J. Cell. Mol. Med. Vol 11, No 5, 2007 pp. 1052-1068

Issues affecting molecular staging in the management of patients with melanoma

G. Palmieri [a, *], M. Casula [a], M. C. Sini [a], P. A. Ascierto [b], A. Cossu [c]

[a] Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Li Punti-Sassari, Italy
[b] Istituto Nazionale Tumori 'Fondazione Pascale', Napoli, Italy
[c] Servizio di Anatomia Patologica, Azienda Unità Sanitaria Locale 1, Sassari, Italy

Received: March 5, 2007; Accepted: July 18, 2007

Abstract

Prediction of metastatic potential remains one of the main goals to be pursued in order to better assess the risk subgroups of patients with melanoma. Detection of occult melanoma cells in peripheral blood (circulating metastatic cells [CMC]) or in sentinel lymph nodes (sentinel node metastatic cells [SNMC]), could significantly contribute to better predict survival in melanoma patients. An overview of the numerous published studies indicate the existence of several drawbacks about either the reliability of the approaches for identification of occult melanoma cells or the clinical value of CMC and SNMC as prognostic factors among melanoma patients. In this sense, characterization of the molecular mechanisms involved in development and progression of melanoma (referred to as melanomagenesis) could contribute to better classify the different subsets of melanoma patients. Increasing evidence suggest that melanoma develops as a result of accumulated abnormalities in genetic pathways within the melanocytic lineage. The different molecular mechanisms may have separate roles or cooperate during all evolutionary phases of melanocytic tumourigenesis, generating different subsets of melanoma patients with distinct aggressiveness, clinical behaviour, and response to therapy. All these features associated with either the dissemination of occult metastatic cells or the melanomagenesis might be useful to adequately manage the melanoma patients with different prognosis as well as to better address the different melanoma subsets toward more appropriate therapeutic approaches.

Keywords: melanoma • occult micrometastasis • RT-PCR assay • pathogenetic mechanism • molecular profile • prognosis

Introduction

The incidence of melanoma is steadily increasing within the Caucasian population [1]. A light phototype (dark-skinned populations show a lower melanoma incidence than fair-skinned populations exposed to similar levels of incident sunlight [2–5]), a large number of acquired common nevi [5–6], the presence of atypical nevi [5–6], or the recurrence of the disease into the family (one-tenth of melanoma patients

*Correspondence to: Giuseppe PALMIERI
Istituto di Chimica Biomolecolare-Sede di Sassari-CNR,
Traversa La Crucca 3-Regione Baldinca, 07040 Li Punti-Sassari (SS), Italy.
Tel.: +39 07 93 96 10 33; Fax: +39 07 93 96 10 36
E-mail: gpalmieri@yahoo.com

doi: 10.1111/j.1582-4934.2007.00091.x

presents a history of at least one additional affected family member [7–8]) have been associated with an increased risk of melanoma.

Melanoma is usually characterized by a high tendency to develop metastasis; considering the small size of most primary lesions, the metastatic potential of melanoma is considerably greater than that of other solid tumours [9]. The most important factor for reducing the melanoma mortality is early diagnosis, allowing treatment to be undertaken at a stage when cure is readily achievable. Dermoscopy has been demonstrated to be a reliable tool for the differential diagnosis of cutaneous pigmented lesions and, therefore, the early diagnosis of cutaneous melanoma [10]. Actually, Breslow tumour thickness [11], tumour ulceration and metastatic involvement of the regional lymph nodes contribute to define the stage of disease, according to the recent American Joint Committee on Cancer (AJCC) guidelines [12]. Although other prognostic factors may also have a role (including level of invasion, mitotic rate, presence of regression, sex and age of the patient, anatomic site of the primary tumour), stage of disease remains the overriding prognostic factor at present and the survival for melanoma patients is progressively worsening as Breslow thickness of primary tumours increases [13]. Despite an earlier onset of the disease and a significantly increased risk for melanoma in kindreds with recurrent melanoma, a positive family history does not act as an independent prognostic factor and does not seem to influence the overall survival or tumour specific survival [14].

Hence, there is a generalized request for improved methods to predict the clinical outcome in melanoma patients. The hypothesis that a more appropriate evaluation of both the biological behaviour and the molecular mechanisms underlying the tumourigenesis could improve prognostic prediction and clinical management of melanoma patients is based on at least two issues. (1) The detection of melanoma cells in peripheral blood (circulating metastatic cells [CMC]) [15–19] or in sentinel lymph nodes (sentinel node metastatic cells [SNMC]) [20–24], which represent the first nodes receiving migrating cells from the primary tumour, has been proposed as a potential tool to select patients with higher risk of relapse at the time of the diagnosis. (2) The characterization of the various molecular mechanisms involved in development and progression of melanoma (referred to as melanomagenesis) [6, 25] could represent the second potential tool to identify the molecular profiles underlying aggressiveness, clinical behaviour or response to therapy as well as to better classify the subsets of melanoma patients with different prognosis.

Role of the RT-PCR in prognostic prediction

Metastatic melanoma cells are not found in either circulation or tissue sections of normal individuals. Consequently, detection of melanoma cells in samples from patients at early-stage disease could indicate a dissemination of the tumour cells and, thus, a high risk of development of distant metastases. However, it is to underline that mobilization of cells from the site of the primary lesion is necessary, but not sufficient, to produce distant metastases [26]. Indeed, physical invasion of blood stream by tumour cells is among the earliest events in the tumour progression cascade and many other steps are required for metastatic colonization of distant parenchymas [26]. Detection of CMC can be thus considered as a surrogate marker of the initial events toward the establishment of distant metastases. In this sense, identification of melanoma cells in histologically negative regional lymph nodes could probably represent a more useful marker for staging melanoma patients (as previously suggested by our group [21]). As here pointed out, several controversial data have been reported about the role of the detection of CMC or SNMC among melanoma patients. To date, the main question to be answered is whether detection of CMC or SNMC could really contribute to predict survival in melanoma patients, providing clinicians with new tools for a more accurate staging, more appropriate follow-up schedules and/or more effective adjuvant therapies.

Melanoma micrometastases in peripheral blood

Reverse transcriptase-polymerase chain reaction (RT-PCR) has been demonstrated to detect a single specific messenger RNA (mRNA) in a mixed cell population, becoming a sensitive method for identification of circulating tumour cells [27–30]. As

Table 1 Published studies on prognostic value of RT-PCR assays in peripheral blood

Author, year [ref.]	No. of patients	RT-PCR markers	Disease stages	Median follow-up (months)	Multivariate analysis
Positive studies					
Hoon, 1995 [37]	119	Tyr, MAGE3, MUC18, p97	I–IV	6	No
Battayani, 1995 [47]	93	Tyr	I–IV	8	No
Kunter, 1996 [48]	64	Tyr	I–IV	20	No
Mellado, 1996 [49]	91	Tyr	I–IV	18	Yes
Curry, 1998 [50]	123	Tyr, MART1	I–III	18	No
Curry, 1999 [51]	186	Tyr, MART1	I–III	24	Yes
Mellado, 1999 [52]	57	Tyr	I–III	27	No
Schittek, 1999 [41]	225	Tyr, MART1	I–IV	4	No
Hoon, 2000 [53]	46	Tyr, MAGE3, MUC18, p97	I–IV	48	Yes
Schrader, 2000 [54]	31	Tyr, MART1, MAGE3	IV	11	No
Negative studies					
Hanekom, 1999 [43]	181	Tyr	I–IV	12	No
Aubin, 2000 [44]	39	Tyr	I–III	10	No
Waldmann, 2001 [45]	20	Tyr	IV	20	No
Strohal, 2001 [46]	76	Tyr, MART1	I–IV	11	No
Brownbridge, 2001 [42]	299	Tyr, MART1	I–IV	12	No
Palmieri, 2003 [40]	200	Tyr, MART1, p97	I–IV	44	Yes
Scoggins, 2006 [19]	1,446	Tyr, MART1, MAGE3, gp100	I–IV	30	Yes

the use of a unique marker could be of limited value in the management of melanoma patients, multi-marker assays including tyrosinase (an enzyme that is involved in the melanin biosynthesis pathway [31]), p97 [32], MUC18 [33], MelanA/MART1 [34], MAGE3 [35] and/or gp100/pMel-17 [36] markers have been proposed, in order to improve sensitivity and specificity of the procedure [37]. Our group has previously reported a positive association between clinical stage and detection of tumour-associated mRNAs in peripheral blood of melanoma patients using a multi-marker RT-PCR assay [38–40]. However, it is strongly debatable whether the use of poorly represented mRNAs may add significant information to tyrosinase as marker highly and specifically expressed in cells of the melanocytic lineage. On the other hand, we cannot exclude that if both the number of positive markers and the probability of developing distant metastases were a function of the amount of circulating cells, a higher number of markers could be more sensitive in identifying the heterogeneous population of metastatic cells. In addition to tyrosinase, previous reports indicated the MelanA/MART1 mRNA as the marker whose RT-PCR amplification may indeed increase sensitivity in CMC detection [40–42].

Many studies have dealt with the possible prognostic value of the presence of a minimal residual disease in melanoma patients who have undergone apparently curative surgery. For CMC, conflicting findings have been reported world-wide (Table 1). About two-fifths of studies were negative, excluding a role for the PCR-based detection of CMC as predictive

factor for clinical outcome (though blood detection of mRNAs corresponding to melanoma-associated molecular markers was significantly correlated with the stage of disease in all analysed series) [19, 40, 42–46]. The majority (about 60%) of published studies presented positive conclusions, supporting the prognostic role of such a procedure [37, 41, 47–54]. In other words, the presence of CMC appeared to be at least a surrogate marker for clinical staging (in negative studies) or a putative predictive factor for disease-specific survival (in positive studies).

