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Review

GENETIC ALTERATIONS IN MELANOMA DEVELOPMENT

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The transition from a normal to a neoplastic cell is a complex process and involves many sequential genetic events. Over the last several years, substantial progress has been made in the understanding of molecular biology of melanoma including its development, progression, and resistance to therapy. An important step in cancer development appears to be senescence — a crucial barrier that prevents the proliferation of cells that are at different stages of malignancy. This review is mainly focused on patterns of molecular changes in the different steps of neoplastic transformation in melanoma and provides an up-to-date view on our understanding of the molecular genetics of melanoma development as well as therapeutic approaches based on this knowledge.

Key words: *cancer, melanoma, genes, mutations, genetics.*

INTRODUCTION

Melanoma is a malignancy of pigment-producing cells (melanocytes) located predominantly in the skin, but also found in eyes, ears, leptomeninges, gastrointestinal tract, oral and genital mucosa. It is one of the deadliest cancers, which causes the greatest number of skin cancer-related deaths worldwide. The incidence of melanoma is increasing at a faster rate than any other malignant tumour. The annual increase in incidence rate varies between populations, but in general has been in the order of 3–7% per year for fair-skinned Caucasian populations (Diepgen *et al*., 2002). The estimates suggest a doubling of melanoma incidence every 10–20 years (Garbe *et al*., 2000). Early detection of thin cutaneous melanoma is the best means of reducing mortality.

The development of melanoma is multifactorial and appears to be related to multiple risk factors, including fair complexion, excessive childhood sun exposure, an increasing number of common and dysplastic moles, a family history of melanoma, the presence of changing mole or evolving lesion on the skin, and, importantly, older age (Rhodes *et al*., 1987; Williams *et al*., 1994). About 40–50% of melanomas develop from pigmented moles, almost all others arise from melanocytes in normal skin.

SUBTYPES OF CUTANEOUS MELANOMA

Melanomas vary in size, shape, and colour (usually pigmented) and in their propensity to invade and metastasise. Four major clinicopathologic subtypes of cutaneous melanoma have been identified: i) lentigo maligna melanoma (LM), ii) superficial spreading malignant melanoma (SSM), iii) nodular melanoma (NM), and iv) acral lentiginous malignant melanoma (ALM).

LM is typically located on the head (cheeks, nose, temple, forehead or ear), neck, and arms (chronically sun damaged skin) of an elderly person. It arises from *lentigo maligna* or Hutchinson's freckle as a 2–6 cm flat lesion with irregular margins. The colours within it vary from light tan to brown or black, and sometimes with patches of red, blue, white or grey. LM grows slowly over 5–20 years. When invasion through the basement membrane into dermis occurs, part of the lesion becomes thickened and nodular. Very rarely, in neglected cases, deep invasion occurs with multiple nodule formation.

SSM is most common on the trunk in men and women and on legs in women. This subtype is most commonly seen in individuals aged 30–50 years and begins as a flat patch of pigmentation that becomes just palpable. SSM spreads laterally and horizontally and has an irregular outline. There are various shades of brown, usually mixed with black, and foci of red, blue and purple. The lesion generally is greater than 6 mm diameter.

NM occurs in 15–30% of melanoma patients. It can appear anywhere, but particularly on the limbs in females and trunk in males. NM manifests as a dark brown–to-black papule or dome-shaped nodule, which may ulcerate and

bleed with minor trauma. It may be clinically amelanotic and tends to lack the typical ABCDE melanoma warning signs and, thus, may elude early detection. This subtype is fast growing (weeks to months) and responsible for most thick melanomas, which may metastasise. Melanoma metastasises via the lymphatics to lymph nodes and via the bloodstream to the brain, lungs, liver, bones and skin.

ALM appears as an irregular pigmentation on the palms and soles, sometimes arising from a naevus. This is the least common subtype of melanoma in white persons (2–8%) but it accounts for 29–72% of melanoma cases in dark skinned individuals and, because of delays in diagnosis, may be associated with a worse prognosis (Byrd *et al*., 2004).

