Clinicopathological and molecular aspects of cutaneous melanoma

Thomas Bogenrieder

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Klinisch-pathologische und molekulare Aspekte des Melanoms der Haut

(mit einer Zusammenfassung in deutscher Sprache)

Proefschrift

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In liefdevolle herinnering aan mijn grootouders

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Es gibt keine Landstraße für die Wissenschaft, und nur diejenigen haben Aussicht, ihre hellen Gipfel zu erreichen, die die Ermüdung beim Erklettern ihrer steilen Pfade nicht scheuen.

Karl Marx

What gets us into trouble
is not what we don’t know,
it’s what we know for sure
that just ain’t so.

Mark Twain

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General Introduction: Cutaneous Melanoma

At the beginning of the new millennium, an assessment of our progress in understanding melanoma pathobiology reveals impressive accomplishments and considerable insight into what melanocytes do to become such an aggressive and often fatal neoplasm. Although our accumulated understanding of melanoma biology, biochemistry and genetics is now considerable, it also has become apparent that many more questions have been generated in that process. Intellectual concepts that once seemed on the verge of comprehension have evolved such that almost elegant complexity (the melanoma), coupled with occasional perplexity (the investigator), better define our current state of knowledge.

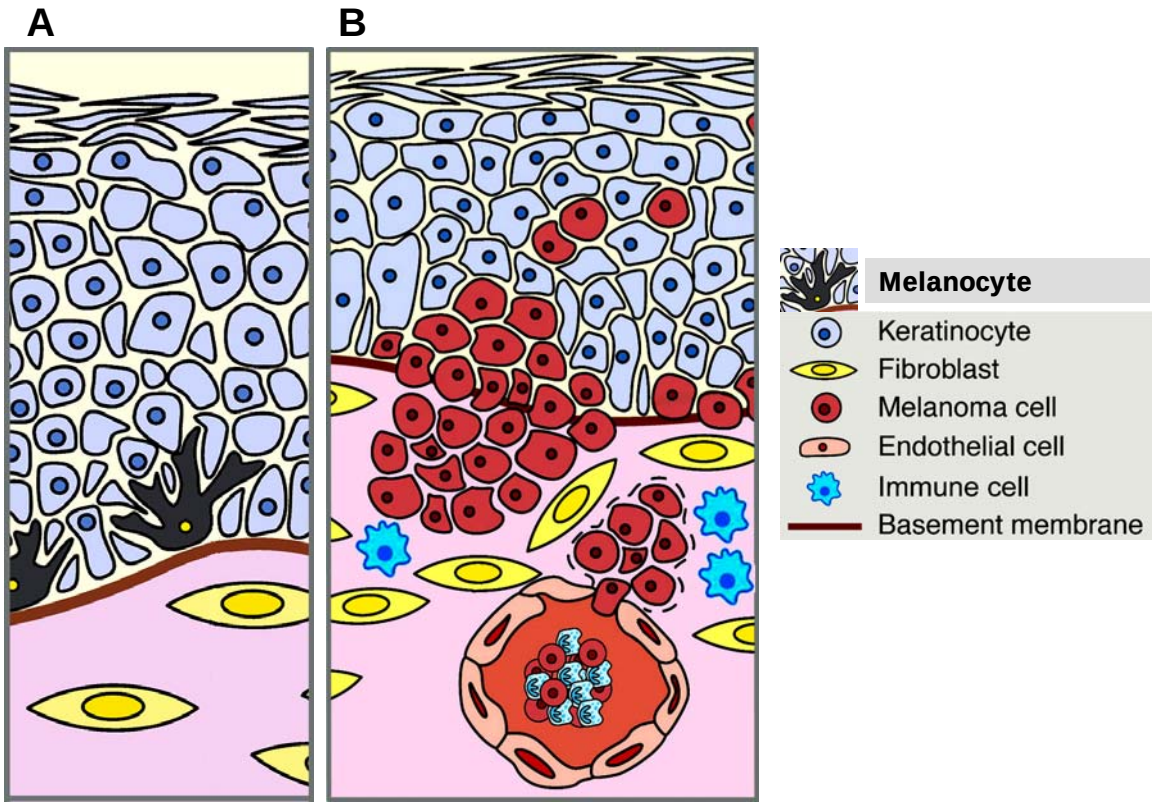

Figure 1. Schematic representation of melanoma development from the pigmented cells of the skin. A) In normal skin, melanocytic homeostasis is maintained by dynamic interactions between the melanocytes and their microenvironment via cell adhesion (cell-cell, cell-matrix) and soluble factors (cytokines, growth factors). B) Melanoma should be seen as a tissue (or an organ) composed of multiple cell types and a structural framework that are connected and interdependent.