Multi-variate analysis assessing the interaction between the disease stage and the other candidate prognostic factors has not been performed in majority of these studies [37, 41–48, 50, 52, 54]. Although identification of circulating melanoma cells at the time of diagnosis may be indeed the first step toward the subsequent development of metastatic disease, it may also simply reflect the stage of disease at diagnosis. In the five studies that used multi-variate analysis (two [19, 40] negative and the remaining ones [49, 51, 53] positive for association with prognosis), the CMC detection did not act as an independent prognostic factor (its strongest correlation was again with stage at diagnosis). However, conclusions of these studies cannot be considered definitive due to the limited number of analysed cases (less then 100 patients [49, 53]), the short time of observation (median follow-up less than 3 years [19, 51]), or the low rates of events for statistical analysis (due to preponderance of patients presenting a AJCC Stage I disease [40]). Moreover, majority of the published studies was focused on progression-free survival, a surrogate end-point that, although reliable, cannot completely substitute the value of overall survival. Nevertheless, all such studies are substantially retrospective, being based on the analysis of patients who underwent CMC assay; again, selection biases cannot be definitively excluded.

The complex biology of metastasis formation could provide possible explanations for all these controversial data. To become invasive, tumour cells need to change their adhesive properties, to loose contact with other cells in the primary tumour, and make new contacts with the extra-cellular matrix of host cells they encounter as they invade [26, 55]. They also need to be able to penetrate into the surrounding host tissue, where the modulation of protease activity in the vicinity of the tumour cells plays a critical role. To migrate away from the primary location, tumour cells also need to gain motility functions. These same properties are also thought to be important when circulating tumour cells exit the circulatory system and start metastatic colonization in secondary organs [55]. Overall, colonization of distant tissues and development of metastasis represent the result of a multi-step cascade of events occurring to cancer cells during tumour dissemination (*i.e.* viability in circulation, capability of exiting blood stream and starting tissue invasion, presence of adequate growth potential for metastasis formation) [26, 55].

In this light, a prolonged presence of melanoma cells in blood stream may indeed contribute to select viable cancer cells with better capacity to begin colonization process at the distant site. All the above-mentioned studies were based on RT-PCR analyses of baseline blood samples obtained from melanoma patients at time of diagnosis (generally, early-stage patients have been enrolled if no more than 4 weeks had elapsed from the surgical treatment, whereas those with advanced disease had baseline blood sample collected before systemic therapy). One could speculate that, after an initial peak of circulating cancer cells (strictly correlated with the tumour burden) at the time of surgical excision of the primary melanoma, progressive disappearance of CMC from peripheral blood (*i.e.* due to lack of their viability in circulation) could be related to the favourable outcome in patients classified as PCR-positive at baseline. Conversely, appearance of CMC in peripheral blood during follow-up (*i.e.* due to the existence of previously quiescent melanoma cells, which might have acquired the capability of entering the blood stream) could justify the observation of relapses in PCR-negative patients at baseline (Fig. 1).

Supporting this hypothesis, recent studies suggest that metastatic cancer cells might be constantly present in blood circulation in a subset of recurrent melanoma patients, before the establishment of distant metastases (as assessed by detection of frequently positive PCR-based assays in peripheral blood samples serially obtained during follow-up visits) [57–58]. In such studies, both disease-free survival and overall survival were significantly higher for patients always showing negative RT-PCR results in comparison with those who tested positive in more than one RT-PCR assay during a prolonged follow-up observation (about 6 years for each of the two series) [57–58]. Similarly, another study defined the existence of changes in CMCs during interferon (IFN)

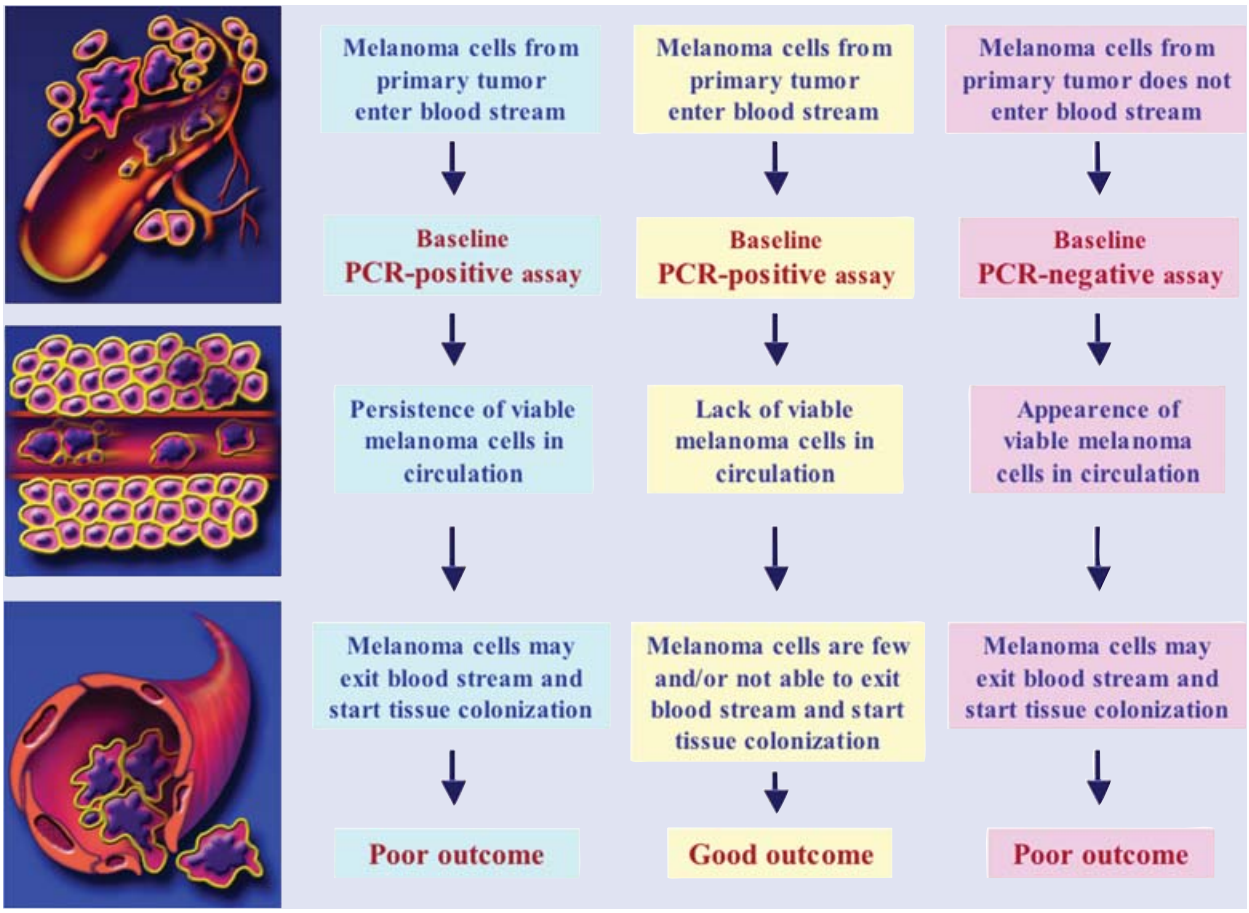

Fig. 1 Comparison between circulating metastatic cells (CMC) detection and prognosis. The different hypotheses to explain the controversial data about prognostic value of RT-PCR assays on peripheral blood of melanoma patients at the time of diagnosis (baseline) are provided. On the left, representation of the cancer cells entering (top), surviving (middle) and exiting (bottom) the blood stream.

therapy among melanoma patients with more advanced disease (AJCC stages II–IV) [59]. Using multi-variate analysis, these authors have shown that patients who became CMC-negative during IFN therapy were significantly associated with better disease-free survival than those who remained or became positive during therapy [59]. Dynamic studies, based on routinely repeated evaluations of the presence of circulating malignant cells by RT-PCR assay during the follow-up period or after the treatment administration among patients with melanoma, could therefore represent a way toward the assessment of the threshold above which sensitivity for clinically significant micrometastases can be optimized and false-positives can be minimized.

To date, the clinical role of CMC detection in melanoma patients remains controversial. Although it seems reasonable to wait for more consistent and definitive results (by performing serial RT-PCR assays in large series of patients, with a longer follow-up in order to observe a higher number of melanoma-related deaths), such procedure should be limited to clinical trials and should not enter clinical practice nor affect treatment-decision making.

Melanoma micrometastases in lymph nodes

Development of regional node metastasis is able not only to change tumour staging but also to significantly

Table 2 Published studies on prognostic value of RT-PCR assays in hystopathologically negative sentinel lymph nodes

Author, year [ref.]	No. of patients	RT-PCR markers	Median follow-up (months)	Multi-variate analysis
Positive studies				
Bostick, 1999 [65]	55	Tyr, MAGE3, MART1	12	Yes
Blaheta, 2000 [66]	101	Tyr	19	Yes
Palmieri, 2001 [21]	61	Tyr, MART1	36	Yes
Denninghoff, 2004 [67]	42	Tyr	37	No
Ulrich, 2004 [68]	288	Tyr	37	Yes
Gradilone, 2004 [69]	129	Tyr	24	Yes
Romanini, 2005 [70]	101	Tyr, MART1	30	No
Negative studies				
Kammula, 2004 [71]	97	Tyr	67	Yes
Mangas, 2006 [72]	142	Tyr	45	Yes

affect patient survival, becoming a negative prognostic factor in melanoma [12–13]. Early identification of occult metastasis, before the development of clinical disease, is thought to improve survival in melanoma patients. In comparison with the CMC detection, identification of occult melanoma metastasis in regional lymph nodes could be more effective for the assessment of the minimal residual disease after surgical excision of the primary melanoma.