HISTOLOGIC EVENTS IN THE PROGRESSION OF MELANOMA

The majority of melanoma subtypes are observed to progress through distinct histologic steps (Clark *et al*., 1984). Five distinct steps have been proposed in the evolution of melanoma: i) common acquired and congenital nevi without displastic changes (benign nevus), ii) dysplastic nevi with structural and architectural atypia, iii) radial-growth phase (RGP) melanoma, iiii) vertical-growth phase (VGP) melanoma and iiiii) metastatic melanoma (Fig. 1). As the presumed precursor to melanoma, both benign and dysplastic nevi are characterised by disruption of the epidermal melanin unit, leading to increased number of melanocytes in relation to keratinocytes. These precursor lesions progress to *in situ* melanoma, which grows laterally and remains largely confined to the epidermis, so this stage is defined as the RGP. VGP melanoma invades both the upper layer of the epidermis and beyond, and penetrates into the underlying dermis and subcutaneous tissue through the basement membrane, forming nodules of malignant cells. It is believed that the transition from radial- to vertical-growth phases is a critical step in the evolution of melanoma that presages the acquisition of metastatic potential and poor clinical outcome (Rusciano, 2000).

MOLECULAR CHANGES IN THE PROGRESSION OF MELANOMA

The histologic progression observed in the growth phases of melanoma is hypothesised to correspond to the accumulation of mutations in different genes critical for cell prolif-

