma patients was inversely correlated with patient survival (Grossman et al., 1999; Gradilone et al., 2003). High expression of Bcl-2 antiapoptotic proteins, such as Bcl-2, Bcl-X\textsubscript{L}, and Mcl-1, may also contribute to melanoma progression and chemoresistance as antisense oligos against these genes can induce death of melanoma cells (Jansen et al., 1998; Heere-Ress et al., 2002; Thallinger et al., 2003). However, the implication of Bcl-2 antiapoptotic proteins as melanoma progression factors is controversial. While some studies indicated that Bcl-2 and Bcl-X\textsubscript{L} gene expression increases with progression of
malignant melanoma (Leiter et al., 2000; Utikal et al., 2002), others found that Bcl-2 and Bcl-X\textsubscript{L} did not correlate to progression of the disease (Gradilone et al., 2003). In the PI3K/AKT/PTEN survival pathway, AKT has been found to be constitutively activated in melanoma, which leads to upregulation of NF-\textkappa B and tumour progression (Dhawan et al., 2002). As the negative regulator of this pathway, PTEN expression was found to be reduced in melanoma biopsies and loss of PTEN can promote tumour growth \textit{in vivo} (Stahl et al., 2003; Tsao et al., 2003). Based on the complexity of the apoptotic and survival pathways that control the fate of a cell, additional studies on the timing of the gene inactivation/overexpression in these pathways from the same set of tumour biopsies and the interdependence among these events will provide a more complete picture of the molecular changes during melanoma initiation and progression. Given the fact that many factors participate in the governance of the fate of melanoma cells, it is worth targeting multiple molecules in different pathways as a therapeutic approach.

Our data that reduced Apaf-1 expression did not correlate with 5-year survival of patients with primary melanoma is consistent with the findings by Fujimoto \textit{et al} (Fujimoto et al., 2004), who showed that Apaf-1 LOH correlated with poorer prognosis of metastatic, but not primary melanoma patients. These data also suggest that the involvement of Apaf-1 in melanoma tumorigenesis is very complex. Loss of Apaf-1 may trigger the initiation of malignant transformation of melanocytes. However, additional genetic changes are required for the vertical growth phase progression of melanoma. Since the vast majority of melanomas have significantly reduced Apaf-1 expression and this reduction occurs in the early stages of melanoma formation, reversion of the reduced
Apaf-1 expression should be considered in the design of novel strategies for the treatment of melanoma patients.
CHAPTER 4*. INCREASED EXPRESSION OF INTEGRIN-LINKED KINASE IS CORRELATED WITH MELANOMA INVASION

4.1 Rationale and Hypothesis

Cell-ECM interactions play an important role in cell survival, growth, differentiation, and migration. ILK, a key component of cell-ECM structures, has been studied extensively since it was cloned in 1996. Increased ILK expression has been shown in several oncogenesis-related processes. Inhibition of ILK suppresses the activation of AKT and induces cell cycle arrest and apoptosis in PTEN-mutant prostate cancer cells (Persad et al., 2000). Recently, Graff et al. (Graff et al., 2001) also showed ILK overexpression correlates with prostate tumour progression.

Melanoma is a life-threatening disease because of its high capability of invasion and rapid metastasis to other organs. Due to the fact that ILK overexpression is closely related to tumour cell migration and invasion, we hypothesized that increased ILK expression may be associated with melanoma progression. We, thus, used TMA and immunohistochemistry to evaluate the ILK expression level in primary human melanoma at different stages.

* A version of this chapter has been published. Dai DL, Makretsov N, Campos EI, Huang C, Zhou Y, Huntsman D, Martinka M, Li G. Increased expression of integrin-linked kinase is correlated with melanoma progression and poor patient survival. Clin Cancer Res. 2003 Oct 1;9(12):4409-14.
4.2 Results

4.2.1 Clinicopathological Features of TMA

In our TMA study, ILK expression was obtained in 12 normal nevi and 67 cases of primary melanoma biopsies. For the primary melanoma cases, 40 were low-risk melanomas (Breslow thickness \( \leq 1.5 \text{ mm} \)) and 17 were high-risk melanomas (Breslow thickness \( >1.5 \text{ mm} \)). Among the 67 cases, 31 were superficial spreading melanoma, 10 were lentigo maligna melanoma, and other 26 cases consisted of desmoplastic melanoma, acrolentiginous melanoma, and nodular melanoma. Fourteen melanomas were located in sun-exposed sites (head and neck), and 53 in sun-protected sites (trunk, arm, leg, and feet). Tumour ulceration was observed in eight cases. Lymph node invasion was observed in six cases. (Table 4.1)