In past years, selective lymphadenectomy has been introduced for the treatment of melanoma by using a lymphatic mapping technique (initially based on intradermic injection of vital blue dye, and then improved by a radio-guided methodology) [60–62]. Intraoperative lymphatic mapping and sentinel node biopsy has become the standard approach for staging the regional lymph nodes in early-stage melanomas [63]. Recently, it has been reported that sentinel-node biopsy may identify subsets of melanoma patients with nodal metastases, whose survival can be prolonged by immediate lymphadenectomy [64]. Moreover, staging of melanoma patients has been revised with the introduction of a distinction between macroscopic (clinical) and microscopic lymph node involvement [12]. To increase the sensitivity of the morphological analysis based on haematoxylin-eosin staining and improve the detection of occult metastases in sentinel nodes, conventional immunohistochemistry using specific melanocytic markers such as HMB-45, S-100, and/or MelanA/MART1 [39, 63–64] should be associated.

The sentinel node metastatic cells (SNMC) could be however identified by application of highly sensitive molecular approaches. Amplification of tissue-specific mRNA by RT-PCR assays on sections of sentinel lymph nodes may be considered as a further attempt to improve the sensitivity in detecting occult melanoma cells [21, 65–66]. The highest sensitivity of the RT-PCR assay has been reported using fresh or frozen sentinel nodes [65–66]. Since this type of tissue samples are not usually available in majority of cases, our group previously defined protocols to amplify total RNA from paraffin-embedded sentinel nodes of melanoma patients (AJCC stages I-III) [21]. Despite a good consistency in detection of RT-PCR products among frozen and paraffin melanoma sections, the multi-marker assay used in our study (including tyrosinase and MelanA/MART1 mRNAs as markers) failed to identify a quite high fraction (about one third) of sentinel nodes containing melanoma cells as assessed by immunohistochemical analysis [21]. This strongly suggests that sensitivity is deeply lowered when RT-PCR is performed on paraffin-embedded specimens. Also taking into account this weakness of the methodology, our study showed that increasing number of PCR-positive markers in histologically proven tumour-free sentinel nodes from melanoma patients was significantly associated with higher rates of relapses after a median follow-up of about 3 years [21]. Several studies have been performed in order to identify subgroups of melanoma patients with RT-PCR-positive sentinel nodes and high risk of disease recurrence (Table 2). A poorer disease-specific survival has been registered in patients with histopathologically negative sentinel nodes that expressed at least one melanoma-associated mRNA marker [65–70]. When an extended follow-up of patients with histologically negative sentinel nodes has been evaluated, detection of occult melanoma cells by RT-PCR assays seems not to select patients with an increased probability of disease recurrence [71–72].

Again, knowledge of the biological behaviour of the melanocytic cells could provide some possible explanation for these conflicting results. It is documented

that nevus cells can be found within the fibrous capsule and trabeculae of lymph nodes, with tendency to form melanocytic aggregates into the lymph node parenchyma [73–75]. The increased frequency of nodal nevi in sentinel nodes might be due to a mechanical transport of nevus cells [75] or to an active migration of melanocytic cells [76]. Therefore, nevi inclusion occurring in lymph nodes of melanoma patients are not uncommon and may mimic metastases [74–76]. Morphological analysis using haematoxylin-eosin staining, for the assessment of the architectural and phenotypic features of the cell clusters into the lymph node, and immunohistochemistry using melanoma-associated markers seem to be able to differentiate melanocytic aggregates from melanoma metastases in large majority of cases [24, 64]. For such a purpose, the tumour suppressor $p16^{CDKN2A}$ protein has been demonstrated to be a useful immunohistochemical marker [77]. When screening of lymph node sections from melanoma patients is instead performed by RT-PCR assay (which detects one specific cell in 10^6–10^7 background cells), using genes specifying for antigens of melanocytic differentiation (mainly, tyrosinase and MelanA/MART1) as markers, it is not possible to determine whether amplified mRNAs come from normal or tumour cells.

In conclusion, RT-PCR analysis has been found to be more sensitive than haematoxylin-eosin staining and immunohistochemistry in detecting cells of the melanocytic lineage within sentinel lymph nodes. Although such a molecular approach could indeed support conventional histopathological analysis (which is retained to underestimate the real incidence of minimal disease) and improve the identification of occult metastases, lack of specificity and limits in the availability of fresh-frozen tissue specimens make this technique impractical for routine use. Therefore, RT-PCR assays on sentinel nodes remain investigational and should not be used to direct adjuvant therapy in melanoma patients at this time. However, studies should be addressed to the enhancement of the PCR-based strategies for detecting occult metastases through either the isolation of more specific molecular targets or a better comprehension of the biological mechanisms underlying the metastasis formation in regional lymph nodes. To date, the Multi-centre Lymphadenectomy Trial II has been designed in order to test the clinical significance of tumour-positive sentinel lymph nodes as inferred by haematoxylin-eosin staining, immunohistochemistry or RT-PCR assay [24]. Results from this study will provide additional clues about the role of regional lymph nodes into the metastatic process of melanoma patients as well as the clinical value, in terms of costs and benefit, of detecting occult node metastases using the PCR-based technique.

Molecular complexity of melanomagenesis

During the recent past years, an increasing number of evidence has pointed out to the importance of correlating the molecular pathways involved into the de-regulation of melanocyte growth with the clinical and pathological aspects of melanoma [6, 78]. The hypothesis that several genetic and molecular abnormalities co-operating in melanomagenesis do generate different subgroups of patients may have important implications in predicting the clinical outcome as well as planning effective therapeutic strategies in each of these subgroups (especially, in the light of a steadily increasing use of new gene-targeted anticancer molecules in combination with conventional drugs for melanoma treatment).

Melanocytic transformation is thought to occur by sequential accumulation of genetic and molecular alterations [6, 25, 79–80]. Although the pathogenetic mechanisms underlying melanoma development are still largely unknown, several genes and metabolic pathways have been shown to carry molecular alterations in melanoma.

Re-arrangements or deletions of the short arm of chromosome 9 represent a common genetic alteration detected in tumour tissues from patients with melanoma [7, 81]. Molecular and cytogenetic investigations have indicated the CDKN genetic locus at the chromosome 9p21 as a candidate region involved in melanoma pathogenesis [81–82]. The *CDKN2A* gene maps at the CDKN locus and encodes two proteins: the cyclin-dependent kinase inhibitor $p16^{CDKN2A}$, which is a component of the CyclinD1-RB pathway, and the tumour suppressor $p14^{CDKN2A}$, which has been functionally linked to the MDM2-p53 pathway [83] (Fig. 2). Alterations of the *CDKN2A* gene have been widely reported as the most common cause of inherited susceptibility to melanoma [84–87]. In melanocytic cells, the $p16^{CDKN2A}$ protein acts as a proliferation inhibitor

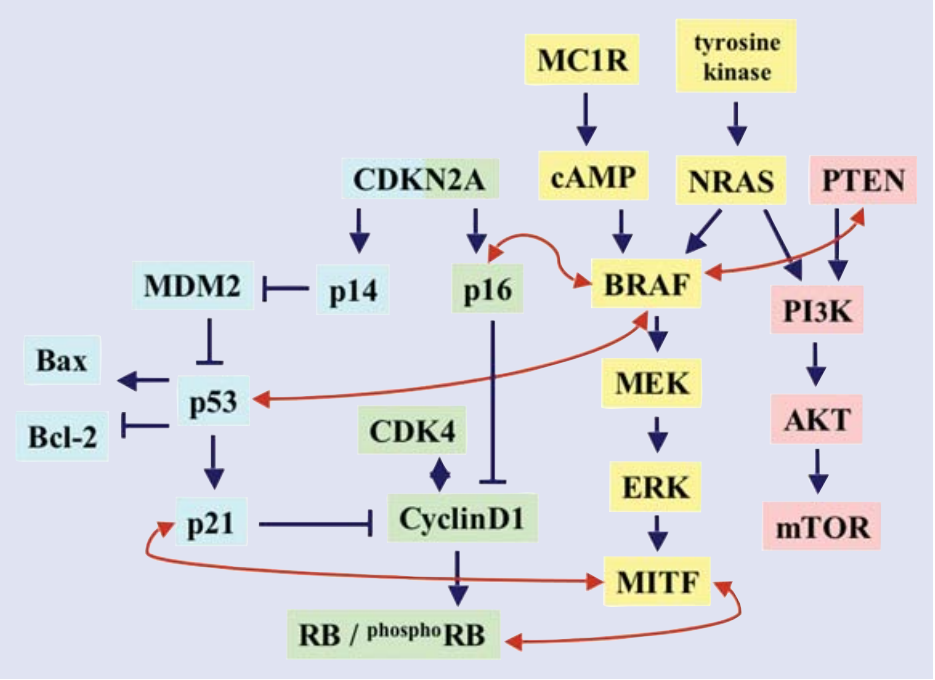

Fig. 2 Molecular pathways involved in melanomagenesis. The proteins have been ordered according to their position into the functional molecular cascade. Straight arrows and barred lines (in blue) indicate induction and inhibition, respectively; bent arrows (in red) indicate interaction between the different pathways.

by binding the CDK4/6 kinases and blocking phosphorylation of the RB protein, which lead cells to cycle arrest [6, 78]. Dysfunction of the proteins involved into the p16^{CDKN2A} pathway has been demonstrated to promote a de-regulation of the cell cycle with an uncontrolled cell growth, which may induce cell proliferation and increase aggressiveness of transformed melanocytic cells [88]. Therefore, genetic (deletion or mutation) [6, 78, 84–87] or epigenetic (gene silencing) [89–90] inactivation of the *CDKN2A* gene may be required for tumour progression and metastasis formation (melanoma cells tend to inactivate both alleles of such a tumour suppressor gene and increase their aggressiveness and malignancy).