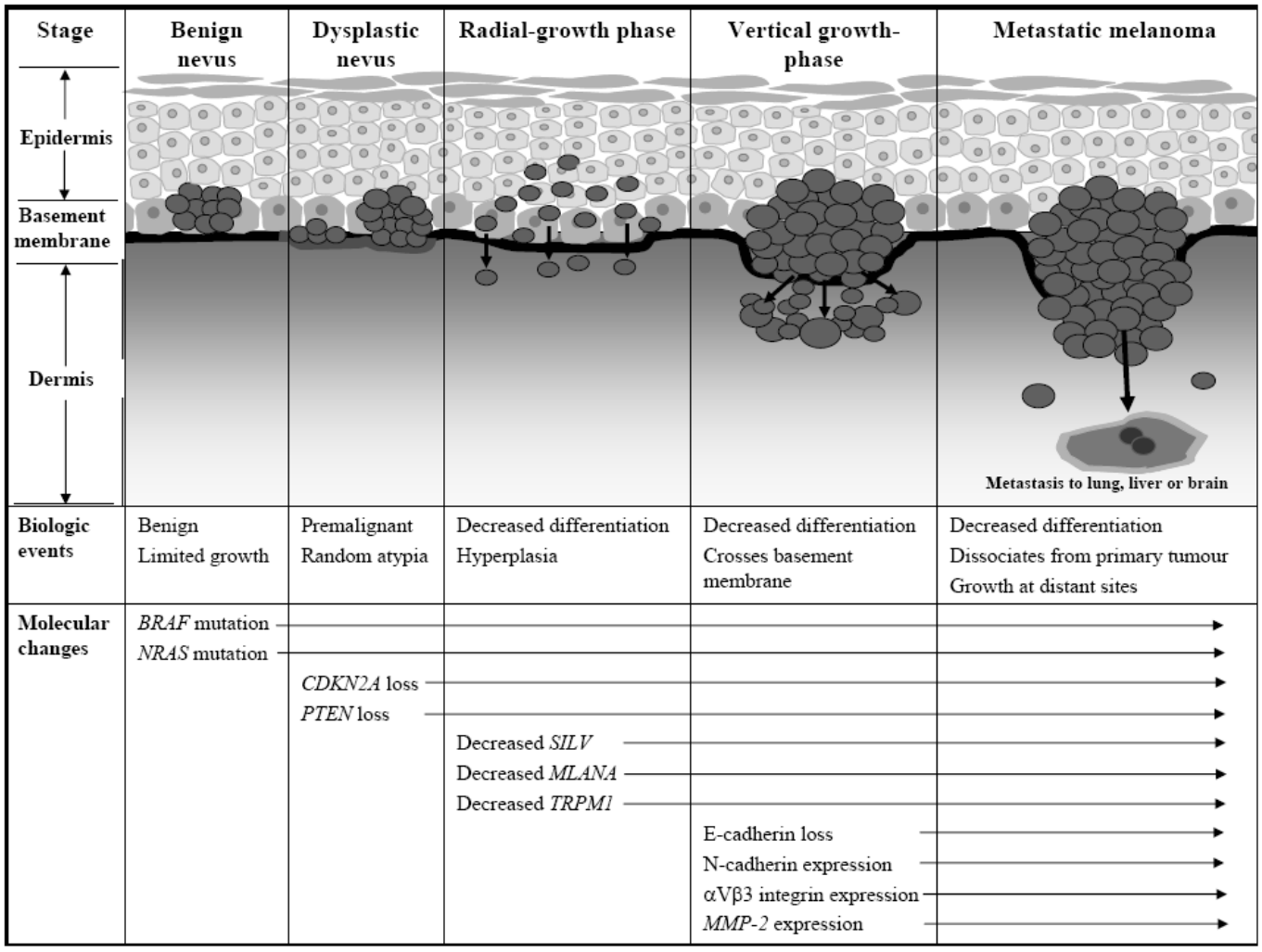

Fig. 1. Biological events and molecular changes in the development of melanoma (adapted from Miller and Mihm, 2006).

eration, differentiation, and cell death (Table 1 lists genes mentioned in the article).

An early event in melanoma development is oncogene activation, most often targeting RAS and its canonical effector pathways RAF-MAPK-ERK and PI3K-Akt. The mutation frequency of the *RAS* gene family, particularly *NRAS*, in human melanoma has been estimated to be 10–20% (Demunter *et al*., 2001; Gorden *et al*., 2003). The more frequently occurring *NRAS* mutations are summarised elsewhere (de Snoo and Hayward, 2005). In particular, the occurrence of *NRAS* mutation varies according to body site and histological type of the lesion. A higher incidence of mutations has been associated with melanomas on chronically sun-exposed body sites, and it has been proposed that they arise as a result of UV-induced mutagenesis (van Elsas *et al*., 1996; Jiveskog *et al*., 1998; Demunter *et al*., 2001). Mutations also appear to be more common in NM and LM than SSM, ALM or mucosal melanomas (van Elsas *et al*., 1996; Jiveskog *et al*., 1998). As mentioned above, there is evidence that *NRAS* mutations occur at an early stage of melanocytic neoplasia — nevus formation. *NRAS* mutations have been found at a frequency of approximately 9% in nevi of various histological types, including compound, junctional (Kumar *et al*., 2004), intradermal (Pollock *et al*., 2003), and Spitz (Saldanha *et al*., 2004) nevi. Mutations are much more common (~35%) in congenital nevi (Papp *et al*., 1999; Pollock *et al*., 2003), but rare in dysplastic nevi (Papp *et al*., 1999), which could indicate that at least two distinct evolutionary paths to melanoma exist, from benign and dysplastic nevi.