The human pigmentary system: the epidermal melanin unit

Under normal physiological conditions, melanocytes and keratinocytes form the 'melanin unit' in the epidermis (Figure 1). In this unit, melanocytes and keratinocytes are aligned along the basement membrane zone at a ratio of 1:5–8. Each melanocyte extends with its dendrites into the upper layers of the epidermis, transporting pigment-containing melanosomes to approximately 35 keratinocytes. The undifferentiated keratinocytes of the basal layer regulate melanocyte growth, number of dendrites and expression of cell surface molecules (1,2), providing

evidence that this highly ordered organizational pattern serves as the structural basis for intercellular regulation. Information is not yet available on how melanocytes and keratinocytes can maintain this lifelong balance, which is only disturbed during transformation into a nevus or a melanoma. When normal melanocytes are isolated from skin and then cultured, they continuously proliferate until senescence. They display a bi- or tripolar morphology, and express cell surface molecules that *in situ* are only found on melanoma cells. When the melanocytes are mixed with undifferentiated keratinocytes to allow cell–cell contact, their growth becomes regulated so that the ratio of melanocytes to keratinocytes remains constant (1-3). The melanocytes develop multiple dendrites, which attach to keratinocytes. Expression of melanoma-associated antigens disappears in co-cultures within three to four days. It remains unclear how signals for phenotypic control of keratinocytes over melanocytes are relayed. Neither differentiated keratinocytes, nor fibroblasts or carcinoma cells can regulate melanocytes. Furthermore, keratinocytes fail to regulate growth or expression of tumor-associated cell surface molecules on the melanoma cells. Based on these biological observations, it can be suggested that the dynamic changes in cell–cell communication play a critical role in cell growth and invasion during transformation.

Epidemiology of cutaneous melanoma

Of all skin neoplasms, melanoma is less common than basal cell cancer or squamous cell cancer, but it is the most fatal. Over the past decades, an increasing trend in incidence has been observed for all Caucasian populations. In the USA, melanoma incidence has increased 15-fold over the past 40 years, and currently the annual increase in incidence is 3–7% per year within the Caucasian population(4). Despite this increase and overall rise in mortality, the survival rate has improved substantially (5). However, melanoma causes disproportionate mortality in those of young and middle age, such that an average of 18.6 potential life years are lost for each melanoma death, one of the highest rates for adult-onset cancers (5). In The Netherlands, incidence rates between 1989 and 1998 increased from 9.5 to 1.5 per 100,000 person years for males, and from 13.3. to 14.8 per 100,000 person years for females (6). In Europe, melanoma is the 7th most commonly diagnosed cancer in males and the 8th most common in females; while in the USA it is the 5th and 7th, and in Australia the 4th and 3rd most commonly diagnosed cancer, respectively(5,7).

Melanoma, skin type and ultraviolet radiation

Melanoma has a complex etiology, which involves both genetic predisposition and exposure to environmental factors. The major environmental etiologic factor is sunlight exposure(8). Melanoma risk depends upon the relationship between the nature of sun exposure and skin type. Individuals that are fair-haired and fair-skinned, with an inability to tan and blue eyes, are more susceptible to develop melanoma during their lives: melanoma is chiefly a disease of white people (5-7). Intermittent sun exposure and a history of severe sunburns, especially during childhood, as well as the use of tanning beds have been associated with an increased risk for melanoma development (9-13). Ultraviolet radiation promotes malignant changes in the skin by a direct mutagenic effect on DNA, by disturbing the immune defence system in the skin, and by promoting reactive oxygen species of melanin which in return cause DNA damage and suppress apoptosis (5,14). Having pigmented lesions, including freckles (15,16) and either common (17,18) or clinically atypical moles (19,20)is also associated with an increased risk of developing cutaneous melanoma. There is also a genetic factor: the risk of developing a

melanoma is greatly increased if there is a positive family history (21,22). Patients with a strong family history and multiple clinically atypical moles are at greatest risk for developing cutaneous melanoma (23-26).

Melanoma development is a multi-step process

Cutaneous melanoma develops from the malignant transformation of melanocytes, the pigment-producing cells that reside in amongst the keratinocytes of the basal epidermal layer of the human skin. Based on clinical and histopathological features, five steps of melanoma progression have been proposed (Figure 2) (27,28): common acquired and congenital nevi with cytologically normal melanocytes; dysplastic nevus with structural and architectural atypia; early radial growth phase (RGP) primary melanoma, which is non-tumorigenic but may be characterized by a tendency to inexorable growth; vertical growth phase (VGP) primary melanoma, which is tumorigenic and may have competence for metastasis; and metastatic melanoma. Despite a wealth of research resources (tissues, cell lines, and antibodies), the exact genetic and biochemical alterations responsible for the development and stepwise progression of melanoma still remain enigmatic. Extensive cytogenetic analyses have failed to identify consistent gene deletions, mutations, translocations, or amplifications in sporadic cases(29,30). Figure 2 summarizes selected genetic and biological events leading to melanoma development and progression. The dynamic progression from a resting melanocyte to a common acquired nevus is very common and does not appear to be accompanied by consistent genetic changes. Nevus cells isolated from common acquired nevi have a finite life span and generally do not carry cytogenetic abnormalities(31-33). Genetic changes are anticipated when dysplastic nevi develop, but the nature of these changes is currently unknown. It is possible that mechanisms leading to persistence and proliferation of dysplastic nevi rest in the dysfunction of the physiological cascade of apoptosis.