4.2.2 ILK Expression in Human Melanomas

To assess whether ILK expression is associated with melanoma progression, we examined ILK expression in melanoma primaries at various stages of invasion by immunohistochemistry. Staining for ILK was uniform in 85% of biopsies with duplicate cores. Various levels of ILK expression were observed in the cytoplasm in the melanoma biopsies (Fig. 4.1). ILK expression was negative (0) in 3 (4.5%), weak (1+) in 17 (25.4%), moderate (2+) in 31 (46.3%), and strong (3+) in 16 primary melanomas (23.9%). In addition, we examined the ILK expression in 12 benign nevi and found that ILK staining was negative in 1 (8.3%), weak (1+) in 4 (33.3%), and moderate (2+) in 7 (58.3%) biopsies. None of the 12 nevi had strong ILK staining. Due to the relative small case
number in normal nevi, our statistical analysis did not reveal a significant difference of ILK staining between melanomas and nevi (Table 4.2) \((P>0.05, \chi^2\) test).

4.2.3 ILK Expression and Clinicopathological Parameters or 5-year Survival

When ILK expression is compared in tumours with different thickness, we find that strong ILK expression significantly correlates with thicker tumours. As shown in Table 4.2, strong ILK expression is significantly more in melanomas >1.5 mm thick (8 of 17, 47%) compared with tumours ≤1.5 mm (8 of 50, 16%; \(P<0.01, \chi^2\) test). Furthermore, strong ILK expression was detected in 83% of the tumours with lymph node invasions compared with only 18% for tumours without lymph node invasions \((P<0.01, \chi^2\) test; Table 4.1). In addition, we did not find correlation between ILK expression with age, sex, tumour subtype, ulceration or location of tumours (sun-protected vs sun-exposed; Table 4.2).

Because strong ILK staining was found in most advanced melanomas, we sought to evaluate whether ILK staining might be related to patient survival. Kaplin-Meier survival curves, constructed using disease-specific survival at 5 years, were evaluated for biopsies that stained negative to moderate and those that stained strongly for ILK expression (Fig. 4.2). Although our data did not reach significance, there still a trend that strong ILK showed a worse 5-year patient survival \((P>0.05, \log-rank\) test).
Table 4.1 ILK expression and clinicopathological characteristics of 67 primary melanomas.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Neg. to Weak</th>
<th>Moderate</th>
<th>Strong</th>
<th>Total</th>
<th>( P ) Value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \leq 58 )</td>
<td>12 (35%)</td>
<td>16 (47%)</td>
<td>6 (18%)</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>&gt;58</td>
<td>8 (24%)</td>
<td>15 (45%)</td>
<td>10 (30%)</td>
<td>33</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10 (26%)</td>
<td>16 (42%)</td>
<td>12 (32%)</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10 (34%)</td>
<td>15 (52%)</td>
<td>4 (14%)</td>
<td>29</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>Tumour thickness (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \leq 1.5 )</td>
<td>15 (30%)</td>
<td>27 (54%)</td>
<td>8 (16%)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>&gt;1.5</td>
<td>5 (29%)</td>
<td>4 (24%)</td>
<td>8 (47%)</td>
<td>17</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Ulceration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>15 (25%)</td>
<td>31 (53%)</td>
<td>13 (22%)</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>5 (63%)</td>
<td>0 (0%)</td>
<td>3 (38%)</td>
<td>8</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>Lymph node invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>19 (31%)</td>
<td>31 (51%)</td>
<td>11 (18%)</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>1 (17%)</td>
<td>0 (0%)</td>
<td>5 (83%)</td>
<td>6</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Tumour subtype&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSM</td>
<td>8 (26%)</td>
<td>15 (48%)</td>
<td>8 (26%)</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>LMM</td>
<td>2 (20%)</td>
<td>7 (70%)</td>
<td>1 (10%)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>10 (38%)</td>
<td>9 (35%)</td>
<td>7 (27%)</td>
<td>26</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>Site&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sun-protected</td>
<td>14 (26%)</td>
<td>24 (45%)</td>
<td>15 (28%)</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Sun-exposed</td>
<td>6 (43%)</td>
<td>7 (50%)</td>
<td>1 (7%)</td>
<td>14</td>
<td>( P &gt; 0.05 )</td>
</tr>
</tbody>
</table>

<sup>a</sup> Chi-square test for negative to moderate versus strong ILK expression.

