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The role of cell cycle regulatory proteins in the pathogenesis of melanoma

WEI LI*†, AMIRA SANKI*‡§, ROOSHDIYA Z. KARIM*†‡, JOHN F. THOMPSON*‡§, C. SOON LEE‡∥, LIQING ZHUANG‡∥, STANLEY W. MCCARTHY*‡∥ and RICHARD A. SCOLYER*†‡

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Summary
The transformation of melanocytes to melanoma cells is characterised by abnormal proliferation resulting from alterations in cell cycle regulatory mechanisms. This occurs through alterations in the two major cell cycle regulatory pathways, the retinoblastoma (Rb) and p53 tumour suppressor pathways. This review summarises the current knowledge of alterations in these two pathways at G1/S transition and specifically the role of the key cell cycle regulatory proteins pRb, p16INK4a (p16), cyclin D1, p27Kip1 (p27), p53 and p21Waf1/Cip1 (p21) in the pathogenesis of melanoma. It also considers their prognostic significance. Current data indicate that alterations of cyclin kinase inhibitor (cdki) levels are implicated in the pathogenesis of melanoma and may be useful prognostic markers. However, large validation studies linked to comprehensive clinical follow up data are necessary to clarify the prognostic significance of cell cycle regulatory proteins in individual patients.

Key words: pRb, p16, cyclin D1, p27, p53, p21, melanoma, pathogenesis, prognosis, immunohistochemistry.

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INTRODUCTION
Epidemiology
Melanoma is a malignant tumour originating from melanocytes, the cells that produce the melanin pigment that colours skin, hair and eyes. Globally, the incidence of melanoma in 2002 was approximately 160 000, with approximately 40 000 people dying of the disease. In Australia and many other Western countries, the incidence of melanoma has increased at an approximate rate of 5% per annum over the past 40 years. This exceeds the increase in the incidence rate of any other solid tumour, with the exception of lung cancer in women. According to the Australian Institute of Health and Welfare (AIHW), Australia has the highest rate of melanoma in the world among males and the second highest rate in females. In the state of New South Wales, Australia, melanoma was the second most common cancer for both men and women in 2003. Mortality from melanoma is lower than for other common cancers and is stable or declining slowly; this is attributed to early diagnosis and prompt definitive treatment. However, it has a disproportionately heavy impact on the most productive years of life because it is the most common cancer in young adults. Melanoma is a major health problem not only because of its incidence and propensity to affect young adults, but also because of its high metastatic potential, aggressive clinical behaviour and notable resistance to currently available chemotherapeutic and immunological treatments.

Risk factors
Exposure to ultraviolet (UV) radiation, and in particular sunburn, has been proposed as a major environmental risk factor for the development of melanoma. UV irradiation is thought to predispose to malignancy by a number of mechanisms including direct mutagenic effects on DNA, promotion of the production of growth factors by skin cells, reduction of immune defenses and stimulation of the production of reactive oxygen species from melanin (which causes DNA damage and suppresses apoptosis). Melanoma develops as a consequence of the accumulation of defects in intracellular genetic pathways, which result in promotion of cell proliferation and impairment of the normal pathways of apoptosis in response to DNA damage. Epidemiological studies have identified some host factors that are also important risk factors for melanoma, including a family history of melanoma, certain melanoma susceptibility genes, number and types of naevi, and skin type and degree of pigmentation. Despite advances in the knowledge of risk factors for melanoma, the underlying molecular mechanisms of melanoma development remain unclear.

CELL CYCLE CONTROL
For eukaryotic cells, the cell cycle has been defined as the interval between the completion of mitosis in a cell and the completion of mitosis by one or both of its daughter cells. Traditionally the cell cycle has been divided into four phases: the G1, S and G2 phases, during which the cell prepares itself for division, and the M phase when the chromosomes are separated and the cell divides (Fig. 1, 2). Cell cycle progression is driven by the sequential activation and inactivation of cyclin dependent kinases (cdks), which are activated when they become bound to

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regulatory proteins (cyclins).9–11 When complexed with D-type cyclins, these kinases drive entry into the cell cycle by phosphorylating the retinoblastoma protein (pRb), which causes the release of E2F transcription factors and expression of E2F, thereby allowing progression from G1 to S phase. This process is negatively regulated by cdki and is also under surveillance at a number of checkpoints.

In the early 1970s, it was recognised that the cell cycle is governed by a series of controlling molecules that provide ‘checkpoints’,12,13 which ensure that the preceding process is accurately completed before allowing the next stage to proceed.14,15 ‘Checkpoints’ are found in at least four stages of the cell cycle: in G1, at the G1/S transition, at the G2/M transition and at the metaphase/anaphase transition.

The cell cycle protein p53, which has been termed ‘the guardian of the genome’,16 is one of the most important cell cycle proteins mediated by regulation of checkpoints at G1. Under normal circumstances, p53 is dormant until activated by DNA damage or other genomic aberrations. p53 then activates the p21 protein to arrest the cell cycle in G1 to allow DNA repair or to induce apoptosis (programmed cell death) if the DNA damage is too severe.

CELL CYCLE CONTROL AND MELANOMA
Enhanced cell proliferation in the absence of external stimuli is a common characteristic of malignant tumours. This observation suggests that a disturbance of normal cell cycle control leading to genomic instability is likely to contribute to the development of many malignancies. Therefore, an extensive knowledge of cell cycle regulatory mechanisms in human cells, including melanocytes, and their alterations in tumour tissues, such as melanomas, will increase an understanding of the process by which tumours develop carcinogenesis and may lead to the identification of new prognostic markers. These markers may also assist in the diagnosis of tumours that are difficult to classify using standard pathological criteria, and in the development of specific, target-directed therapeutic approaches.