The role of the Raf-MEK-ERK cascade in melanoma development has received a great deal of attention in the past five years, due to the identification of mutations in the *BRAF* gene in a high proportion of melanomas. The initial study

Table 1

IMPORTANT GENES IN MELANOMA DEVELOPMENT

Gene	Gene name	Pathway	Function	Changes in melanoma
N-RAS	Neuroblastoma Ras viral (v-ras) oncogene homolog	RAS and MAPK	Oncogene	Sporadic activating mutations
B-RAF	v-raf murine sarcoma viral oncogene homolog B1		Oncogene	Sporadic activating mutation at V600E in melanoma and nevi
MEK	Mitogen-activated protein kinase-extracellular-related kinase		Signal transduction	Increased activity
ERK 1 or *2*	Extracellular-related kinase 1 or 2		Signal transduction	Increased activity
CDKN2A or *INK4A*	Cyclin-dependent kinase inhibitor 2A or inhibitor of kinase 4A	INK4A, CDK, and RB	Tumour suppressor — negative regulation of cell proliferation	Germline mutations in familial melanomas; sporadic deletions, promoter inactivation, loss of heterozygosity
CDK4	Cyclin-dependent kinase 4		Promoter of cell proliferation	Germline mutation R24C or R24H
RB	Retinoblastoma		Tumour suppressor — negative regulation of cell proliferation	Phosphorylation leads to progression from G1 to S phase
ARF	Alternate reading frame	ARF and p53	Tumour suppressor — degrades MDM2	Germline mutations in familial melanoma; sporadic deletions, promoter inactivation
TP53	Tumour protein 53		Tumour suppressor — induces apoptosis and suppresses proliferation after DNA damage	Expression in melanomas
HDM2	Human double minute 2		Targets p53 for ubiquitination and destruction	Up-regulated in presence of ARF mutation
PTEN	Phosphatase and tensin homolog	PTEN and AKT	Tumour suppressor — suppresses PI3K	Sporadic deletion of chromosomal region
PI3K	Phosphatidylinositol 3 kinase		Signal transducer	Active in presence of PTEN mutation
AKT or *PKB*	Protein kinase B		Oncogene	Amplified in some melanomas
MITF	Microphthalmia-associated transcription factor	MITF	Transcription factor	Sporadic amplification of chromosomal region
SILV	Silver homologue		Antigen, melanoma marker	Decreased expression
MLANA	Melan-A		Antigen, melanoma marker	Decreased expression
TRPM1	Melastatin		Unknown	Decreased expression in metastatic melanoma
CDH1	E-cadherin	Cell adhesion	Cell-adhesion molecule	Decreased expression in vertical growth phase
CDH2	N-cadherin		Cell-adhesion molecule	Aberrant expression in vertical growth phase
ITGB3	$\alpha V\beta 3$ integrin		Cell-adhesion molecule	Aberrant expression in vertical growth phase