Progression from dysplasia to RGP primary melanoma is gradual and apparently spontaneous, and may not require additional molecular changes(28). The transition from RGP to VGP is a biologically and clinically critical step, accompanying additional genetic abnormalities. However, the specifics are largely unknown. No major additional genetic changes may be required for further progression to metastatic dissemination since most VGP melanomas can be readily adapted to a metastatic phenotype through selection in growth factor-free medium or by induction of invasion through artificial basement membranes (34). This suggests that micro-environmental factors such as cell-matrix and cell-cell signaling are critical for the metastatic phenotype.

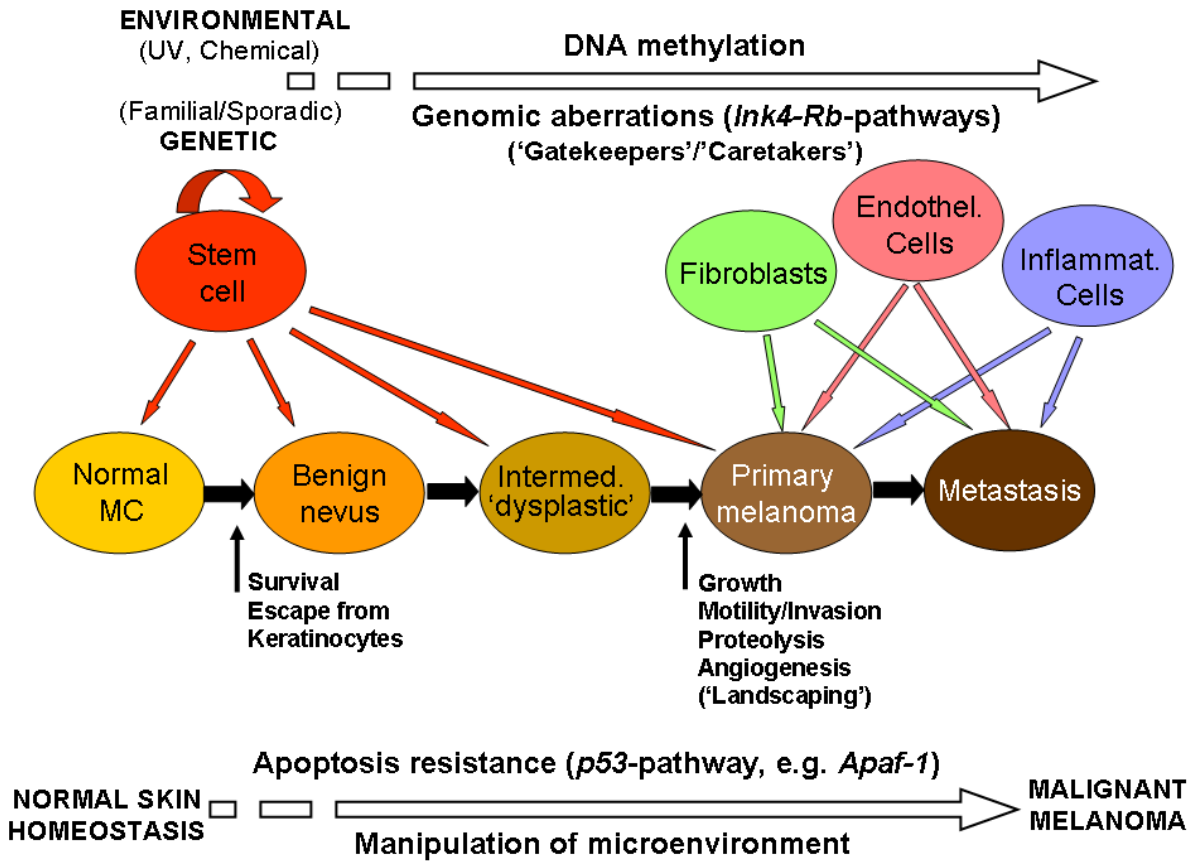

Figure 2. The multistep molecular process of metastatic melanoma development from normal melanocytes. This model implies that melanoma commonly develops and progresses in a sequence of steps from nevic lesions, which can be histologically identified in approximately 35% of cases. The model also acknowledges that melanoma may also develop directly from normal cells. Genetic (such as mutations and deletions of critical genes) and epigenetic (such as DNA-methylation leading to gene silencing) alterations of critical pathways as well as the acquired capability of evading apoptosis, progressively disturb normal skin homeostasis leading to melanoma development. The progression from normal melanocyte to nevus may be initiated by loss of contact between melanocytes and keratinocytes, i.e., the melanocytes escape from keratinocyte (KC) control. Genetic changes are expected at the transition from common acquired (benign) nevus to 'dysplastic' nevus and primary melanoma, allowing cells to persist. At the (tumorigenic) step between intermediary lesion and primary melanoma, increased growth, invasion and stromal 'landscaping' by proteolysis occurs, leading to an increased manipulation of the microenvironment. Additional genetic changes are expected in the progression from primary melanoma to metastatic lesions. The role of tumor initiating or 'stem' cells is still unclear but increasingly appreciated.

Molecular biology

The underlying genetic causes of melanoma are also starting to be elucidated. In a minority of cases, so-called familial melanoma (5–12% of patients), there is an association with mutations in cyclin-dependent kinase (CDK)-N2A ($p16^{INK}$), which is located on chromosome 9 (9p21) and CDK-4, which is located on chromosome 12 (12q13) (35). Both are critical for controlling cell cycle entry. In a recent breakthrough, the possible genetic basis for non-inherited melanoma has also been determined, with over 66% of primary melanomas harboring activating mutations in one of the Raf isoforms, BRAF (36). The mutation, which is a result of a missense mutation, leading to the substitution of glutamate to valine (V600E), directly activates the mitogen-activated protein kinase (MAPK) pathway in melanoma(36). Constitutive activity of

the MAPK pathway in melanoma is known to be responsible for much of the oncogenic behavior of this tumor (37).

The stromal microenvironment

Increased understanding of the underlying biology of melanoma has led to the realization that melanomas are not composed exclusively of tumor cells, and that host cells are also involved intimately in the progression of this disease (38). The careful dissection of the interactions between these multiple cell types and their means of communication offers the possibility of targeting the host-derived component of the melanoma. It is likely that these approaches will be more successful at controlling the spread and dissemination of the melanoma; the stromal cells are more genetically stable and less susceptible to drug resistance.