Detection of 9p21 allelic loss at similar rates in majority of dysplastic nevi, primary tumours and corresponding metastases indicated that such genetic alterations may play a role in melanocytic proliferation and transformation, being maintained in all evolutionary phases of melanocytic tumourigenesis [91–93]. Allelic deletions at CDKN locus and absence of the p16^{CDKN2A} protein expression have been both observed at increased rates moving from early to advanced primary melanomas and to secondary melanoma lesions as well as to melanoma cell lines, being quite completely absent in non-melanoma melanocytic lesions [88, 91–93]. Inactivating epigenetic mechanisms-the 5′ CpG island upstream of *p16*^{CDKN2A} gene has been found methylated in several primary tumours and cell lines [89–90]-may be also responsible for gene silencing. Altogether, data support the hypothesis that inactivation of the *p16*^{CDKN2A} gene by different mechanism might be selected during the tumour progression and, especially, during the establishment and propagation of melanoma cells in culture [94].

Another crucial level of cell cycle regulation in melanoma involves the pathway starting from the *p14*^{CDKN2A} protein, the second product of the *CDKN2A* gene (this is an example of a gene encoding two different proteins which generate two different cascades of functional events) [96]. As for *p16*^{CDKN2A}, the *p14*^{CDKN2A} protein exerts a tumour suppressor effect by inhibiting the oncogenic actions of the downstream MDM2 protein, whose direct interaction with p53 blocks any p53-mediated activity and targets the p53 protein for rapid degradation [95] (Fig. 2). The *MDM2* gene itself has been shown to be amplified in primary tumours [96] and to act as an oncogene in cell cultures [97]. Mutations in *p14*^{CDKN2A} gene are much less frequent than those in *p16*^{CDKN2A} gene [7–8, 79]; they allow degradation of p53 by releasing its binding partner MDM2 [94–95] (Fig. 2). Analogously, the *TP53* gene is mainly inactivated at

functional level and rarely mutated in melanoma [83]. Impairment of the $p14^{CDKN2A}$-MDM2-p53 cascade, whose final effectors are the Bax/Bcl-2 proteins, is implicated in defective apoptotic response to genotoxic damage and, thus, to anticancer agents (in most cases, melanoma cells present concurrent high expression levels of Bax/Bcl-2 proteins, contributing to further increase their aggressiveness and refractoriness to therapy [98]) [94, 98–99] (Fig. 2).

The MAPK-ERK pathway (including the cascade of NRAS, BRAF, MEK1/2 and ERK1/2 gene products) has been also reported to play a major role in both development and progression of melanoma [100–101]. Constitutive activating mutations in *NRAS* occur in about 20% of melanoma cell lines [102–103], whereas oncogenic *BRAF* mutations have been described in 30–60% of primary melanomas [100, 104–106]. The *BRAF* gene codify for a serine/threonine kinase of the RAS/RAF/MEK/ERK mitogen-activated protein kinase (MAPK) pathway, a major signalling cascade involved in the control of cell growth, proliferation and migration [100] (Fig. 2). The ERK1/2 proteins, which represent the final components of such a signalling kinase cascade, have been found to be constitutively activated in melanomas, mostly as a consequence of mutations in upstream components of the pathway [101]. The increased activity of ERK seems to be implicated in rapid melanoma cell growth, enhanced cell survival and resistance to apoptosis [101]. This high basal level of ERK activity may further induce the metastatic potential of melanoma by increasing the expression of invasion-promoting integrins [107]. Presence of *BRAF* mutations in benign and dysplastic nevi supports the hypothesis that activation of the RAF/MEK/ERK pathway is an early event in melanoma progression and that additional co-operating genetic events are required to achieve full malignancy [108].

All these findings clearly indicate the existence of a complex molecular machinery that provides checks and balances in normal melanocytes. Progression from normal melanocytes to malignant metastatic cell in melanoma patients is the result of a combination of down- or up-regulations of the various effectors acting into the different molecular pathways (Fig. 3). The main interactions underlying the melanomagenesis could be summarized as follow.

(1) The 9p21 deletions seem to be set in majority of dysplastic nevi, which have been indicated as pre-neoplastic melanoma lesions, and their incidence does not vary during melanocytic tumourigenesis [88, 91–93]. This implicates that, although an important step for the initiation of neoplastic transformation, 9p21 allelic loss is insufficient for the development of melanoma. Similarly, *BRAF* somatic mutations were demonstrated to be present at quite identical rates in nevi, in primary and metastatic melanomas as well as in cultured melanoma cells [100, 104–106, 108]. This strongly suggests that *BRAF* mutations may co-operate with the 9p21 loss into the initial steps of melanocytic proliferation and melanoma formation.
(2) Activated BRAF may induce formation of melanocyte lesions that rapidly developed into invasive melanomas in transgenic p53-deficient *zebrafish* [109]. This is a further evidence that activating *BRAF* mutations have a role in melanocytic proliferations, being a necessary event for starting the cascade of alterations involved into the melanomagenesis. However, *BRAF* activation alone is not sufficient to induce the malignant process and fully transform proliferating melanocytes, but requires additional, co-operating de-regulative events (such as the inactivation of the p53 pathway) for tumour development.
(3) Activating *BRAF* mutations have been reported to constitutively induce up-regulation of $p16^{CDKN2A}$ (this phenomenon looks like a sort of protective response to an inappropriate mitogenic signal) [110]. As for the p53 deficiency, a genetic or epigenetic inactivation of $p16^{CDKN2A}$ gene may strongly contribute to malignant transformation of the BRAF-driven melanocytic proliferation;
(4) The microphthalmia-associated transcription factor (*MITF)* gene, which is activated by a constitutive induction of the MAPK-ERK pathway, has been demonstrated to participate in regulation of cell cycle progression in normal melanocytes [111]. The MITF protein seems to exert this effect by co-operating with either $p21^{CDKN1A}$, which is a downstream effector of the $p14^{CDKN2A}$-MDM2-p53 cascade, or pRB, which is the final target of the $p16^{CDKN2A}$-CyclinD1 cascade [111]. A MITF gene amplification has been reported in melanoma metastases and cell lines, suggesting a role in melanoma progression [112].

Molecular alterations and disease phenotype

The complexity of the sequential accumulation of the above-mentioned molecular alterations, during the

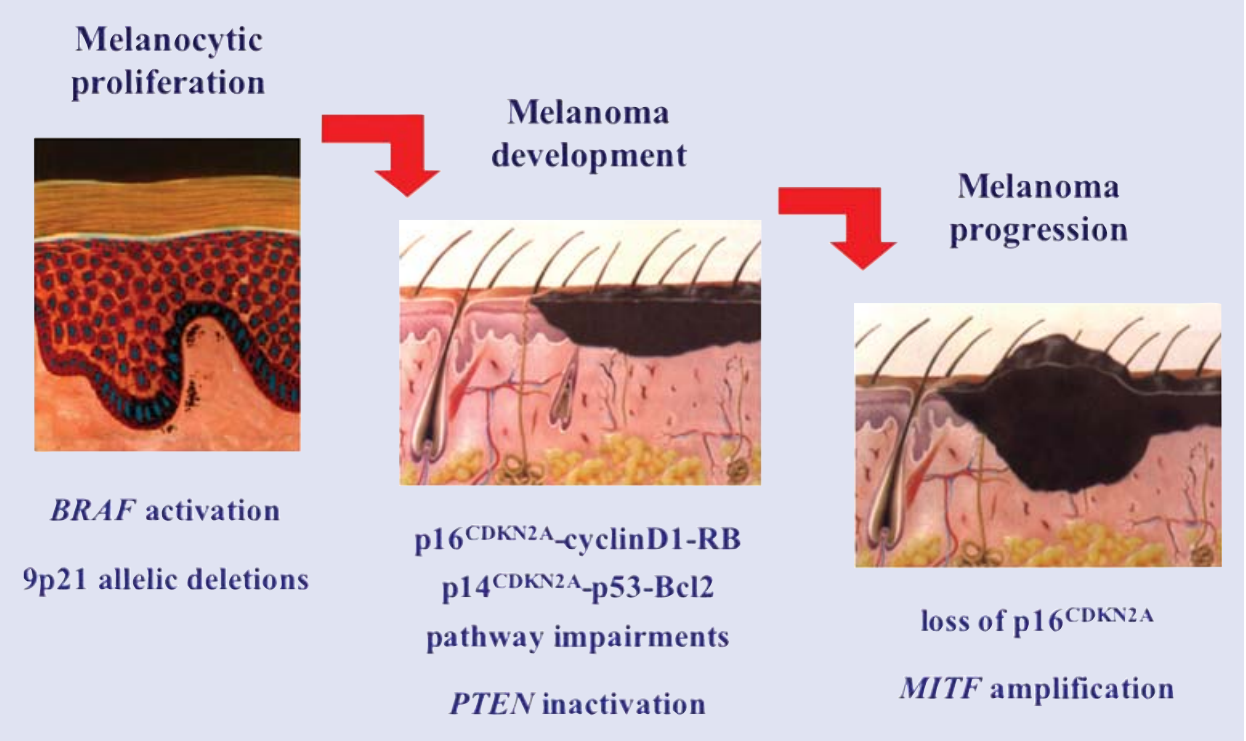

Fig. 3 Proposed model of melanocytic tumourigenesis. The main molecular alterations underlying each step from melanocytic proliferation to advanced melanoma are indicated.

development and progression of melanoma, raises the question whether several distinct types of melanoma might exist.