<sup>b</sup> SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; Other includes desmoplastic melanoma, acrolentigious melanoma, and nodular melanoma.

<sup>c</sup> Sun-protected sites: trunk, arm, leg, and feet. Sun-exposed sites: head and neck.
Table 4.2 Distribution of ILK expression in normal nevi and primary melanomas.

<table>
<thead>
<tr>
<th>ILK Staining</th>
<th>Neg. to Weak</th>
<th>Moderate</th>
<th>Strong</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Nevus</td>
<td>5 (42%)</td>
<td>7 (58%)</td>
<td>0 (0%)</td>
<td>12</td>
</tr>
<tr>
<td>Primary Melanoma</td>
<td>20 (30%)</td>
<td>31 (46%)</td>
<td>16 (24%)</td>
<td>67</td>
</tr>
</tbody>
</table>
Figure 4.1 Representative images of ILK immunohistochemical staining in human primary melanomas. A, Negative ILK expression. B, Weak ILK expression. C, Moderate ILK expression; D, Strong ILK expression. Magnification, X400.
Figure 4.2. Correlation between ILK expression and 5-year patient disease-specific survival. There is a trend that patient with strong ILK expression in the tumours have a worse 5-year survival. ($P>0.05$, log-rank test).
4.3. Discussion

Melanoma is one of the most lethal malignancies among human cancers because of its highly metastatic character and resistance to conventional therapies. Patients with melanoma have a dismal prognosis, and there is still no reliable prognostic marker for this disease. Although ILK has been shown to play an important role in tumorigenesis of a number of human cancers (Tan et al., 2001; White et al., 2001; Attwell et al., 2000; Wang et al., 2001; Marotta et al., 2001), most data are derived from in vitro studies or animal models. The purpose of this study was to gain information on the role of ILK in melanoma progression. We used TMA technology and immunohistochemistry to investigate ILK expression in primary melanoma biopsies. Our results for the first time demonstrate that ILK expression increases as primary melanoma progresses (Table 4.2). In addition, strong ILK expression is also correlated with lymph node invasion (Table 4.2) and patients with strong ILK expression showed a worse 5-year survival (Fig. 4.2). Our data are concordant with the published association of ILK expression with prostate cancer progression (Graff et al., 2001).

The overexpression of ILK in advanced melanoma could be explained by the involvement of ILK in cell survival and death pathways. Previous studies indicate that ILK functions as an effector of the PI3K/Akt cell survival pathway. ILK positively regulates Akt activity and negatively regulates GSK-3 in a PI3K-dependent manner. Not surprisingly, a recent study by Dhawan et al. (2002) showed Akt is constitutively activated in melanoma, which leads to up-regulation of NF-κB and tumour progression. It is conceivable that high expression of ILK in melanoma, as we found in this study, would
result in constitutive activation of Akt. Activation of Akt can also up-regulate vascular endothelial growth factor expression (Banerjee et al., 2003), a key component for angiogenesis, thus stimulating tumour invasion. Indeed, Segrelles et al. (2002) showed that Akt signaling plays an important role in skin tumorigenesis.

In addition to its involvement in PI3K/Akt/NF-κB signaling, ILK is believed to play a role in Wnt and growth factor signaling pathways. ILK-mediated inhibition of GSK-3 activity may lead to down-regulation of E-cadherin expression, nuclear translocation of β-catenin, and activation of the transcription factor AP-1 (Somasiri et al., 2001), resulting in cell cycle progression. In fact, a recent study by Kielhorn et al. (2003) demonstrated that nuclear phospho-β-catenin expression was common in metastatic melanoma and significantly associated with poor overall survival. The increased nuclear phospho-β-catenin in melanoma could be caused by high expression of ILK in these tumours.