Pathologic studies have shown that melanomas are infiltrated by fibroblasts, inflammatory and endothelial cells, and thus consist of a mixture of neoplastic and non-neoplastic cells (38,39). A solid tumor is therefore a dynamic interaction between the neoplasm and host. The classic view of the host–tumor interaction is that the host cells provide the structure via fibroblast-derived stromal proteins and nutrients via endothelial cell-derived microvessels. These assumptions are based on the premise that the stroma is playing a largely passive role in tumor development. However, the emerging view is that the stroma, and in particular the fibroblasts, play an active role in tumor progression and are an integral part of the tumor organ (40). The role of fibroblasts in tumor development was first recognized by pathologists, who observed that most solid tumors were hard and fibrotic in nature, the direct result of fibroblast infiltration and collagen deposition throughout the lesion(39).

As already mentioned, melanocyte growth and behavior is tightly controlled by the surrounding keratinocytes under normal homeostasis (Figure 2). The balance between the two cell types is maintained through the tight regulation of melanocyte proliferation. This control is maintained through a complex web of paracrine growth factors, cell–cell and cell–matrix adhesions.
Downregulation or inactivation of the homotypic adhesion molecule E-cadherin is known to be a major factor in malignant progression (41). In the skin, E-cadherin is one of the major homotypic adhesion molecules linking keratinocytes and melanocytes (42), and loss of this molecule is known to be critical to the escape of melanocytes from keratinocyte control (43,44). The suppressive role of E-cadherin on melanocyte behavior is demonstrated by the fact that, in monoculture, melanocytes proliferate rapidly and upregulate the expression of many melanoma markers, such as Mel-CAM (MCAM, CD146) and integrin αVβ3. All of these markers are lost from the melanocytes when they are cocultured with keratinocytes. Escape from keratinocyte control, via downregulation of E-cadherin and upregulation of N-cadherin, permits the direct interaction of early stage melanoma cells with other cells of the stroma, such as fibroblasts – which also express N-cadherin(44). This switch in cadherin profile leads to survival advantages and increased motility of the melanoma cells through homotypic N-cadherin-mediated signalling(45).
The recruitment and infiltration of fibroblasts into the tumor microenvironment is followed by their activation and a phenotypic switch. There are many parallels between the state of fibroblast activation in tumors and their state during wound healing, which led Harold Dvorak to dub tumors as “wounds that do not heal”(46). Analysis of the tumor-infiltrating fibroblast (TIF) phenotype reveals that these cells lie somewhere in between that of a fibroblast and a smooth muscle cell; for this reason they are often referred to as myofibroblasts. Most commentators agree that there is

much similarity between myofibroblasts and TIFs, and the two cell types are difficult to distinguish on the basis of phenotype.