by Davies *et al*. (2002) estimated that 67% of melanomas harbour *BRAF* mutations. By far the most frequent mutation is V600E (previously reported as position 599). This mutation, found in the activation segment of the B-Raf kinase domain, renders the protein constitutively active and leads to elevated ERK1/2 activation. A great number of follow-up studies (Gorden *et al*., 2003; Pollock *et al*., 2003; Uribe *et al*., 2003; Yazdi *et al*., 2003; Poynter *et al*., 2006) have further examined the frequency of *BRAF* mutations in benign and malignant melanocytic lesions. The resulting estimates have varied from 30% to 70%, and taken together, the published data suggest that approximately 50% of melanomas carry the *BRAF* V600E mutation (Rodolfo *et al*., 2004). Several aspects of the association between *BRAF* mutation and melanoma bear further discussion. *BRAF* mutations are extremely rare in uveal (ocular) and mucosal melanomas (Edwards *et al*., 2004; Sasaki *et al*., 2004; Curtin *et al*., 2005), but are present in approximately 50% of SSM and NM of the skin (Uribe *et al*., 2003; Reifenberger *et al*., 2004; Thomas *et al*., 2004). The latter suggests that sun-related, UV-induced carcinogenesis may play a role in causing these mutations. Importantly, although approximately half of all melanomas carry the *BRAF* V600E mutation, this alteration is also found in 70–80% of common acquired melanocytic nevi (Pollock *et al*., 2003; Yazdi *et al.,* 2003; Kumar *et al*., 2004; Poynter *et al*., 2006). This finding strongly suggests that *BRAF* mutation is an early event in the development of melanocytic neoplasia and alone may not be sufficient to drive melanoma development. It is conceivable that growth arrest of nevi results from oncoge-induced senescence acting as an effective brake against oncogenic signalling. Indeed, human nevi display all of the classical hallmarks of senescence — stable proliferative arrest, induction of acidic β-galactosidase, the remodelling of chromatin with presence of heterochromatin loci, and increased expression of tumour suppressor p16INK4A (Gray-Schopfer *et al*., 2006). Cellular senescence, the growth arrest seen in normal mammalian cells after a limited number of divisions, is controlled by key cell signalling pathways, including the p53 and p16INK4A/RB pathways which both converge in the G1/S cell cycle checkpoint. Oncogene-induced senescence similarly is linked to both, p53 and p16INK4A/RB, signalling pathways (Fig. 2). Aberrant cancer-associated signals in pre-malignant cells activate these pathways and force potential tumour cells into the senescence preventing the progression of cancer. Although formerly thought to be molecularly homogeneous, senescence is now shown to differ depending on the cancer promoting mutation as well as cell type (Sharpless and DePinho, 2005). Oncogene-induced senescence in cultured melanocytes as well as in human nevi involves likely only the p16INK4A/RB pathway and when abolished by disruption of the RB pathway a further p53-dependent growth arrest is observed (Gray-Schopfer *et al*., 2006). Wherewith, cell senescence provides an attractive explanation for the biology of moles, which first grow likely due to mitogenic stimuli (usually an activating *NRAS* or *BRAF* mutations) and then stop growing, often remaining static for decades. To become malignant nevi must acquire additional molecular le-