Another ILK-mediated pathway that may enhance tumour progression is its regulation on MMP expression. During tumour progression, MMPs facilitate the pathological processes of tumour invasion, angiogenesis, and metastasis by breaking down the ECM (Westermarck and Kahari, 1999). It has been shown that overexpression of ILK results in increased MMP-9 expression (Troussard et al., 2000), which is in agreement with our observation that melanoma biopsies with lymph node invasion expressed a significantly higher amount of ILK compared with those without lymph node invasion. Given the fact that increased ILK expression is significantly associated with
primary melanoma progression, ILK may serve as a promising prognostic marker and therapeutic target for malignant melanoma.
5.1. Rationale and Hypothesis

Recently, growing attention has been focused on the role of the oncogene Akt in tumorigenesis. Akt is a serine-threonine kinase which is partially activated through phosphorylation of Thr-308 and reaches its maximum activity after phosphorylation of Ser-473 in tandem with that of Thr-308 (Andjelkovic et al., 1997; Aoki et al., 1998; Bellacosa et al., 1998; Nicholson and Anderson, 2002). As a key effector of PI3-K signaling pathway, Akt has been shown to play a critical role in controlling the balance between cell survival and apoptosis (Franke et al., 1997).

To date, Akt overexpression or activation has been shown to be correlated with poor prognosis in several tumour types, including gastric carcinomas, hepatocellular carcinoma, leukemia, breast cancer, and pancreatic cancer (Nam et al., 2003; Xu et al., 2004; Min et al., 2003; Schmitz et al., 2004; Yamamoto et al., 2004). To investigate the role of Akt activity in melanoma progression, we used TMA and immunohistochemistry to evaluate the Akt activity in different stages of human melanocytic lesions. We hypothesized that increased expression of activated Akt is associated with melanoma progression and poor patient survival.

* A version of this chapter has been published. Dai DL, Martinka M, Li G. Prognostic significance of activated Akt expression in melanoma: a clinicopathologic study of 292 cases. J Clin Oncol. 2005 Mar 1;23(7):1473-82.
5.2 Results

5.2.1 Clinicopathological Features of TMA

Due to loss of biopsy cores or insufficient tumour cells present in the cores, 12 cases of normal nevi, 58 cases of dysplastic nevi, 170 cases of primary melanomas, and 52 cases of melanoma metastases could be evaluated for p-Akt staining. For the 170 primary melanoma cases, there were 95 male and 75 females, with age ranging from 21 to 93 years (median=57). For melanoma staging, we used Breslow thickness and AJCC stages as our criteria for evaluating p-Akt expression: 107 tumours were ≤1.5 mm thick (low-risk melanoma) and 63 were >1.5 mm (high-risk melanoma). Thirty-three melanomas were located in sun-exposed sites (head and neck) and 137 in sun-protected sites (other locations). Ulceration was observed in 28 cases (Table 5.1).

5.2.2 Increased Phospho-Akt Expression Correlates with Melanoma Progression

Various levels of p-Akt staining were observed in nevi and melanoma biopsies (Fig. 5.1). Strong p-Akt staining was recorded in 17%, 43%, 49% and 77% of the biopsies in normal nevi, dysplastic nevi, primary melanoma, and melanoma metastases, respectively (Table 5.2). Significant differences for p-Akt staining pattern were observed between normal nevi and primary melanomas ($P<0.05$, $\chi^2$ test), and between primary melanomas and melanoma metastases ($P<0.001$, $\chi^2$ test). However, there is no significant difference in p-Akt staining between normal nevi and dysplastic nevi ($P>0.05$, $\chi^2$ test), or between dysplastic nevi and primary melanoma ($P>0.05$, $\chi^2$ test).
5.2.3 Phospho-Akt Expression in Melanoma and Clinicopathological Parameters

To assess whether Akt phosphorylation correlates with clinicopathological parameters of the patients, we examined the expression of p-Akt (Ser-473) in 170 primary melanomas at various stages of invasion. First, we analyzed the expression level of p-Akt in tumours with different thickness, as tumour thickness is a well-known prognostic marker for patients with primary melanoma. We found that strong p-Akt expression significantly correlates with advanced stages of primary melanoma. As shown in Table 5.1, while strong p-Akt expression was detected in 60% of high-risk melanomas (Breslow thickness >1.5 mm), only 43% of low-risk melanomas (Breslow thickness ≤1.5 mm) showed strong p-Akt expression ($P<0.05$, $\chi^2$ test). Interestingly, we also found that strong p-Akt staining was detected in 62% of male patients compared with only 33% of female patients who had strong p-Akt expression ($P<0.001$, $\chi^2$ test). No correlation was found between p-Akt expression and patient’s age, tumour subtype, location, or ulceration status of tumours (Table 5.1).