PRB PATHWAY
Overview of pRb pathway
The p16/CDKN2, D-type cyclins, their partners cdk4/cdk6, and pRb constitute a G1 regulatory pathway commonly targeted in tumorigenesis. As a functional unit, deregulation of this pathway appears to represent a common step in the multistep process of melanoma development. The pRb pathway appears to be critically important in the suppression of melanoma development, since nearly all human melanoma cell lines tested have shown disruption of this pathway, via p16 or pRb deficiency, cdk4 mutation or overexpression of cyclin D1.19–21

p16
Overview Located at 9p21, the p16 gene (CDKN2A) encodes a 156 amino acid nuclear protein. The p16 protein belongs to the INK4 (inhibitors of CDK4) family, which has four members, p16INK4a, p15INK4b (p15), p18INK4c (p18) and p19INK4d (p19, in mouse)/p14ARF (p14 in man), all sharing the capacity to control the G1/S transition. By specifically binding to cdk4/6, the p16 proteins inhibit the formation of the cdk4/6/cyclin D enzyme complex, which is required for the phosphorylation of pRb, ensuring that pRb remains in a complex with the E2F transcription factor (Fig. 3).22–24 Since hypophosphorylated pRb binds

Of the many regulatory checkpoints of the cell cycle, the acquisition of abnormalities at the G1/S checkpoint appears to be the most crucial step in the genesis and progression of melanoma.17,18 Inactivation of the pRb and p53 pathways at G1/S transition is a fundamental requirement for the genesis of most human cancers, including melanoma. While alterations in the pRb pathway are relatively common in melanomas, those in the p53 pathway are infrequent. In this article, current knowledge of the role of the key cell cycle regulatory proteins involved in the pRb (p16, cyclin D1, pRb and p27) and p53 pathways (p53 and p21) functioning at G1/S in the pathogenesis and prognosis of melanoma is reviewed.
to and suppresses E2F transcriptional activity and constrains G1 exit, p16 expression results in cell cycle arrest.

Alterations of the p16 gene in the pathogenesis of melanoma

The p16 gene is either mutated or deleted in a large majority of cultured melanoma cell lines (the rate averaging approximately 70% across numerous studies),19–21,25–27 in many uncultured melanoma cells (the rate averaging approximately 36%),25,28–33 and in germ lines of familial melanoma kindreds (in approximately 40%).34–37 In sporadic melanomas, p16 mutations are reported to be rare in most studies,28,38 although a study by Kumar et al. found a mutation frequency of 26% in their series of microdissected cases.31 A number of possible mechanisms, including homozygous deletion,39 loss of heterozygosity (LOH),40,41 intragenic mutation,42 hypermethylation of promoter regions43,44 and microsatellite instability,45 have been found to result in the inactivation of p16, and therefore the loss of p16 expression in melanomas.

Loss of expression of p16 in the pathogenesis of melanoma

Loss of expression of p16 has been demonstrated in almost 50% of primary melanomas40,45–48 (Table 1), at a similar level to that documented in many other cancers, including pancreatic endocrine neoplasms, oesophageal, lung, head and neck, breast, bladder, brain and ovarian cancers.49–52 Moreover, a gradual loss of p16 associated with melanoma progression has been demonstrated in both sporadic and familial melanomas at both protein45,47,53 and mRNA levels.53 Although there is general consensus regarding the loss of p16 expression in locally advanced primary melanomas, whether loss of p16 is also associated with the initiation and early stages of melanoma development is a topic of debate.53,55,54 Existing observations indicate that p16 expression is not altered in naevi,45,47 and is not altered or is reduced in in situ melanomas.53,54 The failure to demonstrate reduced expression in naevi suggests that either these lesions do not represent an early stage in melanoma development or that loss of p16INK4 function is not an initiating event in melanocyte transformation. However, the presence of germline mutations in CDKN2A in a proportion of familial melanoma patients indicates that loss of p16 expression could be involved in the initiation and early development of melanoma in some familial kindreds.55 Chana et al. found a low level of p16 expression in acral lentiginous melanomas (ALM), which was not correlated with tumour thickness, suggesting that loss of p16 expression is an early event in ALM.56

It appears that p16 expression in melanomas depends on the anatomical site of involvement. In one study of cutaneous melanomas, absent or weak staining of p16 was more frequently observed in tumours involving the trunk or extremities than those involving the head or neck.57 However, in a recent study on the expression of p16 in melanomas with or without a contiguous naevus remnant, 85% of melanomas without an associated naevus (mostly from non-sun exposed sites)38 showed loss of nuclear p16 immunoreactivity, whereas only 24% of the

Fig. 3 Diagramatic representation of the p53 and pRb pathways and their regulation of the cell cycle and apoptosis. Arrows indicate positive effects and T bars indicate negative effects.
melanomas associated with naevus remnants showed loss of nuclear p16 staining. These results support the hypothesis that there are two pathways for melanoma development – a UV-B induced pathway and a non-UV-B induced pathway.

Loss of p16 expression and the prognosis of patients with melanomas It has been observed that loss of p16 expression correlates with increasing tumour thickness, higher mitotic rate, increased proliferation rate as measured by immunostaining for Ki-67, and higher Clark level, which are all poor prognostic factors in melanoma patients. However, not all studies have found such correlations.

Reduced expression of p16 in melanomas was associated with a significant risk of relapse by multivariate analysis in one study, and was an independent predictor for decreased patient survival in a number of other studies (Table 1).

Cyclin D1 Overview Cyclin D1 is a 34-kDa nuclear protein of 295 amino acids coded by the CCND1 gene, which is located at 11q13. Synthesised in early G1, cyclin D1 is an important positive regulator of the G1-S cell cycle transition. As the best characterised member of the D-type cyclin family, cyclin D1 binds to and activates its kinase partners cdk4/6, contributing to the phosphorylation and inactivation of pRb, blocking its growth inhibitory activity and promoting the release of bound E2F, leading to cell cycle progression (Fig. 3).