Considering the growth patterns, four histological types of melanoma have been described: superficial spreading, lentigo maligna, nodular and acral melanoma [12]. However, justification for such a distinction is controversial because of histological overlap and lack of prognostic significance [13]. Conversely, different studies have shown that melanomas of the palms and soles (acral melanomas) have distinctive patterns of chromosomal aberrations as compared with those at other sites [113–114]. Comparative genomic hybridization revealed that several genomic regions (mostly, 11q13, 22q11–13, and 5p15) were abnormally amplified in acral melanoma [113–114]; such regions were different from those found altered in superficial spreading or nodular melanoma (mainly, 9p21 and 1p22) [7, 81, 88]. In addition, patients with melanomas of the head and neck differ from patients with melanomas of the trunk in having higher expression levels of p53 protein (TP53-positive melanomas), a higher frequency of associated non-melanoma skin cancers, and lower numbers of melanocytic nevi [25, 80, 94, 115]. By contrast, TP53-negative melanomas were related to high nevus density [78, 115].

Considering the genesis at cutaneous level, most melanomas seem to directly arise from normal melanocytes [6]. However, an increasing number of evidence indicates that (*a)* melanomas progress from pre-existing melanocytic nevi and (*b)* dysplastic nevi may be considered as precursors of melanoma [7, 80, 94]. These observations suggest that melanoma may arise from at least two pathways. Evaluation of the features underlying the risk of developing melanoma provide clues that two additional phenotypic expressions may be associated with the disease. For the same level of fairness of the skin, melanomas can arise either in individuals who are prone to freckle with an inability to tan and few melanocytic nevi or in those who present instead an elevated number of such nevi [25, 115]. Again, the involvement of at least two pathogenetic pathways might be hypothesized.

Finally, recent findings support the existence of a dual pathway for the development of melanoma: one related to chronic exposure to the sun and the other related to melanocyte instability [78, 115]. It has been reported that melanomas on skin not chronically exposed to sun usually carry either a mutated *NRAS* or mutated *BRAF* or concurrently mutated *BRAF* and *PTEN* genes [116–117]. Indeed, recent evidence suggest that *BRAF* and *NRAS* mutation are mutually exclusive at the single-cell level providing further support to the hypothesis that expression of the two mutations may not occur in the same neoplastic cell [117]. However, activating mutations of both *NRAS* and *BRAF* genes may differently segregate into the various cells from the same melanoma; therefore, these mutations can co-exist in the same human melanoma [117]. Since *BRAF*- and *NRAS*-mutated clones have been demonstrated to possess different biological properties in vitro [117], co-existence of the two alterations into the same tumour may result in a heterogeneous response to therapy. In the same group of lesions, *BRAF* mutations have been associated with specific sequence variants of the melanocortin 1 receptor (*MC1R)* gene [118]. In contrast, melanomas on skin chronically exposed to the sun or on acral skin generally present wild-type *BRAF* or *N-RAS* genes with subsequent lack of involvement of the RAS–RAF–ERK pathway [78]. These tumours have instead a genomic instability with an increased number of copies of the proliferation-controlling *CyclinD1* or *CDK4* genes [78], which belong to the $p16^{CDKN2A}$-RB cascade [94–95].

All these evidence represent a strong indication that the different molecular pathways associated with the melanomagenesis may correspond to different subsets of melanoma patients, with distinguished biological and clinical behaviour of the disease. Identification of such different patients' subsets should be introduced in clinical trials by addressing tissue sections from each melanoma patient to molecular analyses: immunohistochemistry using antibodies against the main candidate proteins ($p16^{CDKN2A}$, $p14^{CDKN2A}$, pERK1/2, pRB, p10, p53, CyclinD1), in order to assess any alteration of their expression levels, and/or fluorescence *in situ* hybridization (FISH) analysis, in order to evaluate the existence of pathogenetic gene amplifications (for *MITF, CyclinD1* or *CDK4* genes).

Conclusions

Molecular classification of melanoma patients could be therefore achieved through the assessment of either the molecular profile of primary tumours (indicating which gene or pathway is affected) or the level of disease dissemination (indicating the presence of melanoma cells into blood stream or regional lymph nodes). This however raises a question: 'which is the most appropriate management or the best therapeutic approach for the different subsets of melanoma patients coming from such a molecular staging?'.

Multiple clinical trials on adjuvant therapy have been carried out in melanoma patients using chemotherapy, vaccines, biological drugs, or combinations of these [119]. The only substance yet shown to affect disease behaviour, reproducibly in large randomized controlled clinical trials, is high-dose intravenous IFN-α [119] (low-dose or intermediate-dose IFN offered no overall survival benefit in several trials [120]). However, after several years in which IFN has been utilized in melanoma therapy the real mechanism of action is as yet unknown [121]. None of the trials based on different dosage, route of administration and duration of IFN contained biological end-points aimed to better understand the activity of the molecule [119–121]. Also for this specific purpose, a new patient classification, which would take into account the molecular profile of each melanoma, could be indeed helpful to determine patients who may most benefit from IFN therapy, deeply contributing to shed light on how to overcome IFN resistance or enhance IFN effectiveness [122].

To speak in more general terms, the recent introduction of gene-targeted anticancer molecules in combination with conventional drugs into the clinical practice further support the idea that is time for a more appropriate selection of patients to be addressed to the various innovative therapies [123]. It is unlikely that targeting a single component in the signalling pathway will yield significant antitumour responses. For this purpose, analysis of all known molecular targets could help us to make a prediction, identifying the subsets of patients who would be expected to be more or less likely to respond to specific therapeutic interventions. Nevertheless, several new genes and molecular pathways are being discovered through gene expression profiling based on microarray technology [124–128], making correlations between molecular signatures and clinical outcome [126–128]. These findings along with the advancements of the biotechnologies will do provide even more reliable tools for detailed gene-based analyses, allowing to better characterize molecular biomarkers which may predict prognosis and response to treatment in patients with melanoma.

Acknowledgements

Authors are grateful to patients for their co-operation. Work was partially funded by Associazione Italiana Ricerca sul Cancro (AIRC), Regione Autonoma della Sardegna, and Ricerca Finalizzata Ministero della Salute.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. **Parkin DM, Whelan SL, Ferlay J, Teppo L, Thomas DB** (eds). Cancer incidence in five continents, Vol. VIII. Lyon, France: IARC Press, 2003.
2. **Gilchrest BA, Eller MS, Geller AC, Yaar M.** The pathogenesis of melanoma induced by ultraviolet radiation. *New Engl J Med.* 1999; 340:1341–8.
3. **Jhappan C, Noonan FP, Merlino G.** Ultraviolet radiation and cutaneous malignant melanoma. *Oncogene.* 2003; 22: 3099–112.
4. **Chaudru V, Chompret A, Bressac-de Paillerets B, Spatz A, Avril MF, Demenais F.** Influence of genes, nevi, and sun sensitivity on melanoma risk in a family sample unselected by family history and in melanoma-prone families. *J Natl Cancer Inst.* 2004; 96: 785–95.
5. **Whiteman DC, Stickley M, Watt P, Hughes MC, Davis MB, Green AC.** Anatomic site, sun exposure, and risk of cutaneous melanoma. *J Clin Oncol.* 2006; 24: 3172–7.
6. **Thompson JF, Scolyer RA, Kefford RF.** Cutaneous melanoma. *The Lancet.* 2005; 365: 687–701.
7. **Haluska FG, Hodi FS.** Molecular genetics of familial cutaneous melanoma. *J Clin Oncol.* 1998; 16: 670–82.
8. **Bataille V. Genetic epidemiology of melanoma.** *Eur J Cancer.* 2003; 39: 1341–7.
9. **Miller AJ, Mihm MC.** Melanoma. *N Engl J Med.* 2006; 355: 51–65.
10. **Simionescu O, Costache M, Testori A.** Cutaneous melanoma: digital dermoscopy-essential tool for positive diagnosis. *J Cell Mol Med.* 2006; 10: 991–4.
11. **Breslow A.** Thickness, cross-sectional area and depth of invasion in the prognosis of cutaneous melanoma. *Ann Surg.* 1970; 172: 902–8.
12. **Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, Coit DG, Fleming ID, Gershenwald JE, Houghton A Jr, Kirkwood JM, McMasters KM, Mihm MF, Morton DL, Reintgen DS, Ross MI, Sober A, Thompson JA, Thompson JF.** Final version of the American Joint Committee on Cancer Staging System for Cutaneous Melanoma. *J Clin Oncol.* 2001; 19: 3635–48.
13. **Balch CM, Soong SJ, Gershenwald JE, Thompson JF, Reintgen DS, Cascinelli N, Urist M, McMasters KM, Ross MI, Kirkwood JM, Atkins MB, Thompson JA, Coit DG, Byrd D, Desmond R, Zhang Y, Liu PY, Lyman GH, Morabito A.** Prognostic factors analysis of 17,600 melanoma patients: Validation of the American Joint Committee on Cancer Melanoma Staging System. *J Clin Oncol.* 2001; 19: 3622–34.
14. **Florell SR, Boucher KM, Garibotti G, Astle J, Kerber R, Mineau G, Wiggins C, Noyes RD, Tsodikov A, Cannon-Albright LA, Zone JJ, Samlowski WE, Leachman SA.** Population-based analysis of prognostic factors and survival in familial melanoma. *J Clin Oncol.* 2005; 23: 7168–77.
15. **Jung FA, Buzaid AC, Ross MI, Woods KV, Lee JJ, Albitar M, Grimm EA.** Evaluation of tyrosinase mRNA as a tumor marker in the blood of melanoma patients. *J Clin Oncol* 1997; 15: 2826–31.
16. **Ghossein RA, Bhattacharya S.** Molecular detection and characterisation of circulating tumour cells and micrometastases in solid tumours. *Eur J Cancer.* 2000; 36: 1681–94.
17. **Taback B, Morton DL, O'Day SJ, Nguyen DH, Nakayama T, Hoon DS.** The clinical utility of multimarker RT-PCR in the detection of occult metastasis in patients with melanoma. *Recent Results Cancer Res.* 2001; 158: 78–92.
18. **Max N, Willhauck M, Wolf K, Thilo F, Reinhold U, Pawlita M, Thiel E, Keilholz U.** Reliability of PCR-based detection of occult tumour cells: lessons from real-time RT-PCR. *Melanoma Res.* 2001; 11: 371–8.
19. **Scoggins CR, Ross MI, Reintgen DS, Noyes RD, Goydos JS, Beitsch PD, Urist MM, Ariyan S, Davidson BS, Sussman JJ, Edwards MJ, Martin RC, Lewis AM, Stromberg AJ, Conrad AJ, Hagendoorn L, Albrecht J, McMasters KM.** Prospective multi-institutional study of reverse transcriptase polymerase chain reaction for molecular staging of melanoma. *J Clin Oncol.* 2006; 24: 2849–57.
20. **Max N, Keilholz U.** Minimal residual disease in melanoma. *Semin Surg Oncol.* 2001; 20: 319–28.
21. **Palmieri G, Ascierto PA, Cossu A, Mozzillo N, Motti ML, Satriano SM, Botti G, Caraco C, Celentano E, Satriano RA, Lissia A, Tanda F, Pirastu M, Castello G.** Detection of occult melanoma cells in paraffin-embedded histologically negative sentinel lymph nodes using a reverse

transcriptase polymerase chain reaction assay. *J Clin Oncol.* 2001; 19: 1437–43.