Tumor stem cells or tumor-initiating cells

Another *leitmotiv* in cancer research in recent years is the involvement of tumor stem cells or tumor-initiating cells in carcinogenesis. There is growing evidence to suggest that not all cancer cells have similar levels of malignant potential and that tumor progression may be driven by specialized sub-sets of tumor-initiating cells. It is likely that as tumor-initiating cells have lower proliferation rates and enhanced survival mechanisms they may also drive drug resistance. Such cells have also been proposed to exist for melanoma. Cells with stem-cell markers and features have recently been identified in melanoma tissues and cell lines. These melanoma stem-like cells possess many traits of tumor-initiating or tumor stem cells including self-renewal capacity, high tumorigenicity, and differentiation into various mesenchymal lineages, including melanocytic cells.

Stem cells exist within a specialized microenvironment termed as stem cell niche. The niche plays a critical role in maintaining the undifferentiated state of stem cell pool through the provision of paracrine and extracellular matrix signals. In human and murine skin, melanocyte stem cells reside in the hair follicle bulge of the lower permanent portion of the hair follicle (47,48). During the growth phase of the hair follicle or wound healing, signals from the hair bulge niche provide the melanocyte stem cells with the necessary signals for growth and differentiation. Although the exact nature of these signals is not known, recent work has shown that WNT-3a may play a role in this process (49).

Several subpopulations of melanoma-initiating cells have been distinguished: $CD20^+$, $CD133^+$, $ABCB5^+$ (a novel member of the ABC drug transporter family), label-retaining or slow-cycling cells, and side-population cells with high efflux activities. The biologic and molecular characterization of melanoma stem cells is still in an early phase and the origin of melanoma stem cells has yet to be determined. Whether melanoma stem cells are derived from melanocyte stem cells, melanocyte progenitors, or more mature melanocytes that have de-differentiated also remains unclear at this point. It is hoped that the targeting of melanoma initiating cells may be one novel approach to overcome the incredible therapy resistance of this tumor (reviewed in (37,50)).

Prognosis of melanoma

The prognosis of melanoma patients depends chiefly on two factors: firstly, the thickness of the primary tumor (Breslow thickness) and, secondly, the presence or absence of metastatic cells in the regional lymph nodes. Additional factors, which should also be taken into account are tumor ulceration, lymphatic invasion, male sex, age, and site of the primary melanoma (5) Five-year overall survival decreases from 93% for stage I patients Breslow thickness ≤ 1mm), 68% for stage II patients (Breslow thickness > 2mm), 45% for stage III patients (regional lymph node metastasis), to 11% for stage IV patients (systemic metastasis) (51-53).

Lymphatic spread of melanoma and elective lymph node dissection

It has long been recognised that melanoma metastasizes mostly via the lymphatics. It first metastasizes to the regional lymph nodes, where the metastatic cells settle Subsequently, they are thought to metastasize systemically after occult growth. Primary tumors can also metastasize directly into the circulation, leading to rapid systemic dissemination. Most patients have no clinical signs of metastatic disease at

the time of initial diagnosis. However, approximately 20% of these patients do have occult (micro-)metastases in the regional lymph nodes. The optimal treatment of melanoma with regard to the regional lymph node basin has been one of the most important controversies for many years. Until the early 1990s, there were two basic therapeutic options: On the one hand, elective lymph node dissection, by which all regional lymph nodes were removed surgically in every patient after the diagnosis of a primary melanoma; on the other hand, a watch-and-wait approach, in which a delayed lymph node dissection was performed at the time when clinical (i.e. palpable) lymph node metastases occur.
A series of studies compared elective lymph node dissection with the watch-and-wait approach, yet failed to offer a definitive optimal strategy (54-59). However, investigators of the *Intergroup Melanoma Trial* recently demonstrated a survival benefit for elective lymph node dissection in prospectively stratified subset of patients (60). The World Health Organization trial no. 14 showed that survival was significantly better for patients with resected microscopic nodal disease by elective lymph node dissection compared with patients who did not undergo elective lymph node dissection and subsequently developed gross nodal disease (55). From these latter findings, one can deduce that lymph node dissection may be curative in some patients with nodal disease.
A major disadvantage of elective lymph node dissection is that this procedure is applied to all patients with clinically negative lymph nodes. Hence, the majority of patients without nodal disease (approximately 80%) are subjected to the potentially considerable morbidity of elective lymph node dissection without any therapeutic benefit (61). Morbidity can be significant and includes wound infection, seroma formation and lymphedema. Ideally, therefore, only those patients with existing lymph node metastases would be identified for therapeutic lymph node dissection. This is conceptually feasible by removing only the lymph node(s) from the draining basin most likely to be affected by metastatic cells, the so-called sentinel lymph node(s).