Amplification of the CCND1 gene in the pathogenesis of melanoma Compared with CDKN2a, the role of the CCND1 gene in the pathogenesis of melanoma is less well established. In one recent study, amplification of CCND1 was found in 47% of primary tumours and 35% of metastases. These studies suggest a possible role for the CCND1 gene in the pathogenesis of melanoma. However, in a series of 61 melanoma metastases, no CCND1 amplification was found and in another study of ten melanoma cell lines only one showed an amplification of chromosomal region 11q13, which contains the cyclin D1 locus.

Amplification of CCND1 has been identified in up to 44% of acral lentiginous...
of melanomas, but was much less frequently observed in melanoma subtypes.

All cases with an increased copy number of the CCND1 gene were found to over-express cyclin D1 when assessed immunohistochemically. Strikingly, about 25% of melanomas that over-expressed cyclin D1 were found to have a normal copy number of the CCND1 gene, which suggests that expression levels of cyclin D1 are modulated by mechanisms other than gene copies.

The expression of cyclin D1 in the pathogenesis of melanoma Evidence supporting an oncogenic role for cyclin D1 includes the results of experiments demonstrating that antisense treatment of mice bearing melanoma xenografts over-expressing cyclin D1 leads to apoptosis and tumour shrinkage. Under normal conditions, the expression of cyclin D1 is stringently regulated by extracellular mitogenic stimuli as well as in melanoma metastases. In immunohistochemical studies of human melanomas, increased expression of cyclin D1 has been reported in both uveal and cutaneous melanomas, suggesting that it has an oncogenic role in melanoma pathogenesis (Table 2). However, other studies found no immunoreactivity for cyclin D1 in more than two-thirds of melanomas. In contrast to melanomas, naevi and normal melanocytes in skin adjacent to tumours were found to have absent or weak expression of cyclin D1. Seykora et al. reported that the expression of cyclin D1 was 2.7 times higher in melanomas than in naevi.

Expression of cyclin D1 and the prognosis of patients with melanoma Several studies have assessed the prognostic value of cyclin D1 in patients with melanomas. Studies on uveal melanomas found that cyclin D1 positivity was associated with an unfavourable outcome and was an independent risk factor for the development of metastases by multivariate analysis. In a study of cutaneous melanomas, a high level of cyclin D1 was found to be significantly associated with thinner lesions. However, Bachmann et al. and Alonso et al. found no association between increased expression of cyclin D1 and patient outcome.

pRb

Overview Located at chromosome 13q14, Rb gene encodes a nuclear protein of 928 amino acids. The pRb protein acts as a gatekeeper at the G1 phase by preventing cells from entering into the S phase. There are three members in the retinoblastoma family of nuclear pocket proteins, which include Rb itself, pRb2/p130 and p107. In non-dividing cells, the pRb protein in its underphosphorylated form is bound to the E2F family of transcription factors (E2F1–E2F5). The E2F-pRb complex represses transcription of E2F-responsive genes, leading to a block in the cell cycle. During late G1, phosphorylation of pRb by cdk4/6-cyclin

### Table 2: Studies of cyclin D1 expression in melanocytic lesions

<table>
<thead>
<tr>
<th>Study, year</th>
<th>No. cases</th>
<th>Site of tumour</th>
<th>Types of tumour</th>
<th>Expression of cyclin D1</th>
<th>Measure of prognosis</th>
<th>Results of prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coupland et al., 1998</td>
<td>66</td>
<td>Uveal melanoma</td>
<td>Primary melanoma</td>
<td>65% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Florenes et al., 2000</td>
<td>255</td>
<td>Cutaneous melanoma</td>
<td>Naevi</td>
<td>0% expression</td>
<td>Survival</td>
<td>No correlation</td>
</tr>
<tr>
<td>Coupland et al., 2000</td>
<td>96</td>
<td>Uveal melanoma</td>
<td>Primary melanoma</td>
<td>62% expression</td>
<td>(&gt;15% positive cells)</td>
<td>Survival</td>
</tr>
<tr>
<td>Brantley et al., 2000</td>
<td>79</td>
<td>Cutaneous melanoma</td>
<td>Naevi</td>
<td>41% expression</td>
<td>(&gt;20% positive cells)</td>
<td>NT</td>
</tr>
<tr>
<td>Georgieva et al., 2004</td>
<td>51</td>
<td>Cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>8% expression</td>
<td>(&gt;25% positive cells)</td>
<td>NT</td>
</tr>
<tr>
<td>Sauter et al., 2002</td>
<td>127</td>
<td>Cutaneous melanoma</td>
<td>Melanocytes</td>
<td>6% expression</td>
<td>(&gt;10% positive cells)</td>
<td>NT</td>
</tr>
<tr>
<td>Errico et al., 2003</td>
<td>45</td>
<td>Uveal melanoma</td>
<td>Primary melanoma</td>
<td>51% expression</td>
<td>Recurrence</td>
<td>More with high expression</td>
</tr>
<tr>
<td>Buchmann et al., 2004</td>
<td>186</td>
<td>Cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>64% over-expression</td>
<td>Staining index &gt;4</td>
<td>Survival</td>
</tr>
<tr>
<td>Alonso et al., 2004</td>
<td>165</td>
<td>Cutaneous melanoma</td>
<td>Naevi</td>
<td>0% expression</td>
<td>Survival</td>
<td>No correlation</td>
</tr>
<tr>
<td>Ramirez et al., 2005</td>
<td>126</td>
<td>NS</td>
<td>Primary melanoma</td>
<td>6% expression</td>
<td>(&gt;20% positive cells)</td>
<td>NT</td>
</tr>
</tbody>
</table>

NS, not stated; NT, not tested.
D leads to the release of the E2F transcription factors that positively regulate the transcription of genes whose products are required for S phase progression31 (Fig. 3). pRb remains hyperphosphorylated for the remainder of the cell cycle and cdk2-cyclin E participates in maintaining this hyperphosphorylated state.