22. **Davids V, Kidson SH, Hanekom GS.** Accurate molecular detection of melanoma nodal metastases: an assessment of multimarker assay specificity, sensitivity, and detection rate. *Mol Pathol.* 2003; 56: 43–51.
23. **Kuo CT, Hoon DS, Takeuchi H, Turner R, Wang HJ, Morton DL, Taback B.** Prediction of disease outcome in melanoma patients by molecular analysis of paraffin-embedded sentinel lymph nodes. *J Clin Oncol.* 2003; 21: 3566–72.
24. **Essner R.** Sentinel lymph node biopsy and melanoma biology. *Clin Cancer Res.* 2006; 12: 2320s–5s.
25. **Rivers JK.** Is there more than one road to melanoma? *Lancet.* 2004; 363: 728–30.
26. **Steeg PS.** Tumor metastasis: mechanistic insights and clinical challenges. *Nat Med.* 2006; 12: 895–904.
27. **Brossart P, Schmier JW, Kruger S, Willhauck M, Scheibenbogen C, Mohler T, Keilholz U.** A PCR-based semiquantitative assessment of malignant melanoma cells in peripheral blood. *Cancer Res.* 1995; 55: 4065–8.
28. **Foss AJ, Guille MJ, Occleston NL, Hykin PG, Hungerford JL, Lightman S.** The detection of melanoma cells in peripheral blood by reverse transcription-polymerase chain reaction. *Br J Cancer.* 1995; 72: 155–9.
29. **Pittman K, Burchill S, Smith B, Southgate J, Joffe J, Gore M, Selby P.** Reverse transcriptase-polymerase chain reaction for expression of tyrosinase to identify malignant melanoma cells in peripheral blood. *Ann Oncol.* 1996; 7: 297–301.
30. **Sarantou T, Chi DD, Garrison DA, Conrad AJ, Schmid P, Morton DL, Hoon DS.** Melanoma-associated antigens as messenger RNA detection markers for melanoma. *Cancer Res.* 1997; 57: 1371–6.
31. **Brichard V, Van Pel A, Wolfel T, Wolfel C, De Plaen E, Lethe B, Coulie P, Boon T.** The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med.* 1993; 178: 489–95.
32. **Rose TM, Plowman GD, Teplow DB, Dreyer WJ, Hellstrom KE, Brown JP.** Primary structure of the human melanoma-antigen p97 (melanotransferrin) deduced from the mRNA sequence. *Proc Natl Acad Sci USA.* 1986; 83: 1261–5.
33. **Lehmann JM, Riethmuller G, Johnson JP.** MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily. *Proc Natl Acad Sci USA.* 1989; 86: 9891–5.
34. **Coulie PG, Brichard V, Van Pel A, Wolfel T, Schneider J, Traversari C, Mattei S, De Plaen E, Lurquin C, Szikora JP, Renauld JC, Boon T.** A new gene coding for a differentiation antigen recognized by autologous cytolytic T limphocytes on HLA-A2 melanomas. *J Exp Med.* 1994; 180: 35–42.
35. **Gaugler B, Van den Eynde B, van der Bruggen P, Romero P, Gaforio JJ, De Plaen E, Lethe B, Brasseur F, Boon T.** Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J Exp Med.* 1994; 179: 921–30.
36. **Bakker AB, Schreurs MW, de Boer AJ, Kawakami Y, Rosenberg SA, Adema GJ, Figdor CG.** Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J Exp Med.* 1994; 179: 1005–9.
37. **Hoon DS, Wang Y, Dale PS, Conrad AJ, Schmid P, Garrison D, Kuo C, Foshag LJ, Nizze AJ, Morton DL.** Detection of occult melanoma cells in blood with a multiple-marker polymerase chain reaction assay. *J Clin Oncol.* 1995; 13: 2109–16.
38. **Palmieri G, Strazzullo M, Ascierto PA, Satriano SMR, Daponte A, Castello G.** Polymerase chain reaction-based detection of circulating melanoma cells as an effective marker of tumor progression. J. Clin. *Oncol.* 1999; 17: 304–11.
39. **Palmieri G, Pirastu M, Strazzullo M, Ascierto PA, Satriano SMR, Motti ML, Botti G, Mozzillo N, Castello G, Cossu A, Lissia A, Tanda F.** Clinical significance of PCR-positive mRNA markers in peripheral blood and regional nodes of malignant melanoma patients. Melanoma Cooperative Group. *Recent Results Cancer Res.* 2001; 158: 200–3.
40. **Palmieri G, Ascierto PA, Perrone F, Satriano SMR, Ottaiano A, Daponte A, Napolitano M, Caracò C, Mozzillo N, Melucci MT, Cossu A, Tanda F, Gallo C, Satriano RA, Castello G.** Prognostic value of circulating melanoma cells detected by reverse transcriptase-polymerase chain reaction. *J Clin Oncol.* 2003; 21: 767–73.
41. **Schittek B, Bodingbauer Y, Ellwanger U, Blaheta HJ, Garbe C.** Amplification of MelanA messenger RNA in addition to tyrosinase increases sensitivity of melanoma cell detection in peripheral blood and is associated with the clinical stage and prognosis of malignant melanoma. *Br J Dermatol.* 1999; 141: 30–6.
42. **Brownbridge GG, Gold J, Edward M, Mackie RM.** Evaluation of the use of tyrosinase-specific and melanA/MART-1-specific reverse transcriptase-coupled-polymerase chain reaction to detect melanoma cells in peripheral blood samples from 299 patients with malignant melanoma. *Br J Dermatol.* 2001; 144: 279–87.
43. **Hanekom GS, Stubbings HM, Johnson CA, KIdson SH.** The detection of circulating melanoma cells correlates with tumor thickness and ulceration

but is not predictive of metastasis for patients with primary melanoma. *Melanoma Res.* 1999; 9: 465–73.