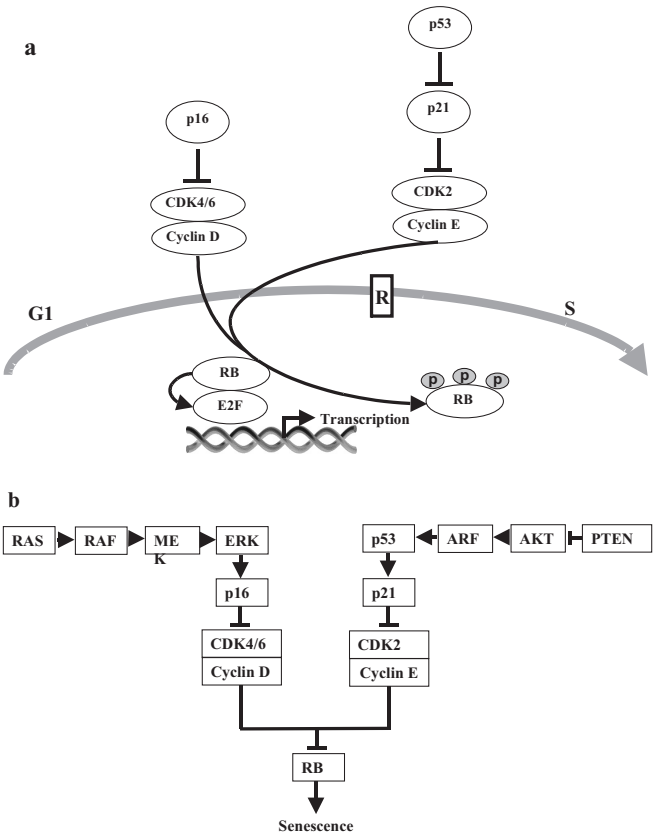

Fig. 2. Signaling pathways leading to cell proliferation and senescence. **a**, the G1/S cell cycle checkpoint controls the passage of eukaryotic cells from the first "gap" phase (G1) into the DNA synthesis phase (S) and is mainly controlled by cyclin D associated with cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) at its early phase and cyclin E associated with cyclin-dependent kinase 2 (CDK2) at the later restriction phase. Phosphorylation of Rb by CDK4/6-cyclin D and CDK2-cyclin E complexes releases Rb from its inhibitory interaction with the E2F transcription factor, thereby allowing the expression of E2F-related genes and cell proliferation. The cyclin-dependent kinase inhibitor p16INK4A and protein 53 (p53) via activation of other cyclin-dependent kinase inhibitor p21 inhibit CDK4/6 and CDK2 respectively thereby stopping the cell cycle and cell proliferation. **b**, pathways involved in oncogene-induced senescence. Induction of p16INK4A and p21 by senescence-inducing signals results in inhibition of activity of CDK4/6 and CDK2.

sions to free themselves from growth restraints. Indeed, the next step toward melanoma is the development of cytologic atypia in the dysplastic nevi, which may arise from existing benign nevi or as a new lesion. The molecular abnormalities at this stage of progression affect cell cycle regulation, particularly the G1/S cell cycle checkpoint and involve tumour suppressor genes *CDKN2A* (encodes the above mentioned p16INK4A) and *PTEN* (Fig. 1).

CDKN2A is a single gene that through alternative splicing and transcription of the products in different reading frames encodes two diverse tumour suppressor proteins, which are transcribed from different first exons but utilise the same second and third exons (Fig. 3). The α-transcript, comprising exons 1α, 2, and 3, encodes p16INK4a protein of 156 amino acids, a negatively regulator of the cell cycle mentioned above. Therefore, the arrest of cell cycle caused by p16INK4A can, however, be overcome by mutations in

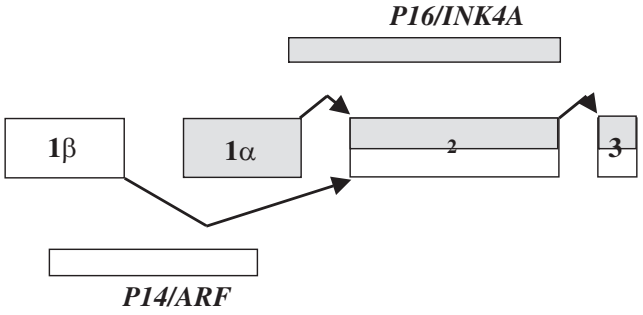

Fig. 3. The *CDKN2A* locus encoding p16INK4A and p14ARF.

p16INK4A, leading to uncontrolled cell proliferation and growth.

The smaller β-transcript is encoded by exon 1β, located approximately 20 kb centromeric to exon 1α, and exons 2 and 3, and specifies the alternative product p14ARF (alternative reading frame). Protein p14ARF is also involved in cell cycle regulation by binding to HDM2 (human double minute 2) protein (MDM2 in mouse) (Zhang *et al*., 1998), which in turn inhibits HDM2-induced p53 degradation, resulting in stabilisation and accumulation of the p53 protein as well its downstream target p21 (Ortega *et al*., 2002).

Germline *CDKN2A* mutations have been identified in 20–57% of familial melanoma kindreds (Goldstein *et al*., 2007). The increased susceptibility to melanoma that is associated with the loss of the germline *CDKN2A* suggests that this genetic event increases the probability of displastic nevi becoming malignant or increases the rate of the development of new melanoma without the precursor. However, familial melanoma represents approximately 5–10% of all melanoma cases. For most cancers, inherited susceptibility is identified by positive family history, multiple primaries and an early age of onset. Hence, a number of studies have been sought for germline *CDKN2A* mutations in patients with multiple primary melanomas and early-onset melanoma, however, in the majority of studies the mutation-positive cases had also documented family history of melanoma (Blackwood *et al*., 2002). Sporadic melanomas rarely show the same molecular derangements as familial cases. Therefore, other molecular events are involved in the pathogenesis of most melanomas. In 25–50% of non-familial melanoma, a different tumour suppressor gene, phosphatase and tensin homolog (*PTEN*) is inactivated by mutation. *PTEN* negatively regulates the phosphatidylinositol 3-kinase (PI3K)-Akt signalling pathway, which conveys potent cell proliferation and survival signals (Wu *et al*., 2003). Loss of heterozygosity on regions of chromosome 10q, which harbours the *PTEN* locus, was demonstrated in 30–50% of malignant melanomas. Summarising various reports, approximately 3% of primary melanomas, 8% of melanoma metastases, and 31% of melanoma cell lines were shown to have *PTEN* mutations (de Snoo and Hayward, 2005). Information on specific mutations is summarised elsewhere (Wu *et al*., 2003).