<u>The sentinel lymph node concept</u>
The sentinel lymph node concept is based on the theory of an orderly progression of tumor cells through the lymphatic system in the initial phase of the metastatic cascade (62). It assumes that early lymphatic metastases, if present, are always found first within the first-line tumor draining lymph node, the sentinel lymph node. The rationale of the sentinel lymph node concept is based on the premise that if the sentinel lymph node is not involved, one can reasonably presume that the remaining lymph nodes of the affected regional basin are free from additional metastases as well.
These principles have been described in the mid 19th century by the German pathologist Rudolf Virchow (“Virchow’s node” in stomach cancer) and L.R. Braithwaite, a surgeon from Leeds, in 1923, the latter using the term “glands sentinel” and drawing on observations made in appendicitis (63). In the 1930s, the anatomist J.H. Gray injected Thorotrast into freshly resected human tissues and then obtained X-rays demonstrating detailed lymphatic anatomy. A number of new anatomical observations contributed to a better understanding of lymphatic anatomy. His conclusions declare an early understanding of what we now take as common knowledge: ‘‘On the grounds of anatomy and physiology, the focal lymph glands will be the first and most likely seat of glandular metastases’’(64).
In the early 1950s Weinberg at UCLA reported the mapping of lymph nodes with blue dye in cancer patients. He injected blue dye in the stomach during surgery for gastric malignancy with the specific goal of ‘‘targeting the primary nodes’’ (65)}.

Subsequently, he continued this approach with lung cancer and described ‘‘mapping of the nodes by vital staining’’ (66). In 1960, Gould and co-workers where the first to use the term “sentinel node” in a publication on cancer of the parotid (67). These authors suggested that the examination of the sentinel lymph node could be instrumental in deciding to perform a radical neck dissection.
This technique was applied by several investigators in the 1960s for testicular cancer, routinely demonstrating that a limited number of nodes received drainage from the tumor. Recognition of this phenomenon began to generate a new language and tumor draining nodes were termed primary nodes and the next nodes to receive drainage were called secondary nodes (68). By 1966, this technology led to the observation that ‘‘a small sentinel node… is immediately visualized before the main lateral nodes on each side are seen’’ (69). In 1976, the Paraguayan surgeon Ramon Cabanas reported on a sentinel lymph node in penile carcinoma to the Society of Surgical Oncology. This work, published the following year articulated our current concept or sentinel lymph node surgery: Firstly, “evidence of a specific lymph center, the so called sentinel lymph node”; secondly, biopsy the sentinel lymph node first and followed by selective lymphadenectomy based on the status of the sentinel lymph node; thirdly, the “status of the sentinel lymph node as a basis for a new classification’’(70).

It was Donald Morton and co-workers from Santa Monica who took the practice of taking a sentinel lymph node biopsy in melanoma patients a major step further in the early 1990s. In 1992, this group published the first results of a study in which the sentinel lymph node was identified in 194 melanoma patients by use of a blue dye (61). Others subsequently confirmed the accuracy of this procedure in studies in which the sentinel lymph node procedure was followed by immediate completion lymph node dissection, so that all lymph nodes of the draining basin could be examined histologically (62,71). Initially, the sentinel lymph node procedure was performed by means of a vital blue dye which was intradermally injected around the primary melanoma or scar. Nowadays, a higher detection rate is achieved by means of specific radiotracers, through pre-operative lymphoscintigraphy and intra-operative detection of the sentinel lymph node using a handheld gamma-detection probe. (61,72-75). Identification of the sentinel node is most accurate when a “triple technique” is employed, i.e., encompassing pre-operative lymphoscintigraphy, intradermal injection of blue dye around the primary melanoma site immediately pre-operatively, and the use of a handheld gamma-detection probe intra-operatively (76-78).
The prognostic value of the sentinel lymph node status has been demonstrated in several large studies (79-86). All these studies reported a large difference in five-year survival between patients who are sentinel node-negative *versus* –positive; independently of other prognostic variables: Five-year overall survival for sentinel lymph node-positive patients is around 55-60%, whereas five-year overall survival for sentinel lymph node-negative patients is around 90%.

<u>Treatment of melanoma</u>
Current treatment of cutaneous melanoma exemplifies not only the need for increased translational research but also many of the challenges of moving cancer research from bench to bedside. Melanoma remains unique among solid tumors in that its treatment primarily is surgical. Radiation is only of limited benefit, and chemotherapy has been disappointing in both the adjuvant and metastatic settings.

This leaves clinicians with few options for reducing the chance of recurrence after surgery and for treating unresectable, metastatic disease.
From a therapeutic standpoint, early treatment of melanoma is essential; removal of the nondisseminated primary lesion is usually curative, whereas the median survival rate for patients with disseminated disease is 8.1 months, with only approximately 2% surviving for 5 years (87). To date, all major chemotherapy drugs, immunotherapies and radiotherapies, even modern targeted compounds, have failed in large-scale clinical phase III trials. Only one drug, the alkylating agent dacarbazine (DTIC), is currently approved for the treatment of metastatic melanoma. Unfortunately, even responses to DTIC are poor, with clinical response rates of 5–10% and cure rates of 1%. Other regimens, combining DTIC with cisplatin, vinblastine, tamoxifen or carmustine, show little clinical benefit over DTIC alone (88). As melanoma has the dubious distinction of being almost completely resistant to every known therapy, new approaches to treating melanoma are needed urgently.