Alterations of Rb gene in the pathogenesis of melanoma

Despite the fact that inherited mutations in Rb predispose humans to melanomas and other tumors,84,85 mutations in Rb genes are rare in melanomas.19,86,87 The finding that pRb was expressed strongly in most melanomas also suggests that the Rb gene is not commonly mutated in this kind of cancer.82,88–90 However, there may be a functional inactivation along its pathway and it may be important in the pathogenesis of melanoma.89,91 Instead of gene mutations, the function of pRb in human melanoma tumours and cell lines is in fact commonly inhibited by hyperphosphorylation, and to a lesser extent by underexpression of the gene.19,92,93 The mechanisms for the inhibition of normal pRb function include sustained phosphorylation of pRb by deregulated cdks,94,95 sequestration of Rb by viral transforming protein such as the human papillomavirus E7 protein,96 the adenoviral E1A protein,97,98 or the SV40 large T-antigen.99

Expression of pRb in the pathogenesis of melanoma

Aberrant function of pRb is a fundamental element of uncontrolled growth in melanoma. Inhibition of the normal, suppressive function of the Rb family of pocket proteins has been induced in melanomas in vivo in some experimental mouse models,101,102 suggesting a role for pRb in the pathogenesis of melanoma. In studies of melanoma cell lines, melanoma cells have been shown to exhibit over-expression of hyperphosphorylated pRb (which means that pRb was active but unable to bind to E2F) or a marked decrease in or lack of pRb expression, compared with normal melanocytes.19,74 Inactivation of pRb in melanomas may occur as a result of phosphorylation of sites in the C-terminal region of pRb protein by cyclin/cdk complexes.89

The persistent phosphorylation of pRb commonly observed in melanomas is expected to relieve E2F-pRb transcriptional repression and to generate free E2F activity. Indeed, free E2F activity, mostly E2F2 and E2F4, is highly abundant in melanoma cells compared with normal melanocytes in one study.92 Another possible explanation for the lack of binding of E2F to pRb is that E2F is affected by mutational change. However, alterations in the E2F gene family are infrequent and no mutations have been found in the Rb binding site of E2F in melanomas in one study.103

Reduced pRb expression is significantly correlated with melanocytic tumour progression in two studies. Higher levels of expression were observed in naevi and lower levels in melanoma metastases90,104 (Table 3). However, another study found that pRb was progressively increased in advanced and metastatic melanoma in vivo.105 This increase was associated with increased pRb inactivation due to protein phosphorylation. Furthermore, some other studies have found no pRb expression in normal melanocytes or in the majority of naevus cells106 and undetectable expression in a rapidly growing uveal melanoma.107 These differences may be attributable to the use of different antibodies in the various studies or to differences in the types of tissue involved by the tumours. The use of frozen sections is reported to increase the sensitivity of the method used and allow the detection of some proteins that may have been partially destroyed by fixation in formalin and embedding in paraffin.108

The expression of pRb and the prognosis of patients with melanomas

The prognostic significance of altered pRb expression in melanoma is unclear. Aberrant staining of pRb was found to correlate strongly with failed radiotherapy before enucleation in patients with uveal melanoma.91 Higher expression of pRb2/p130 was found to correlate with a better prognosis in patients with choroidal melanoma90 (Table 3). However, larger studies are required comparing the prognostic value of pRb with other well-characterised markers.109

Table 3 Studies of pRb expression in melanocytic lesions

<table>
<thead>
<tr>
<th>Study, year</th>
<th>No. cases</th>
<th>Site of tumour</th>
<th>Types of tumour</th>
<th>Expression of pRb</th>
<th>Measure of prognosis</th>
<th>Results of prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bachmann et al., 200442</td>
<td>219</td>
<td>Cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>44% low expression</td>
<td>staining index&lt;4</td>
<td>Survival</td>
</tr>
<tr>
<td>Britney et al., 200046</td>
<td>32</td>
<td>Uveal melanoma</td>
<td>Metastatic melanoma</td>
<td>68% low expression</td>
<td>&gt;20% positive cells</td>
<td>NT</td>
</tr>
<tr>
<td>Massaro-Giordano et al., 199990</td>
<td>55</td>
<td>Choroidal melanoma</td>
<td>Primary melanoma</td>
<td>94% strong staining</td>
<td></td>
<td>Survival</td>
</tr>
<tr>
<td>Korabiowska et al., 2001104</td>
<td>209</td>
<td>Cutaneous melanoma</td>
<td>Naevi</td>
<td>98% low or no expression</td>
<td></td>
<td>Survival</td>
</tr>
<tr>
<td>Saenz-Santamaria et al., 1993106</td>
<td>31</td>
<td>Cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>100% expression</td>
<td></td>
<td>NT</td>
</tr>
<tr>
<td>Alonso et al., 2004108</td>
<td>165</td>
<td>Cutaneous melanoma</td>
<td>Naevi</td>
<td>0% expression</td>
<td></td>
<td>Survival</td>
</tr>
</tbody>
</table>

NT, not tested.
p27

Overview  p27 is both a nuclear and cytoplasmic 22-kDa protein (198 amino acids) located on chromosome 12p12–12p13.10,11 However, only the nuclear form of p27 protein functions as a cdki. Its structure shares 44% homology with p21 at the N-terminal portion.11,12 p27 has been demonstrated to play an important role in regulating progression through G1 and entrance into the S phase of the cell cycle by binding to and preventing premature activation of cdk4/cyclin D and cdk2/cycle E complexes113,114 (Fig. 3).