44. **Aubin F, Chtourou M, Teyssier JR, Laubriet A, Mougin CH, Blanc D, Humbert P.** The detection of tyrosinase mRNA in the peripheral blood of stage I melanoma patients is not of clinical relevance in predicting metastasis risk and survival. *Melanoma Res.* 2000; 10: 113–8.
45. **Waldmann V, Wacker J, Deichmann M, Jäckel A, Bock M, Näher H.** Prognosis of metastatic melanoma: no correlation of tyrosinase mRNA in bone marrow and survival time. *Recent Result Cancer Res.* 2001; 158: 118–25.
46. **Strohal R, Mosser R, Kittler H, Wolff K, Jansen B, Brna C, Stingl G, Pehamberger H.** MART-1/Melan-A and tyrosinase transcripts in peripheral blood of melanoma patients: PCR analyses and follow-up testing in relation to clinical stage and disease progression. *Melanoma Res.* 2001; 11: 543–8.
47. **Battayani Z, Grob JJ, Xerri L, Noe C, Zarour H, Houvaeneghel G, Delpero JR, Birmbaum D, Hassoun J, Bonerandi JJ.** PCR detection of circulating melanocytes as a prognostic marker in patients with melanoma. *Arch Dermatol.* 1995; 131: 443–7.
48. **Kunter U, Buer J, Probst M, Duensing S, Dallmann I, Grosse J, Kirchner H, Schluepen EM, Volkenandt M, Ganser A, Atzpodien J.** Peripheral blood tyrosinase messenger RNA detection and survival in malignant melanoma. *J Natl Cancer Inst.* 1996; 88: 590–4.
49. **Mellado B, Colomer D, Castel T, Munoz M, Carballo E, Galan M, Mascaro JM, Vives-Corrons JL, Grau JJ, Estape J.** Detection of circulating neoplastic cells by reverse-transcriptase polymerase chain reaction in malignant melanoma: association with clinical stage and prognosis. *J Clin Oncol.* 1996; 14: 2091–7.
50. **Curry BJ, Myers K, Hersey P.** Polymerase chain reaction detection of melanoma cells in the circulation: relation to clinical stage, surgical treatment, and recurrence from melanoma. *J Clin Oncol.* 1998; 16: 1760–9.
51. **Curry BJ, Myers K, Hersey P.** MART-1 is expressed less frequently on circulating melanoma cells in patients who develop distant compared with locoregional metastases. *J Clin Oncol.* 1999; 17: 2562–71.
52. **Mellado B, Gutierrez L, Castel T, Colomer D, Fontanillas M, Castro J, Estape J.** Prognostic significance of the detection of circulating malignant cells by reverse transcriptase-polymerase chain reaction in long-term clinically disease-free melanoma patients. *Clin Canc Res.* 1999; 5: 1843–8.
53. **Hoon DS, Bostick P, Kuo C, Okamoto T, Wang HJ, Elashoff R, Morton DL.** Molecular markers in blood as surrogate prognostic indicators of melanoma recurrence. *Cancer Res.* 2000; 60: 2253–7.
54. **Schrader AJ, Probst-Kepper M, Grosse J, Kunter U, Schenk F, Franzke A, Atzpodien J, Buer J.** Molecular and prognostic classification of advanced melanoma: a multi-marker microcontamination assay of peripheral blood stem cells. *Melanoma Res.* 2000; 10: 355–62.
55. **Christofori G.** New signals from the invasive front. *Nature.* 2006; 441: 444–50.
56. **Leiter U, Meier F, Schittek B, Garbe C.** The natural course of cutaneous melanoma. *J Surg Oncol.* 2004; 86:172–8.
57. **Voit C, Kron M, Rademaker J, Schwurzer-Voit M, Sterry W, Weber L, Ozdemir C, Proebstle T, Keilholz U.** Molecular staging in stage II and III melanoma patients and its effect on long-term survival. *J Clin Oncol.* 2005; 23: 1218–27.
58. **Palmieri G, Satriano SM, Budroni M, Cossu A, Tanda F, Canzanella S, Caraco C, Simeone E, Daponte A, Mozzillo N, Comella G, Castello G, Ascierto PA.** Serial detection of circulating tumour cells by reverse transcriptase-polymerase chain reaction assays is a marker for poor outcome in patients with malignant melanoma. *BMC Cancer.* 2006; 6: 266.
59. **Mellado B, Del Carmen Vela M, Colomer D, Gutierrez L, Castel T, Quinto L, Fontanillas M, Reguart N, Domingo-Domenech JM, Montagut C, Estape J, Gascon P.** Tyrosinase mRNA in blood of patients with melanoma treated with adjuvant interferon. *J Clin Oncol.* 2002; 20: 4032–9.
60. **Morton DL, Foshag LJ, Hoon DS, Nizze JA, Famatiga E, Wanek LA, Chang C, Davtyan DG, Gupta RK, Elashoff R.** Prolongation of survival in metastatic melanoma after active specific immunotherapy with a new polyvalent melanoma vaccine. *Ann Surg.* 1992; 216: 463–82.
61. **Glass LF, Messina JL, Cruse W, Wells K, Rapaport D, Miliotes G, Berman C, Reintgen D, Fenske NA.** The use of intraoperative radiolymphoscintigraphy for sentinel node biopsy in patients with malignant melanoma. *Dermatol Surg.* 1996; 22: 715–20.
62. **Joseph E, Messina J, Glass FL, Cruse CW, Rapaport DP, Berman C, Reintgen DS.** Radioguided surgery for the ultrastaging of the patient with melanoma. *Cancer J Sci Am.* 1997; 3: 341–5.
63. **Smart KR, Cahoon BW, Dale PS.** Sentinel lymphadenectomy for staging patients with intermediate-level melanoma. *Am Surg.* 2000; 66: 280–3.
64. **Morton DL, Thompson JF, Cochran AJ, Mozzillo N, Elashoff R, Essner R, Nieweg OE, Roses DF, Hoekstra HJ, Karakousis CP, Reintgen DS, Coventry BJ, Glass EC, Wang HJ, MSLT Group.**

Sentinel-node biopsy or nodal observation in melanoma. *N Engl J Med.* 2006; 355: 1307–17.

65. **Bostick PJ, Morton DL, Turner RR, Huynh KT, Wang HJ, Elashoff R, Essner R, Hoon DS.** Prognostic significance of occult metastases detected by sentinel lymphadenectomy and reverse transcriptase-polymerase chain reaction in early-stage melanoma patients. *J Clin Oncol.* 1999; 17: 3238–44.
66. **Blaheta HJ, Ellwanger U, Schittek B, Sotlar K, MacZey E, Breuninger H, Thelen MH, Bueltmann B, Rassner G, Garbe C.** Examination of regional lymph nodes by sentinel node biopsy and molecular analysis provides new staging facilities in primary cutaneous melanoma. *J Invest Dermatol.* 2000; 114: 637–42.
67. **Denninghoff VC, Kahn AG, Falco J, Curutchet HP, Elsner B.** Sentinel lymph node: detection of micrometastases of melanoma in a molecular study. *Mol Diagn.* 2004; 8: 253–8.
68. **Ulrich J, Bonnekoh B, Bockelmann R, Schon M, Schon MP, Steinke R, Roessner A, Schmidt U, Gollnick H.** Prognostic significance of detecting micrometastases by tyrosinase RT/PCR in sentinel lymph node biopsies: lessons from 322 consecutive melanoma patients. *Eur J Cancer.* 2004; 40: 2812–9.
69. **Gradilone A, Ribuffo D, Silvestri I, Cigna E, Gazzaniga P, Nofroni I, Zamolo G, Frati L, Scuderi N, Agliano AM.** Detection of melanoma cells in sentinel lymph nodes by reverse transcriptase-polymerase chain reaction: prognostic significance. *Ann Surg Oncol.* 2004; 11: 983–7.
70. **Romanini A, Manca G, Pellegrino D, Murr R, Sarti S, Bianchi F, Alsharif A, Orlandini C, Zucchi V, Castagna M, Gandini D, Salimbeni G, Ghiara F, Barachini P, Mariani G.** Molecular staging of the sentinel lymph node in melanoma patients: correlation with clinical outcome. *Ann Oncol.* 2005; 16: 1832–40.
71. **Kammula US, Ghossein R, Bhattacharya S, Coit DG.** Serial follow-up and the prognostic significance of reverse transcriptase-polymerase chain reaction–staged sentinel lymph nodes from melanoma patients. *J Clin Oncol.* 2004; 22: 3989–96.
72. **Mangas C, Hilari JM, Paradelo C, Rex J, Fernandez-Figueras MT, Fraile M, Alastrue A, Ferrandiz C.** Prognostic significance of molecular staging study of sentinel lymph nodes by reverse transcriptase-polymerase chain reaction for tyrosinase in melanoma patients. *Ann Surg Oncol.* 2006; 13: 910–8.
73. **Abrahamsen HN, Sorensen BS, Nexo E, Hamilton-Dutoit SJ, Larsen J, Steiniche T.** Pathologic assessment of melanoma sentinel nodes: a role for molecular analysis using quantitative real-time reverse transcription-PCR for MART-1 and tyrosinase messenger RNA. *Clin Cancer Res.* 2005; 11: 1425–33.
74. **Biddle DA, Evans HL, Kemp BL, El-Naggar AK, Harvell JD, White WL, Iskandar SS, Prieto VG.** Intraparenchymal nevus cell aggregates in lymph nodes: a possible diagnostic pitfall with malignant melanoma and carcinoma. *Am J Surg Pathol.* 2003; 27: 673–81.
75. **Cook MG.** Benign melanocytic lesions mimicking melanomas. *Pathology.* 2004; 36: 414–8.
76. **Holt JB, Sangueza OP, Levine EA, Shen P, Bergman S, Geisinger KR, Creager AJ.** Nodal melanocytic nevi in sentinel lymph nodes. Correlation with melanoma-associated cutaneous nevi. *Am J Clin Pathol.* 2004; 121: 58–63.
77. **Mihic-Probst D, Saremaslani P, Komminoth P, Heitz PU.** Immunostaining for the tumour suppressor gene p16 product is a useful marker to differentiate melanoma metastasis from lymph-node nevus. *Virchows Arch.* 2003; 443: 745–51.
78. **Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KH, Aiba S, Brocker EB, LeBoit PE, Pinkel D, Bastian BC.** Distinct sets of genetic alterations in melanoma. *N Engl J Med.* 2005; 353: 2135–47.
79. **Hayward NK.** Genetics of melanoma predisposition. *Oncogene.* 2003; 22: 3053–62.
80. **Wolchok JD, Saenger YM.** Current topics in melanoma. *Curr Opin Oncol.* 2007; 19: 116–20.
81. **Casorzo L, Luzzi C, Nardacchione A, Picciotto F, Pisacane A, Risio M.** Fluorescence in situ hybridization (FISH) evaluation of chromosomes 6, 7, 9 and 10 throughout human melanocytic tumorigenesis. *Melanoma Res.* 2005; 15: 155–60.
82. **Rao UN, Jones MW, Finkelstein SD.** Genotypic analysis of primary and metastatic cutaneous melanoma. *Cancer Genet Cytogenet.* 2003; 140: 37–44.
83. **Sharpless E, Chin L.** The INK4a/ARF locus and melanoma. *Oncogene.* 2003; 22: 3092–98.
84. **Casula M, Ascierto PA, Cossu A, Sini MC, Tore S, Colombino M, Satta MP, Manca A, Rozzo C, Satriano SMR, Castello G, Lissia A, Tanda F, Palmieri G.** Mutation analysis of candidate genes in melanoma-prone families: evidence of different pathogenetic mechanisms at chromosome 9p21. *Melanoma Res.* 2003; 13: 571–9.
85. **Begg CB, Orlow I, Hummer AJ.** Lifetime risk of melanoma in CDKN2A mutation carriers in a population-based sample. *J Natl Cancer Inst.* 2005; 97: 1507–15.
86. **Puig S, Malvehy J, Badenas C.** Role of the CDKN2A locus in patients with multiple primary melanomas. *J Clin Oncol.* 2005; 23: 3043–51.