Over the past decades there has been a fervent attempt to elucidate melanoma biology and to identify novel approaches to more efficient melanoma therapy and translate them into routine clinical use. A detailed review of novel compounds in clinical testing is beyond the scope of this thesis and the reader is referred to recent excellent review articles (89-91),

With this general outline of our current medical understanding of melanoma in mind, this thesis aims to address several important molecular and clinicopathological aspects that have arisen over the past decade.

References

1. Valyi-Nagy IT, Hirka G, Jensen PJ et al. Undifferentiated keratinocytes control growth, morphology, and antigen expression of normal melanocytes through cell-cell contact. Lab Invest. 1993; 69:152-159.

2. Shih IM, Elder DE, Hsu MY et al. Regulation of Mel-CAM/MUC18 expression on melanocytes of different stages of tumor progression by normal keratinocytes. Am J Pathol. 1994; 145:837-845.

3. Scott GA, Haake AR. Keratinocytes regulate melanocyte number in human fetal and neonatal skin equivalents. J Invest Dermatol. 1991; 97:776-781.

4. Diepgen TL, Mahler V. The epidemiology of skin cancer. Br J Dermatol. 2002; 146 Suppl 61:1-6.

5. Thompson JF, Scolyer RA, Kefford RF. Cutaneous melanoma. Lancet. 2005; 365:687-701.

6. de VE, Schouten LJ, Visser O et al. Rising trends in the incidence of and mortality from cutaneous melanoma in the Netherlands: a Northwest to Southeast gradient? Eur J Cancer. 2003; 39:1439-1446.

7. de VE, Coebergh JW. Cutaneous malignant melanoma in Europe. Eur J Cancer. 2004; 40:2355-2366.

8. Marks R. Epidemiology of melanoma. Clin Exp Dermatol. 2000; 25:459-463.

9. Whiteman DC, Whiteman CA, Green AC. Childhood sun exposure as a risk factor for melanoma: a systematic review of epidemiologic studies. Cancer Causes Control. 2001; 12:69-82.

10. Tucker MA, Goldstein AM. Melanoma etiology: where are we? Oncogene. 2003; 22:3042-3052.

11. Armstrong BK, Kricker A. The epidemiology of UV induced skin cancer. J Photochem Photobiol B. 2001; 63:8-18.

12. Elwood JM, Jopson J. Melanoma and sun exposure: an overview of published studies. Int J Cancer. 1997; 73:198-203.

13. Tsao H, Sober AJ. Ultraviolet radiation and malignant melanoma. Clin Dermatol. 1998; 16:67-73.

14. Meyskens FL, Jr., Farmer PJ, nton-Culver H. Etiologic pathogenesis of melanoma: a unifying hypothesis for the missing attributable risk. Clin Cancer Res. 2004; 10:2581-2583.

15. Bliss JM, Ford D, Swerdlow AJ et al. Risk of cutaneous melanoma associated with pigmentation characteristics and freckling: systematic overview of 10 case-control studies. The International Melanoma Analysis Group (IMAGE). Int J Cancer. 1995; 62:367-376.

16. Marrett LD, King WD, Walter SD et al. Use of host factors to identify people at high risk for cutaneous malignant melanoma. CMAJ. 1992; 147:445-453.

17. Grulich AE, Bataille V, Swerdlow AJ et al. Naevi and pigmentary characteristics as risk factors for melanoma in a high-risk population: a case-control study in New South Wales, Australia. Int J Cancer. 1996; 67:485-491.

18. Berwick M, Halpern A. Melanoma epidemiology. Curr Opin Oncol. 1997; 9:178-182.

19. Newton JA, Bataille V, Griffiths K et al. How common is the atypical mole syndrome phenotype in apparently sporadic melanoma? J Am Acad Dermatol. 1993; 29:989-996.

20. Tucker MA, Halpern A, Holly EA et al. Clinically recognized dysplastic nevi. A central risk factor for cutaneous melanoma. JAMA. 1997; 277:1439-1444.

21. Ford D, Bliss JM, Swerdlow AJ et al. Risk of cutaneous melanoma associated with a family history of the disease. The International Melanoma Analysis Group (IMAGE). Int J Cancer. 1995; 62:377-381.