Alterations of the p27 gene in the pathogenesis of melanoma
Specific alterations of the p27 gene, including mutations and homozygous deletions, are exceedingly rare in melanoma. Instead p27 is predominantly regulated post-transcriptionally/post-translationally.115,116 Methylation of the p27 gene is another mechanism for altering the expression levels of p27 in melanomas.116 p27 methylation is found to be associated with transcriptional silencing in melanoma in situ.116

The expression of p27 in the pathogenesis of melanoma
The expression of p27 is highest in quiescent cells and declines as cells re-enter the cell cycle. Constitutive over-expression of p27 arrests the cell cycle at the G1 phase. p27 is degraded at the late G1 phase.117 The degradation of p27 allows for the activation of cyclin E/cdk2 and promotes S phase entry (Fig. 3).

The pattern of p27 expression in melanocytic tumours, as evidenced by immunohistochemistry, is highly heterogeneous118–122 (Table 4). Expression of p27 was found to be progressively lost in the transition from benign naevi to metastatic melanomas and in the transition from thin primary melanomas (AJCC 2002 stage I) to thicker primary melanomas (AJCC 2002 stage II).118,119,120,123,124

While the majority of metastatic melanomas had a loss of p27 expression, one-third of metastatic melanomas showed an increase in p27 expression of greater than 20%.115,119 This suggests that subsets of melanoma cells maintain an ability to up-regulate p27, perhaps in response to unfavourable changes in the tissue micro-environment (e.g., hypoxia and/or low pH). p27 up-regulation may represent a survival response to protect tumour cells from injury by preventing excessive cell proliferation and apoptosis.125

The expression of p27 has been identified as essential for the observed hypoxia-induced cell cycle arrest in a variety of human tumours, including melanoma.74,115,126–130 In these studies, a significant increase in p27 expression was found in tumour cells in hypovascular, necrotic areas compared with perivascular areas. An inverse relationship between Ki-67 and p27 was also identified in these tumours, supporting the hypothesis that p27 up-regulation may play a role in hypoxia-mediated cell cycle arrest in these tumours.118 The parallel increase in proliferation and decrease in p27 expression may be explained by a constant increase in the expression of SKP2, a protein involved in the ubiquitination and degradation of p27 during different stages of melanoma progression.18 However, some studies have failed to demonstrate an inverse relationship between Ki-67 and p27.119

Unlike the other cell cycle proteins, which display distinct nuclear immunoreactivity, some melanomas demonstrate additional p27 cytoplasmic positivity.118,131 The increase of p27 expression in the cytoplasm may partially explain the reduction of p27 nuclear expression in melanomas by its translocation into the cytoplasm and increased degradation in highly mitotically active cells.119,121,132 This finding supports the hypothesis that sequestration of p27 in the cytoplasm blocks p27 activity and its nuclear expression, and plays an important role in promoting oncogenesis.132

Table 4  Studies of p27 expression in melanocytic lesions

<table>
<thead>
<tr>
<th>Study, year</th>
<th>No. cases</th>
<th>Site of tumour</th>
<th>Types of tumour</th>
<th>Expression of p27</th>
<th>Measure of prognosis</th>
<th>Results of prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flores et al., 1998119</td>
<td>162</td>
<td>Cutaneous melanoma</td>
<td>Naevi Primary melanoma Metastatic melanoma</td>
<td>100% expression (&gt;50% positive cells)</td>
<td>More with low expression (≤5% positive cells)</td>
<td>No correlation</td>
</tr>
<tr>
<td>Bales et al., 199921</td>
<td>43</td>
<td>NS</td>
<td>Primary melanoma Metastatic melanoma Compound naevi Spitz naevi Primary melanoma Primary melanoma</td>
<td>72% high expression 50% high expression LI=40.1 ± 4.8 LI=38.4 ± 4.0 LI=42.3 ± 5.1</td>
<td>Survival NT</td>
<td>NT</td>
</tr>
<tr>
<td>Morgan et al., 199922</td>
<td>63</td>
<td>NS</td>
<td>Primary melanoma Metastatic melanoma</td>
<td>28% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Mouriaux et al., 200019</td>
<td>32</td>
<td>Choroidal melanoma</td>
<td>Naevi Melanoma in situ Primary melanoma Metastatic melanoma</td>
<td>LI=85 ± 15 LI=45 ± 20 LI=30 ± 25 LI=15 ± 20</td>
<td>Survival NT</td>
<td>NT</td>
</tr>
<tr>
<td>Li et al., 200420</td>
<td>198</td>
<td>Cutaneous melanoma</td>
<td>Naevi Melanoma in situ Primary melanoma Metastatic melanoma Radial melanoma Vertical melanoma Metastatic melanoma Naevi Dysplastic naevi Primary melanoma Metastatic melanoma</td>
<td>70% expression 76% expression 45% expression 37% expression 88% expression 95% expression 50% expression 13% expression</td>
<td>Survival No correlation</td>
<td>NT</td>
</tr>
<tr>
<td>Alonso et al., 200424</td>
<td>165</td>
<td>Cutaneous melanoma</td>
<td>Naevi Radial melanoma Vertical melanoma Metastatic melanoma</td>
<td>76% expression 45% expression 37% expression 88% expression</td>
<td>Survival No correlation</td>
<td>NT</td>
</tr>
<tr>
<td>Ivan et al., 200421</td>
<td>94</td>
<td>Cutaneous melanoma</td>
<td>Naevi Primary melanoma Metastatic melanoma</td>
<td>95% expression 50% expression 13% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

NS, not stated; NT, not tested; LI, labelling index.
The expression of p27 and the prognosis of patients with melanomas There have been very few studies reporting the association of p27 with clinical outcome in melanoma patients. Florenes et al. found that decreased expression of p27 was significantly associated with increasing Breslow thickness and that low p27 expression was correlated with reduced disease-free survival in primary nodular melanomas, but not in the superficial spreading subtype of melanomas. The mechanisms of protein that drives expression of the downstream cells. This leads to the accumulation of wild-type p53 protein is then released from mdm2 and becomes double minute 2 homologue (mdm2) (Fig. 3). The p53 induction of cell cycle arrest is mediated by Fig. 3, the p53 induction of cell cycle arrest is mediated by apoptosis, through inactivation of cyclin-dependent kinases. (Fig. 3).