87. **Goldstein AM, Chan M, Harland M, Hayward NK, Demenais F, Timothy Bishop D, Azizi E, Bergman W, Bianchi-Scarra G, Bruno W, Calista D, Cannon Albright LA, Chaudru V, Chompret A, Cuellar F, Elder DE, Ghiorzo P, Gillanders EM, Gruis NA, Hansson J, Hogg D, Holland EA, Kanetsky PA, Kefford RF, Teresa Landi M, Lang J, Leachman SA, Mackie RM, Magnusson V, Mann GJ, Newton Bishop J, Palmer JM, Puig S, Puig-Butille JA, Stark M, Tsao H, Tucker MA, Whitaker L, Yakobson E.** Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. *J Med Genet.* 2007; 44: 99–106.
88. **Haluska FG, Tsao H, Wu H, Haluska FS, Lazar A, Goel V.** Genetic alterations in signaling pathways in melanoma. *Clin Cancer Res.* 2006; 12: 2301–7.
89. **Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC.** 5´ CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. *Nature Med.* 1995; 1: 686–92.
90. **Costello JF, Berger MS, Huang HS, Cavenee WK.** Silencing of p16/CDKN2 expression in human gliomas by methylation and chromatin condensation. *Cancer Res.* 1996; 56: 2405–10.
91. **Fujimoto A, Morita R, Hatta N, Takehara K, Takata M.** p16INK4a inactivation is not frequent in uncultured sporadic primary cutaneous melanoma. *Oncogene.* 1999; 18: 2527–32.
92. **Palmieri G, Cossu A, Ascierto PA, Botti G, Strazzullo M, Lissia A, Colombino M, Casula M, Floris C, Tanda F, Pirastu M, Castello G.** Definition of the role of chromosome 9p21 in sporadic melanoma through genetic analysis of primary tumors and their metastases. *Br J Cancer.* 2000; 83: 1707–14.
93. **Ruben A, Babilas P, Baron JM, Hofheinz A, Neis M, Sels F.** Analysis of tumor cell evolution in a melanoma: evidence of mutational and selective pressure for loss of p16ink4 and for microsatellite instability. *J Invest Dermatol.* 2000; 114: 14–20.
94. **Vogelstein B, Kinzler KW.** Cancer genes and the pathways they control. *Nat Med.* 2004; 10: 789–99.
95. **Pomerantz J, Schreiber-Agus N, Lie´ geois NJ.** The Ink4a tumor suppressor gene product, 19Arf, interacts with MDM2 and neutralizes DM2's inhibition of p53. *Cell.* 1998; 92: 713–23.
96. **Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B.** Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature.* 1992; 358: 80–3.
97. **Finlay CA.** The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth. *Mol Cell Biol.* 1993; 13: 301–6.
98. **Soengas MS, Lowe SW.** Apoptosis and melanoma chemoresistance. *Oncogene.* 2003; 22: 3138–52.
99. **Bowen AR, Hanks AN, Allen SM, Alexander A, Diedrich MJ, Grossman D.** Apoptosis regulators and responses in human melanocytic and keratinocytic cells. *J Invest Dermatol.* 2003; 120: 48–55.
100. **Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho J, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA.** Mutations of the BRAF gene in human cancer. *Nature.* 2002; 417: 949–54.
101. **Smalley KSM.** A pivotal role for ERK in the oncogenic behaviour of malignant melanoma? *Int J Cancer.* 2003; 104: 527–32.
102. **van Elsas A, Zerp SF, van der Flier S, Kruse KM, Aarnoudse C, Hayward NK, Ruiter DJ, Schrier PI.** Relevance of ultraviolet-induced N-Ras point mutations in development of human cutaneous melanoma. *Am J Pathol.* 1996; 149: 883–93.
103. **Tsao H, Zhang X, Benoit E, Haluska FG.** Identification of PTEN/MMAC1 alterations in uncultured melanomas and melanoma cell lines. *Oncogene.* 1998; 16: 3397–402.
104. **Dong J, Phelps RG, Qiao R, Yao S, Benard O, Ronai Z, Aaronson SA.** BRAF oncogenic mutations correlate with progression rather than initiation of human melanoma. *Cancer Res.* 2003; 63: 3883–5.
105. **Shinozaki M, Fujimoto A, Morton DL, Hoon DS.** Incidence of BRAF oncogene mutation and clinical relevance for primary cutaneous melanomas. *Clin Cancer Res.* 2004; 10: 1753–7.
106. **Casula M, Colombino M, Satta MP, Cossu A, Ascierto PA, Bianchi-Scarrà G, Castiglia D, Budroni M, Rozzo C, Manca A, Lissia A, Carboni A, Petretto E, Satriano SMR, Botti G, Mantelli M, Ghiorzo P, Stratton MR, Tanda F, Palmieri G.** BRAF gene is somatically mutated but does not make a major contribution to malignant melanoma susceptibility. *J. Clin. Oncol.* 2004; 22: 286–92.
107. **Petitclerc E, Stromblad S, von Schalscha TL, Mitjans F, Piulats J, Montgomery AM, Cheresh DA, Brooks PC.** Integrin alpha(v)beta3 promotes M21 melanoma growth in human skin by regulating tumor cell survival. *Cancer Res.* 1999; 59: 2724–30.
108. **Pollock PM, Harper UL, Hansen KS, Yudt LM, Stark M, Robbins CM, Moses TY, Hostetter G, Wagner U, Kakareka J, Salem G, Pohida T, Heenan P, Duray P, Kallioniemi O, Hayward NK, Trent JM,**

Meltzer PS. High frequency of BRAF mutations in nevi. *Nat Genet.* 2003; 33:19–20.

109. **Patton EE, Widlund HR, Kutok JL, Kopani KR, Amatruda JF, Murphey RD, Berghmans S, Mayhall EA, Traver D, Fletcher CD, Aster JC, Granter SR, Look AT, Lee C, Fisher DE, Zon LI.** BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma. *Curr Biol.* 2005; 15: 249–54.
110. **Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS.** BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature.* 2005; 436: 720–4.
111. **Carreira S, Goodall J, Aksan I, La Rocca SA, Galibert MD, Denat L, Larue L, Goding CR.** Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. *Nature*. 2005; 433: 764–9.
112. **Garraway LA, Widlund HR, Rubin MA, Getz G, Berger AJ, Ramaswamy S, Beroukhim R, Milner DA, Granter SR, Du J, Lee C, Wagner SN, Li C, Golub TR, Rimm DL, Meyerson ML, Fisher DE, Sellers WR.** Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature.* 2005; 436: 117–22.
113. **Bastian BC, Kashani-Sabet M, Hamm H, Godfrey T, Moore DH 2nd, Brocker EB, LeBoit PE, Pinkel D.** Gene amplifications characterize acral melanoma and permit the detection of occult tumor cells in the surrounding skin. *Cancer Res.* 2000; 60:1968–73.
114. **Bastian BC, Olshen AB, LeBoit PE, Pinkel D.** Classifying melanocytic tumors based on DNA copy number changes. *Am J Pathol.* 2003; 163: 1765–70.
115. **Whiteman DC, Parsons PG, Green AC.** p53 Expression and risk factors for cutaneous melanoma: a case-control study. *Int J Cancer.* 1998; 77: 843–8.
116. **Tsao H, Zhang X, Fowlkes K, Haluska FG.** Relative reciprocity of NRAS and PTEN/MMAC1 alterations in cutaneous melanoma cell lines. *Cancer Res.* 2000; 60: 1800–4.
117. **Sensi M, Nicolini G, Petti C, Bersani I, Lozupone F, Molla A, Vegetti C, Nonaka D, Mortarini R, Parmiani G, Fais S, Anichini A.** Mutually exclusive NRASQ61R and BRAFV600E mutations at the single-cell level in the same human melanoma. *Oncogene.* 2006; 25: 3357–64.
118. **Landi MT, Bauer J, Pfeiffer RM, Elder DE, Hulley B, Minghetti P, Calista D, Kanetsky PA, Pinkel D, Bastian BC.** MC1R germline variants confer risk for BRAF-mutant melanoma. *Science.* 2006; 313: 521–2.
119. **Hersey P.** Adjuvant therapy for high-risk primary and resected metastatic melanoma. *Intern Med J.* 2003; 33: 33–43.
120. **Wheatley K, Ives N, Hancock B, Gore M, Eggermont A, Suciu S.** Does adjuvant interferon-alpha for high-risk melanoma provide a worthwhile benefit? A meta-analysis of the randomised trials. *Cancer Treat Rev.* 2003; 29: 241–52.
121. **Eggermont AM.** The role interferon-alpha in malignant melanoma remains to be defined. *Eur J Cancer.* 2001; 37: 2147–53.
122. **Borden EC, Smith TJ.** Melanoma: adjuvant therapy with interferons. *ASCO Educational Book* 1999; 120–5.
123. **Verweij J, de Jonge M.** Multitarget tyrosine kinase inhibition: "And the winner is. . .". *J Clin Oncol.* 2007; 25: 2340–2.
124. **Carr KM, Bittner M, Trent JM.** Gene-expression profiling in human cutaneous melanoma. *Oncogene.* 2003; 22: 3076–80.
125. **Talantov D, Mazumder A, Yu JX, Briggs T, Jiang Y, Backus J, Atkins D, Wang Y.** Novel genes associated with malignant melanoma but not benign melanocytic lesions. *Clin Cancer Res.* 2005; 11: 7234–42.
126. **Meltzer PS.** Genetic diversity in melanoma. *N Engl J Med.* 2005; 353: 2104–7.
127. **Winnepenninckx V, Lazar V, Michiels S, Dessen P, Stas M, Alonso SR, Avril MF, Ortiz Romero PL, Robert T, Balacescu O, Eggermont AM, Lenoir G, Sarasin A, Tursz T, van den Oord JJ, Spatz A;** Melanoma Group of the European Organization for Research and Treatment of Cancer. Gene expression profiling of primary cutaneous melanoma and clinical outcome. *J Natl Cancer Inst.* 2006; 98: 472–82.
128. **Jaeger J, Koczan D, Thiesen HJ, Ibrahim SM, Gross G, Spang R, Kunz M.** Gene expression signatures for tumor progression, tumor subtype, and tumor thickness in laser-microdissected melanoma tissues. *Clin Cancer Res.* 2007; 13: 806–15.