Interestingly, patients with disorders such as Cowden disease, which arise from inherited *PTEN* mutations and are characterised by the presence of benign hamartomatous tumours, are not widely reported to display an increased susceptibility to melanoma (Liaw *et al*., 1997). More recently, two studies directly demonstrated that Akt is constitutively activated in more than 60% of melanomas, with higher frequencies of activation at later stages of the disease (Dhawan *et al*., 2002; Stahl *et al*., 2004). The latter study, by Stahl *et al*., 2004, identified amplification of the *AKT3* gene as an additional mechanism, distinct from *PTEN* loss, which may be responsible for these high frequencies of Akt activation.

Finally, it is important to note that loss or mutation of *PTEN* may occur concurrently with *BRAF* mutation, but neither *BRAF* nor *PTEN* alterations are found together with *NRAS* mutations (Tsao *et al*., 2000; Daniotti *et al*., 2004; Tsao *et al*., 2004). Thus, melanomas that have arisen in the absence of *NRAS* mutations nearly always harbour activated *BRAF*, inactivating alterations of *PTEN*, or both. Conversely, tumours with oncogenic *NRAS* mutations typically retain wild-type *BRAF* and *PTEN*. These results reiterate the importance of both the PI3K-Akt and Raf-MEK-ERK cascades in melanoma development. They also suggest that each of these pathways plays a significant role in melanoma development and that, in a subset of melanomas the two pathways may cooperate in promoting cancer progression.

Further progression of melanoma is associated with decreased differentiation of melanocytes and the decreased expression of melanoma markers regulated mainly by microphthalmia transcription factor (MITF). MITF, normally, causes differentiation and cell cycle arrest in normal melanocytes. However, melanoma cell do not have these characteristics. The progression from nevus to melanoma is accompanied by the decreased or absent expression of such melanoma markers as the melanoma-specific genes silver homologue (*SILV*) and the melan-A (*MLANA*) (Du *et al*., 2003), both controlled by MITF. Similarly, expression of the melastatin 1 (*TRPM1*) gene, whose function is unknown, is also controlled by MITF and melanomas that are deficient in melastatin (Duncan *et al*., 2001, Hammock *et al*., 2006) as well as in SILV and MLANA have a poor prognosis (Takeuchi *et al*., 2003). A large-scale search for genomic changes in melanoma with the use of high-density single nucleotide polymorphisms (SNPs) found an increased copy number of a region in chromosome 3 which harbours the *MITF* locus. This increase was accompanied by the evaluated expression of MITF protein, implicating *MITF* as an oncogene (Garraway *et al*., 2005).

The most important nature of malignant melanoma is its extremely high potential to develop metastasis. Histologically, invasive characteristics appear in the vertical growth phase, when melanoma cells penetrate the basement membrane and start to grow intradermally as an expanding nodule. Metastatic melanoma develops when tumour cells dissociate from the primary lesion, migrate through the surrounding stroma and invade blood and lymphatic vessels to form a tumour at a distant site. Clinically, the depth of local inva-

sion, measured directly by histopathologic analysis (the Breslow index), is the principal prognostic factor and primary criterion in melanoma staging (Balch *et al*., 2001).