p53
Overview p53 is derived from a 53-kDa protein encoded by the TP53 gene. The TP53 gene is located on the short arm (p) of chromosome 17 and consists of 11 exons. The human TP53 gene spans 20 kb and codes for a nuclear phosphoprotein (393 amino acid in length). As an oncosuppressive protein, p53 acts as ‘the guardian of the genome’. Under normal circumstances, p53 is dormant until activated by DNA damage or other genomic aberrations. p53 then activates its downstream effectors, such as p21, to arrest the cell cycle in G1, which inhibits replication of damaged DNA and allows DNA repair to occur. Alternatively, if damage is too severe, apoptosis is induced (Fig. 3). Loss of the guardianship function of p53 allows continued replication of cells with damaged DNA, and in turn leads to an accumulation of genetic changes that contribute to malignant progression. As depicted in Fig. 3, the p53 induction of cell cycle arrest is mediated by p14, which transmits the stress signal to the ‘mouse of double minute 2’ homologue (mdm2) (Fig. 3). The p53 protein is then released from mdm2 and becomes phosphorylated, resulting in the stabilisation of p53 in the cells. This leads to the accumulation of wild-type p53 protein that drives expression of the downstream components and induces G1 arrest. The mechanisms of p53 induction of apoptosis are shown in Fig. 3.

TP53 gene mutations in the pathogenesis of melanoma Over 600 papers have been published to date on TP53 gene alterations in melanoma, but these have not yielded consistent results. Several studies reported a prevalence of TP53 gene alterations ranging from 1 to 29% and 11 to 29% in primary and metastatic melanomas, respectively. However, other studies found an absence of TP53 mutations, or reported that they are uncommon events, suggesting that they have an insignificant role in the development of melanoma. In general, TP53 gene mutations are likely to be rare events in melanoma, especially when compared with internal cancers. TP53 mutations in melanoma may be UV radiation related. Melanomas in patients with xeroderma pigmentosum (XP), whose tumours are mainly located on sun-exposed parts of the body and are not as aggressive as melanomas occurring in non-XP patients, show a very high frequency (60%) of TP53 mutations. Furthermore, there are marked differences in the sites of TP53 gene mutations between melanomas and internal cancers. In internal cancers, mutational hot spots are more frequent and coincide with the four highly conserved regions of the TP53 gene, and TP53 mutations usually result from C:C substitutions at the CpG sites. In contrast, mutations in melanomas are not localised to hot spot regions, and usually affect the C:C base pairing by substitution with T:A pairs. This further suggests a role of UV exposure as the underlying aetiology in the genesis of p53 mutations in melanomas.

The few studies that have detected mutational changes of the TP53 gene in naevi found that they occur at a lower frequency (0–18%) than in primary and metastatic melanomas; and include C:C to T:A transition-type mutations related to UV radiation. Interestingly, most TP53 mutation-positive naevi were found in patients with a family/personal history of melanoma.

The differences in the frequency of TP53 mutations in melanoma between the different studies may be attributed to variations in the detection methods applied, bias in the selection of patients at various developmental stages of tumour progression, different anatomical sites, and genetic heterogeneity of the tumours. To summarise, TP53 gene mutations occur in at least a subset of melanomas, but at a low frequency. TP53 mutations are more common in metastatic melanomas and less common in naevi than in primary melanomas. However, the absence of TP53 mutations in a subset of melanomas does not preclude the possibility that these tumours may have sustained defects at upstream or downstream points along the p53 pathway, which inactivate the p53 protein in response to DNA damage. This indicates that the entire p53 pathway is a more critical determinant of the fate of these lesions than the status of the TP53 gene itself.

Over-expression of p53 protein in the pathogenesis of melanoma While the TP53 gene mutations are rare events in melanoma, the p53 proteins are widely expressed in melanomas, with variable frequency. This observation is consistent with the finding that mdm2 promotes p53 degradation, suggesting that an imbalance of p53 and mdm2 may be involved in the pathogenesis of melanoma. However, mdm2 expression was found to correlate with an increased p53 expression and mutations in other studies.

It has been reported that the expression of p53 may be related to the anatomical site of the lesion. Straume et al. reported that p53 expression was generally stronger and more frequent in lesions arising in sites chronically exposed to the sun, such as head and neck melanomas, compared with other sites (32 versus 6%), further implicating UV...
exposure in dysregulation of p53. However, other studies have demonstrated no difference in the expression of p53 between chronically sun-exposed cutaneous sites and concealed mucosal sites. 164

Most studies have reported an absence or a very low level (<15%) of p53 expression in melanocytic naevi. 159,177,178 Furthermore, most studies have shown that the expression of p53 progressively increases with tumour progression (benign naevi→dysplastic naevi→radial growth phase melanomas→vertical growth phase melanomas→metastatic melanomas), 149,177,179,181 and with increased depth of tumour invasion. 168,171,178,181–183 Therefore, the expression of p53 protein may be a late event in melanoma progression.