5.2.4 Survival Analysis

To evaluate whether strong p-Akt expression in human primary melanomas correlates with a worse prognosis, Kaplan-Meier survival curves were constructed using disease-specific 5-year survival to evaluate the biopsies stained negative to moderate versus those stained strong for p-Akt expression. Our data revealed that strong p-Akt expression in primary melanoma tissue is inversely correlated with disease-specific 5-year survival ($P<0.05$, log-rank test) (Fig. 5.2). Since p-Akt showed higher expression levels in high-risk melanomas (thickness >1.5 mm), we further divided primary
melanoma cases into two subgroups according to tumour thickness and performed survival analysis separately. Although there is still a trend that strong p-Akt correlates with poor disease-specific patient survival in both groups, our statistic analysis did not reveal a significant difference ($P>0.05$ for both, log-rank test) (Fig. 5.3). This is probably due to the decreased number of patients after dividing the whole population. In addition, although male patients showed a much higher p-Akt levels than female patients, our Kaplan-Meier survival curve showed strong p-Akt expression has a similar effect on 5-year survival in male and female groups ($P=0.056$ and 0.074, respectively, log-rank test) (Fig. 5.4).

Next, we examined whether strong p-Akt expression is an independent prognostic marker for melanoma. We performed Cox Regression analysis including p-Akt expression, age, gender, thickness, ulceration and location of the tumours for 170 primary melanomas. Our results indicate that p-Akt expression reached a borderline significance for predicting the patient outcome independently of other clinicopathological parameters ($P=0.071$). Tumour thickness, location and ulceration were most significant prognostic markers for disease-specific survival (Table 5.3). Thus, p-Akt may represent a new independent prognostic marker for melanomas.

5.2.5 Correlations Between ILK and p-Akt Expression in Human Melanomas

Previous studies indicated that ILK plays an essential role for Akt activation (Persad et al., 2001; Troussard et al., 2003). In our melanoma TMA study, expression levels of ILK and p-Akt were both obtained in 62 cases of primary melanomas. A linear association between ILK expression and Akt activity was observed in these cases.
(P=0.05, χ² test; P=0.005, linear association) (Table 5.4). In the following survival
analysis, our CART model indicated that patients without increased ILK or p-Akt
expression showed a 5-year survival rate of 91%, whereas in the patients with increased
ILK or p-Akt expression, the 5-year survival rate dropped to 74% (Fig. 5.5A). A
borderline significance was obtained when comparing these two groups using Kaplan-
Meier method (Fig. 5.5B) (P=0.059, log-rank test).
Table 5.1 Phospho-Akt expression and clinicopathological characteristics of 170 primary melanomas.

<table>
<thead>
<tr>
<th>Variable</th>
<th>p-Akt Staining</th>
<th></th>
<th></th>
<th></th>
<th>Total</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neg. to Weak</td>
<td>Moderate</td>
<td>Strong</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤57</td>
<td>7 (8%)</td>
<td>36 (42%)</td>
<td>43 (50%)</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;57</td>
<td>10 (12%)</td>
<td>33 (39%)</td>
<td>41 (49%)</td>
<td>84</td>
<td>P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5 (5%)</td>
<td>31 (33%)</td>
<td>59 (62%)</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12 (16%)</td>
<td>38 (51%)</td>
<td>25 (33%)</td>
<td>75</td>
<td>P&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Tumour thickness (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤1.5</td>
<td>12 (11%)</td>
<td>49 (45%)</td>
<td>46 (43%)</td>
<td>107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1.5</td>
<td>5 (8%)</td>
<td>20 (32%)</td>
<td>38 (60%)</td>
<td>63</td>
<td>P&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Ulceration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>16 (11%)</td>
<td>60 (42%)</td>
<td>66 (46%)</td>
<td>142</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>1 (4%)</td>
<td>9 (32%)</td>
<td>18 (64%)</td>
<td>28</td>
<td>P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Tumour subtypeb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSM</td>
<td>7 (9%)</td>
<td>31 (39%)</td>
<td>41 (52%)</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMM</td>
<td>5 (19%)</td>
<td>13 (48%)</td>
<td>9 (33%)</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>5 (8%)</td>
<td>25 (39%)</td>
<td>34 (53%)</td>
<td>64</td>
<td>P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Sitec</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sun-protected</td>
<td>13 (9%)</td>
<td>57 (42%)</td>
<td>67 (49%)</td>
<td>137</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sun-exposed</td>
<td>4 (12%)</td>
<td>12 (36%)</td>
<td>17 (52%)</td>
<td>33</td>
<td>P&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