At the molecular level, spread of melanoma is related to changes in cell adhesion. Normally, cell adhesion controls cell recognition, motility, and tissue integrity, but disturbances in cell adhesion contribute to the tumour progression and altered cell signalling. Cadherins, particularly E-cadherins, in normal human skin, are multifunctional transmembrane proteins that sustain cell-to-cell contacts. E-cadherin expression occurs in melanocytes and keratinocytes in the epidermis and causes melanocytes to associate with keratinocytes. Progression from the radial-growth phase to the vertical-growth phase of melanoma is associated with the loss of E-cadherin, suggesting that escape of epidermal melanocytes from E-cadherin mediated regulatory control by keratinocytes is an important event relevant to the development of melanoma (Hsu *et al*., 2000a). Another hallmark of melanoma progression from the radial-growth phase to the vertical-growth phase is the increase of N-cadherin expression. N-cadherin is characteristic for invasive carcinomas and in the case of melanoma enables metastatic spread by allowing melanoma cells to interact with other N-cadherin expressing cell, such as dermal fibroblasts and the vascular endothelium (Hsu *et al*., 2000b).

An additional group of proteins that mediates cell contacts, particularly cell contacts with the extracellular matrix, are integrins. Melanoma in the transition from radial to vertical growth is associated with the expression of $\alpha V\beta 3$ integrin (Dannen *et al*., 1994). This integrin induces expression of matrix metalloproteinase 2 (MMP2), an enzyme that degrades the collagen in basement membrane (Hofmann *et al*., 2000; Felding-Habermann *et al*., 2002). In addition $\alpha V\beta 3$ integrin increases the expression of the pro-survival gene *BCL-2* (Petitclerc *et al*., 1999) and stimulates the motility of melanoma cells (Li *et al*., 2001).

TREATMENT OF MELANOMA

Surgery of cutaneous malignant melanoma is the main treatment for localised melanoma. Radio- and chemotherapy have a limited (if any) survival impact on the control of late-stage melanoma. To some extent, biotherapy (i.e. interferon-alpha) appears to improve patient survival, however, under certain clinical circumstances. Therefore, numerous agents developed on the basis of knowledge for molecular changes in melanoma have been used in the treatment of advanced disease, but to date no single agent has significantly changed survival rates in melanoma.

CONCLUSIONS

A genomic profiling of human melanomas has revealed a highly rearranged melanoma genome, again attesting molecular heterogeneity of this disease (Bittner *et al*., 2000; Curtin *et al*., 2005; Haqq *et al*., 2005). More, the implementation on a large scale of novel technologies such a high-throughput methods (e.g. microarrays) and RNA interference has further increased the amount of discoveries in the field of molecular oncology, including melanoma. The amount of data presently available has already required the development of dedicated statistical and mathematical models to analyse and integrate the information generated. Moreover, the interactions between single molecules, different pathways, normal and malignant cells is making the understanding of cancer biology so complicated that a new discipline called "systems biology" has been brought out to the integration of this information (Hornberg *et al*., 2006).

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ĢENĒTISKĀS IZMAIŅAS MELANOMAS ATTĪSTĪBĀ

Normālas šūnas pārvēršanās par audzēja šūnu ir komplicēts process, kas ietver daudzas secīgas ģenētiskas izmaiņas. Pēdējo gadu laikā melanomas molekulārās ģenētikas jomā, ietverot slimības attīstību, progresiju un rezistenci pret terapiju, ir panākts ievērojams progress. Viens no nozīmīgiem posmiem audzēja attīstībā ir šūnu proliferatīvā novecošanās jeb senescence, kas aptur audzēja šūnu proliferāciju dažādās tā attīstības stadijās. Rakstā ir apkopoti zināmie dati par molekulārajām izmaiņām melanomas attīstības procesā un to iespējamo lomu melanomas terapijā.