For some cancers, such as astrocytic tumours, follicular lymphomas and colorectal cancers, there is a clear concordance between the presence of TP53 mutations and the over-expression of p53 protein. 155,184,185 However, this has not been observed in melanomas. The presence of p53 protein expression in melanomas either with low frequency or with no TP53 gene mutations suggests that the expression of p53 protein may have resulted from increased activation and the stabilisation of the protein after stress arising from DNA damage, oncogene activation or hypoxia, rather than TP52 mutation. 143

The expression of p53 and the prognosis of patients with melanomas The prognostic significance of p53 alterations in melanoma is controversial. An expression of p53 protein has been reported to be significantly associated with a tumour thickness over 1.5 mm, Clark levels IV and V, the presence of ulceration, lymphatic infiltration, high mitotic rate, and a low 5-year survival rate, but to be inversely correlated with disease-free interval in some studies. 81,171,186,187 (Table 5). Nonetheless, other studies have found no relationship between p53 expression and clinical outcome, 37,170,173,181,188 or even that p53 over-expression was significantly associated with improved survival. 189

Table 5 Studies of p53 expression in melanocytic lesions

<table>
<thead>
<tr>
<th>Study, year</th>
<th>No. cases</th>
<th>Site of tumour</th>
<th>Types of tumour</th>
<th>Expression of p53</th>
<th>Measure of prognosis</th>
<th>Results of prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lassam et al., 1993 178</td>
<td>153</td>
<td>Cutaneous melanoma</td>
<td>Naevi</td>
<td>0% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Cristofolini et al., 1993 180</td>
<td>122</td>
<td>Cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>5% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Florenes et al., 1995 176</td>
<td>156</td>
<td>NS</td>
<td>Naevi</td>
<td>70% expression</td>
<td>Survival</td>
<td>NT</td>
</tr>
<tr>
<td>Yamamoto et al., 1995 171</td>
<td>60</td>
<td>Cutaneous melanoma</td>
<td>Metastatic melanoma</td>
<td>15% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Kanoko et al., 1996 183</td>
<td>89</td>
<td>NS</td>
<td>Naevi</td>
<td>30% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Ross et al., 1997 187</td>
<td>60</td>
<td>Cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>0% expression</td>
<td>Disease free interval</td>
<td>NT</td>
</tr>
<tr>
<td>Straume et al., 1997 180</td>
<td>102</td>
<td>Cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>81% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Healy et al., 1998 180</td>
<td>70</td>
<td>Cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>0.0–10 (median 0.03) expression</td>
<td>Disease free survival</td>
<td>NT</td>
</tr>
<tr>
<td>Hiicken et al., 1999 188</td>
<td>111</td>
<td>Cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>0% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Chana et al., 1999 185</td>
<td>71</td>
<td>Uveal melanoma</td>
<td>Primary melanoma</td>
<td>65% high expression</td>
<td>Survival</td>
<td>No correlation</td>
</tr>
<tr>
<td>Karjalainen et al., 1999 180</td>
<td>284</td>
<td>Stage I cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>0% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Coupland et al., 2000 181</td>
<td>96</td>
<td>Uveal melanoma</td>
<td>Primary melanoma</td>
<td>68% (staining index &gt;0%) expression</td>
<td>Survival</td>
<td>No correlation</td>
</tr>
<tr>
<td>Ragnarsson-Olding et al., 2004 184</td>
<td>56</td>
<td>Mucosal melanoma</td>
<td>Primary melanoma</td>
<td>22% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Hussein et al., 2005 186</td>
<td>60</td>
<td>Uveal melanoma</td>
<td>Primary melanoma</td>
<td>70% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

The expression of p53 and the prognosis of patients with melanomas The prognostic significance of p53 alterations in melanoma is controversial. An expression of p53 protein has been reported to be significantly associated with a tumour thickness over 1.5 mm, Clark levels IV and V, the presence of ulceration, lymphatic infiltration, high mitotic rate, and a low 5-year survival rate, but to be inversely correlated with disease-free interval in some studies 81,171,186,187 (Table 5). Nonetheless, other studies have found no relationship between p53 expression and clinical outcome, 37,170,173,181,188 or even that p53 over-expression was significantly associated with improved survival. 189

p21

Overview p21 is a 21-kDa protein product of approximately 166 amino acids encoded by the ras gene (chromosome 6p21) and it has been reported to be located in both the nucleus and cytoplasm of cells. 190 However, only the nuclear form of p21 protein functions as a cdki. p21 is a direct transcriptional target of p53, and is strongly induced by wild-type p53 in response to DNA damage. It mediates the growth suppression effects of p53 by arresting the cell cycle at the G1/S checkpoint and by inducing apoptosis 191 (Fig. 3). In addition, p21 has also been

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demonstrated to be involved in cellular senescence, terminal differentiation, and apoptosis through p53-independent mechanisms.\textsuperscript{192,193} p21 has both positive and negative effects on G1 progression, with the inhibitory effects predominating.\textsuperscript{194} When present at low concentrations within the cell, p21 facilitates the attachment of cyclin D1 to cdk4/6 kinases, which results in the inactivation of pRb and progression of the cell cycle.\textsuperscript{195} Higher concentrations of p21 lead to an increase in its stoichiometry in p21-cyclin D1-cdk4 complexes, which results in the inhibition of cyclinD1-cdk4 activity.\textsuperscript{196} The p21 protein binds a broad range of cyclin/cdk complexes, with a preference for those containing cdk2.\textsuperscript{197}

### Alterations of p21 gene in the pathogenesis of melanoma

The gene encoding p21 has been cloned and identified as a melanoma-differentiating antigen (mda6), the expression of which is up-regulated during melanoma differentiation.\textsuperscript{198} Mutations in the p21 gene are infrequent in human cancers,\textsuperscript{199} including melanoma.\textsuperscript{136,200} Although polymorphisms of p21 have been found in melanomas,\textsuperscript{201} it is unclear whether they are involved in the genesis of melanomas.

The expression of p21 in the pathogenesis of melanoma

As a cdk inhibitor, a decreased expression of p21 is expected to increase the proliferation of tumour cells. However, only a small number of studies have confirmed that loss of p21 may contribute to an increased tumorigenic potential by showing a decreased p21 mRNA and protein levels either in melanoma cell lines\textsuperscript{202} or in human melanomas\textsuperscript{169,203} (Table 6). In most studies, an increased expression of p21 is found\textsuperscript{118,204,205} and is associated with melanoma differentiation, growth arrest and metastatic suppression.\textsuperscript{169,198} p21 levels are found to be low or undetectable in the majority of naevi, with greater p21 expression seen in primary and metastatic melanomas.\textsuperscript{47,118,169,198,203–205} The expression of p21 is lower in the metastases compared with the corresponding primary lesions in most studies,\textsuperscript{119,203} suggesting that down-regulation of p21 expression is associated with development of a metastatic phenotype. The exact mechanism causing the increased expression of p21 in melanoma is unclear, although possible hypotheses include micro-environmental signals, checkpoint adaptation, p21 mutation, altered or inhibited p21 binding to cyclin/CDK complexes, and abnormal protein degradation.