a Chi-square test for negative to moderate versus strong Apaf-1 expression.
b SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; Other includes desmoplastic melanoma, acrolentigous melanoma, and nodular melanoma.
c Sun-protected sites: trunk, arm, leg, and feet. Sun-exposed sites: head and neck.
Table 5.2 Distribution of p-Akt expression in normal nevi, dysplastic nevi, primary melanomas and metastatic melanomas.

<table>
<thead>
<tr>
<th></th>
<th>p-Akt Staining</th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neg. to Weak</td>
<td>Moderate</td>
<td>Strong</td>
<td></td>
</tr>
<tr>
<td>Normal nevus</td>
<td>8 (67%)</td>
<td>2 (17%)</td>
<td>2 (17%)</td>
<td>12</td>
</tr>
<tr>
<td>Dysplastic nevus</td>
<td>4 (7%)</td>
<td>29 (50%)</td>
<td>25 (43%)</td>
<td>58</td>
</tr>
<tr>
<td>Primary melanoma</td>
<td>17 (10%)</td>
<td>69 (41%)</td>
<td>84 (49%)</td>
<td>170</td>
</tr>
<tr>
<td>Metastatic melanoma</td>
<td>6 (12%)</td>
<td>6 (12%)</td>
<td>40 (77%)</td>
<td>52</td>
</tr>
</tbody>
</table>
Table 5.3  Multivariate Cox regression analysis of parameters including p-Akt, tumour thickness, location and ulceration, age, and gender on patient 5-year disease-specific survival in 170 primary melanomas.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative risk</th>
<th>95%CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Akt</td>
<td>2.41</td>
<td>0.93-6.28</td>
<td>0.071</td>
</tr>
<tr>
<td>Thickness</td>
<td>5.72</td>
<td>1.78-18.38</td>
<td>0.003</td>
</tr>
<tr>
<td>Ulceration</td>
<td>2.73</td>
<td>1.12-6.68</td>
<td>0.027</td>
</tr>
<tr>
<td>Location</td>
<td>3.08</td>
<td>1.36-7.00</td>
<td>0.007</td>
</tr>
<tr>
<td>Age</td>
<td>1.32</td>
<td>0.53-3.32</td>
<td>0.554</td>
</tr>
<tr>
<td>Gender</td>
<td>1.87</td>
<td>0.79-4.42</td>
<td>0.158</td>
</tr>
</tbody>
</table>

a Coding of variables: p-Akt was coded as 1: negative to moderate expression; and 2: strong expression. Thickness was coded as 1: ≤1.5 mm; and 2: >1.5 mm. Ulceration was coded as 1: absent; and 2: present. Location was coded as 1: extremities and trunk; and 2: head and neck. Age was coded as 1: ≤57 years; and 2: >57 years. Gender was coded as 1: male; and 2: female.

b CI: confidence interval.
Table 5.4 Correlation between ILK and p-Akt expression in 62 primary melanomas.