As a downstream effector of p53, the expression of p21 is found to be inversely correlated with the expression of p53 in a number of studies.\textsuperscript{76,169,206} However, p21 expression exhibited no relationship to p53 immunoactivity in other studies.\textsuperscript{133,198,200,207} These findings support the hypothesis that over-expression of p21 in human melanoma may occur by either p53-dependent or p53-independent pathways. In the p53-independent pathway, the over-expression of p21 occurs in response to extracellular signals such as hypoxia and nutrient deprivation.\textsuperscript{198,208,209} Thus, the relationship between p21 and p53 expression is complex and may involve other genes that are transcriptionally activated by TP53, such as mdm2 proteins.\textsuperscript{17}

### Table 6 Studies of p21 expression in melanocytic lesions

<table>
<thead>
<tr>
<th>Study, year</th>
<th>No. cases</th>
<th>Site of tumour</th>
<th>Types of tumour</th>
<th>Expression of p21</th>
<th>Measure of prognosis</th>
<th>Results of prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maelandsmo et al., 1996\textsuperscript{203}</td>
<td>176</td>
<td>Cutaneous melanoma</td>
<td>Naevi</td>
<td>0% expression</td>
<td>Recurrence</td>
<td>Survival</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Primary melanoma</td>
<td>69% expression</td>
<td></td>
<td>No correlation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Metastatic melanoma</td>
<td>57% expression</td>
<td></td>
<td>No correlation</td>
</tr>
<tr>
<td>Trotter et al., 1997\textsuperscript{205}</td>
<td>60</td>
<td>Cutaneous melanoma</td>
<td>Naevi</td>
<td>1.8 ± 0.3% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Primary melanoma</td>
<td>8.9 ± 1.7% expression</td>
<td></td>
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<tr>
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<td>Metastatic melanoma</td>
<td>29 ± 3% expression</td>
<td></td>
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</tr>
<tr>
<td>Sparrow et al., 1998\textsuperscript{47}</td>
<td>110</td>
<td>Cutaneous melanoma</td>
<td>Naevi</td>
<td>28% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Melanoma in situ</td>
<td>66% expression</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td>Primary melanoma</td>
<td>61% expression</td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td>Metastatic melanoma</td>
<td>48% expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Karjalainen et al., 1999\textsuperscript{39}</td>
<td>267</td>
<td>Stage I cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>16% (staining index &lt;1%)</td>
<td>Recurrence</td>
<td>More with low expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Metastatic melanoma</td>
<td>39% (staining index 1-10%)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Primary melanoma</td>
<td>22% (staining index 10-20%)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Metastatic melanoma</td>
<td>23% (staining index &gt;20%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouriaux et al., 2001\textsuperscript{118}</td>
<td>32</td>
<td>Choroidal melanoma</td>
<td>Primary melanoma</td>
<td>37% expression</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Baldi et al., 2004\textsuperscript{204}</td>
<td>60</td>
<td>Cutaneous melanoma</td>
<td>Primary melanoma</td>
<td>36% expression (&gt;10% positive cells)</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Poyraz et al., 2004\textsuperscript{204}</td>
<td>48</td>
<td>Cutaneous melanoma</td>
<td>Metastatic melanoma</td>
<td>80% expression (&gt;10% positive cells)</td>
<td>NT</td>
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</tr>
<tr>
<td>Alonso et al., 2004\textsuperscript{18}</td>
<td>165</td>
<td>Cutaneous melanoma</td>
<td>Naevi</td>
<td>0% expression</td>
<td>Survival</td>
<td>Worse with low expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Radial melanoma</td>
<td>32% expression</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Vertical melanoma</td>
<td>27% expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Metastatic melanoma</td>
<td>42% expression</td>
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</tbody>
</table>

NT, not tested.
associated with melanoma progression and a metastatic phenotype. Expression of p21 has been reported to be inversely associated with high AJCC stage and older age (>55 years) at diagnosis, and positively correlated with the presence of scleral invasion in choroidal melanomas. Reduced p21 expression is found to be associated with reduced recurrence free survival in one study, but longer overall survival in another (Table 6). However, other studies have demonstrated no significant relationship between the expression of p21 and clinical outcome of melanoma patients.

CONCLUSIONS

There is now convincing evidence that alterations in cell cycle proteins and the resultant dysregulation of the cell cycle are important causes of uncontrolled cell proliferation in melanoma. These alterations in the proteins have emerged as novel tumour markers with potential for use in estimation of prognosis, assessment of treatment response, and in the development of effective anti-melanoma therapies. However, most studies have shown that these alterations are complex, with most tumours showing abnormal expression of greater than one protein. In addition, a number of unanswered questions remain such as whether the over-expression or decreased expression occurred within the same tumour cell, if the alterations of cell cycle proteins happen separately or jointly, and if jointly, how they interact in melanoma. Perhaps the answers to these questions may improve our knowledge of the molecular mechanisms that take place in neoplastic melanocytes.

Published studies correlating the alterations in cell cycle proteins with clinical outcomes, such as recurrence and survival, have reported conflicting results, and their predictive value in relation to therapy responses have not been investigated extensively. A large scale study is required, focusing on the expression of a range of cell cycle proteins in different stages of melanocytic lesions linked to thorough, accurate, and long-term follow-up data. The results of such a study would improve our understanding of the neoplastic transformation of melanocytes and help to clarify the prognostic significance of alterations in the expression of these cell cycle proteins.

ACKNOWLEDGEMENT

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