<table>
<thead>
<tr>
<th>ILK Staining</th>
<th>p-Akt Staining</th>
<th></th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neg. to Weak</td>
<td>3</td>
<td>11</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>4</td>
<td>17</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Strong</td>
<td>0</td>
<td>6</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>7</td>
<td>34</td>
<td>21</td>
<td>62</td>
</tr>
</tbody>
</table>
Figure 5.1 Representative images of p-Akt immunohistochemical staining in human melanocytic lesions. A, Normal nevus with weak p-Akt expression. B, Dysplastic nevus with weak p-Akt expression. C, Primary melanoma with moderate p-Akt expression. D, Melanoma metastasis with strong p-Akt expression. Magnification, X400.
Figure 5.2. Correlation between p-Akt expression and 5-year disease-specific survival of primary melanoma patients. Patients with strong p-Akt expression have a significantly worse 5-year survival than those with negative to moderate p-Akt expression. $P<0.05$, log-rank test.
Figure 5.3 Correlation between p-Akt expression and 5-year disease-specific survival in subgroups of primary melanoma patients. **A,** Disease-specific 5-year survival of patients with thin melanomas. \((P=0.181,\) log-rank test). **B,** Disease-specific 5-year survival of patients with thick melanomas. \((P=0.239,\) log-rank test)
Figure 5.4 Correlation between p-Akt expression and 5-year disease-specific survival in subgroups of male and female melanoma patients. 

A, Disease-specific 5-year survival of male patients ($P=0.054$, log-rank test). 

B, Disease-specific 5-year survival of female patients ($P=0.071$, log-rank test).
Figure 5.5 Correlation between ILK/p-Akt expression and 5-year disease-specific survival. A, CART analysis of 62 primary melanoma patients based on ILK and p-Akt expression. B, Respective Kaplan-Meier curve for each leaf ($P=0.059$, log-rank test).
5.3 Discussion

The role of Akt in tumorigenesis has been studied extensively in recent years. Elevated Akt activity has been observed in numerous types of human cancers (Fresno Vara et al., 2004). In this study, to better understand the role of Akt activity in melanoma progression, we used TMA technology and immunohistochemistry to investigate Akt activity in 292 cases of pigmented skin lesions at different stages using an antibody specific for phosphorylated Akt at Ser-473. Our results demonstrated that p-Akt expression increases as melanoma progresses (Table 5.2). Furthermore, strong p-Akt expression is significantly correlated with tumour invasion (Table 5.1) and inversely correlated with patient 5-year survival in primary melanomas (Fig. 5.2 and Table 5.3). To our knowledge, this is the first study to analyze the phosphorylation of Akt in primary melanoma tumours and its association with tumour progression and patient survival.

Our result of a significant correlation between increased p-Akt expression and melanoma progression is consistent with the findings by Dhawan et al. (2002) who showed an increased phospho-Akt expression in more advanced melanocytic lesions. However, the authors in that study only included 16 cases of nevi, one case of lentigo maligna, and 12 cases of metastatic melanoma. Since no primary melanomas were included in their study, it is impossible to draw conclusions on the role of activated Akt in melanoma progression. In order to study the role of Akt in melanoma pathogenesis, we selected 292 cases of melanocytic lesions at different stages: normal nevi, dysplastic nevi, primary melanomas, and metastatic melanomas. A linear trend of increased Akt
phosphorylation was observed following progression of melanocytic lesions and significant differences were recorded between normal nevi and primary melanomas, and between primary and metastatic melanomas (Table 5.2). These stage-specific expression patterns suggest that increased Akt activity might be a common requirement for the transformation from benign neoplasia to malignancies as well as from primary tumours to metastatic disease in melanoma. Dysplastic nevus is often considered to represent intermediate steps in melanoma tumorigenesis and may share some of the genetic alterations with primary melanoma (Hussein and Wood, 2002). In our study, there is a clear trend that more p-Akt is expressed in dysplastic nevi compared to normal nevi (43% vs. 17%, $P=0.087$, $\chi^2$ test). This difference did not reach significance probably due to the small number of normal nevi cases. The increased p-Akt staining in dysplastic nevi compared to normal nevi is not due to TMA sampling. Sixty-two percent of our dysplastic nevi samples contain both junctional and dermal components. Eighty-three percent of these samples showed identical p-Akt level between two components, whereas only 11% cases showed higher p-Akt expression in junctional component and 6% cases showed higher p-Akt expression in dermal component. On the other hand, p-Akt expression pattern in dysplastic nevi was similar to primary melanomas (43% vs. 49%, $P=0.406$, $\chi^2$ test) (Table 5.2), suggesting that Akt activation is a very early event in melanoma tumorigenesis.

Interestingly, there was a higher percentage of strong p-Akt expression in male patients rather than female patients (Table 5.1). Despite the different p-Akt expression pattern observed in genders, Kaplan-Meier survival curve showed strong p-Akt
expression has a similar effect on 5-year disease-specific survival in male or female groups (Fig. 5.4). One possible explanation of higher p-Akt expression in male patients is that male patients may have thicker tumours at diagnosis than female patients, as shown by many studies (Hersey et al., 1991; Osborne and Hutchinson, 2001; Melia et al., 2001). However, in our study, there is no statistical difference on average tumour thickness between genders (average thickness: 2.0 mm for male and 1.8 mm for female, \( P=0.453 \), t-test). Also, when we compared the number of cases \( \leq 1.5 \text{ mm} \) or \( >1.5 \text{ mm} \), there is no difference between male and female groups (\( P=0.566 \), \( \chi^2 \) test). Another possible reason of higher p-Akt expression in male is that sex-related hormones may contribute to PI3K signaling. It has been reported that both androgen and estrogen have the ability to activate PI3K through interaction between their receptors and p85α subunit of PI3-K (Simoncini et al., 2000; Sun et al., 2003), thus leading to the activation of Akt.

However, it seems that the differential expression of p-Akt between genders is tissue-specific. For example, it has been shown that in male rats, Akt activity is higher in lacrimal gland but lower in neuronal cells derived from cortical plate compared with female rats (Zhang et al., 2003; Rocha et al., 2002). The mechanisms of higher p-Akt expression in male melanoma patients and its clinical significance remain to be determined.

The correlation between strong p-Akt expression and tumour invasion (Table 5.1) and a poorer 5-year patient survival (Fig. 5.2 and Table 5.3) are concordant with the previous studies describing elevated Akt signaling in melanoma. As a key player in PI3K cell survival pathway, activated Akt modulates the function of numerous substrates
involved in the regulation of cell cycle progression and cell survival. In melanoma, constitutively activated Akt has been shown to lead to upregulation of NF-κB (Dhawan et al., 2002). As the negative regulator of Akt pathway, PTEN expression was reduced in melanoma biopsies and loss of PTEN can promote melanoma tumour growth in vivo (Tsao et al., 2003; Stahl et al., 2003). Moreover, blocking Akt activity can inhibit cell proliferation and reduces the sensitivity of melanoma cells to apoptosis both in vitro and in vivo (Yang et al., 1999; Jetzt et al., 2003; Krasilnikov et al., 2004). These evidences point to the key role of Akt in melanoma tumorigenesis.
CHAPTER 6. GENERAL CONCLUSIONS

Malignant melanoma is a life-threatening skin cancer due to its highly metastatic character and resistance to radio- and chemo-therapy. It has been proposed that melanoma progression involves a multistep process from normal nevi, dysplastic nevi, primary melanoma to metastatic melanoma. Although clinical and histological variables such as Breslow's thickness and ulceration that predict patient survival have been identified in malignant melanoma, the potential relevance of biological variables remains largely unknown. In current study, using tissue microarrays and immunohistochemistry, we evaluated the expression patterns of three genes potentially involved in melanoma pathogenesis, including the tumour suppressor Apaf-1, oncogenes ILK and the activated form of Akt (phospho-Akt Ser-473), in melanocytic lesions, and analyzed the correlation between the expression of these genes and 5-year patient survival and other clinicopathological data such as tumour thickness, ulceration, subtype, patient's gender and age.

Our data showed that Apaf-1 expression in melanoma is significantly reduced compared with normal nevi. Our results also revealed that reduction of Apaf-1 was not associated with the primary tumour invasion and 5-year disease-specific survival, which suggested that Apaf-1 may only be involved in the initiation of melanoma development.

Our study suggested that survival signaling through PI3K-Akt may play a key role in melanoma tumorigenesis. Our data showed that expression of both p-Akt and its
upstream regulator ILK correlates with tumour invasion in primary melanomas, and p-Akt expression is significantly increased with progression of human melanoma. Most strikingly, our data indicate that increased p-Akt expression correlates with a worse 5-year survival of primary melanoma patients and may serve as an independent prognostic factor for primary melanomas. These data, coupled with a number of functional studies demonstrating an essential role of Akt activity in melanomagenesis (Jetzt et al., 2003; Bedogni et al., 2004), implicate that Akt may serve as a promising prognostic marker and therapeutic target for malignant melanoma.
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