22. Goldstein AM, Tucker MA. Genetic epidemiology of familial melanoma. Dermatol Clin. 1995; 13:605-612.

23. Clark WH, Jr., Reimer RR, Greene M et al. Origin of familial malignant melanomas from heritable melanocytic lesions. 'The B-K mole syndrome'. Arch Dermatol. 1978; 114:732-738.

24. Lynch HT, Frichot BC, III, Lynch JF. Familial atypical multiple mole-melanoma syndrome. J Med Genet. 1978; 15:352-356.

25. Kraemer KH, Tucker M, Tarone R et al. Risk of cutaneous melanoma in dysplastic nevus syndrome types A and B. N Engl J Med. 1986; 315:1615-1616.

26. Greene MH, Clark WH, Jr., Tucker MA et al. High risk of malignant melanoma in melanoma-prone families with dysplastic nevi. Ann Intern Med. 1985; 102:458-465.

27. Clark WH, Jr., Elder DE, Guerry D et al. A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. Hum Pathol. 1984; 15:1147-1165.

28. Meier F, Satyamoorthy K, Nesbit M et al. Molecular events in melanoma development and progression. Front Biosci. 1998; 3:D1005-D1010.

29. Kamb A, Herlyn M. Malignant melanoma. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic & molecular basis of inherited diseases / ed.: Charles R. Scriver .. 2001:967-977.

30. Bogenrieder T. CM, Elder DE, Herlyn M. Molecular and cellular Biology. In: Balch CM, Houghton AN, Sober AJ, Soong SJ, eds. Cutaneous melanoma. 2003:751.

31. Balaban GB, Herlyn M, Clark WH, Jr. et al. Karyotypic evolution in human malignant melanoma. Cancer Genet Cytogenet. 1986; 19:113-122.

32. Balaban G, Herlyn M, Guerry D et al. Cytogenetics of human malignant melanoma and premalignant lesions. Cancer Genet Cytogenet. 1984; 11:429-439.

33. Mancianti ML, Gyorfi T, Shih IM et al. Growth regulation of cultured human nevus cells. J Invest Dermatol. 1993; 100:281S-287S.

34. Kath R, Jambrosic JA, Holland L et al. Development of invasive and growth factor-independent cell variants from primary human melanomas. Cancer Res. 1991; 51:2205-2211.

35. Bishop DT, Demenais F, Goldstein AM et al. Geographical variation in the penetrance of CDKN2A mutations for melanoma. J Natl Cancer Inst. 2002; 94:894-903.

36. Davies H, Bignell GR, Cox C et al. Mutations of the BRAF gene in human cancer. Nature. 2002; 417:949-954.

37. Smalley KS. A pivotal role for ERK in the oncogenic behaviour of malignant melanoma? Int J Cancer. 2003; 104:527-532.

38. Labrousse AL, Ntayi C, Hornebeck W et al. Stromal reaction in cutaneous melanoma. Crit Rev Oncol Hematol. 2004; 49:269-275.

39. Ruiter D, Bogenrieder T, Elder D et al. Melanoma-stroma interactions: structural and functional aspects. Lancet Oncol. 2002; 3:35-43.

40. Bissell MJ, Radisky D. Putting tumours in context. Nat Rev Cancer. 2001; 1:46-54.

41. Hajra KM, Fearon ER. Cadherin and catenin alterations in human cancer. Genes, chromosomes & cancer. 2002; 34:255-268.

42. Haass NK, Smalley KSM, Herlyn M. The Role of Altered Cell-Cell Communication in Melanoma Progression. Histochem J. 2004; 35:309-318.

43. Tang A, Eller MS, Hara M et al. E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes in vitro. J Cell Sci. 1994; 107 (Pt 4):983-992.

44. Hsu MY, Meier FE, Nesbit M et al. E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. Am J Pathol. 2000; 156:1515-1525.

45. Li G, Satyamoorthy K, Herlyn M. N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. Cancer Res. 2001; 61:3819-3825.

46. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing
1. N Engl J Med. 1986; 315:1650-1659.

47. Nishimura EK, Granter SR, Fisher DE. Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. Science. 2005; 307:720-724.

48. Tumbar T, Guasch G, Greco V et al. Defining the epithelial stem cell niche in skin. Science. 2004; 303:359-363.

49. Fang D, Leishear K, Nguyen TK et al. Defining the conditions for the generation of melanocytes from human embryonic stem cells. Stem Cells. 2006; 24:1668-1677.

50. Zabierowski SE, Herlyn M. Melanoma stem cells: the dark seed of melanoma. J Clin Oncol. 2008; 26:2890-2894.

51. Balch CM, Buzaid AC, Atkins MB et al. A new American Joint Committee on Cancer staging system for cutaneous melanoma. Cancer. 2000; 88:1484-1491.

52. Balch CM, Soong SJ, Gershenwald JE et al. Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. J Clin Oncol. 2001; 19:3622-3634.

53. Balch CM, Buzaid AC, Soong SJ et al. Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. J Clin Oncol. 2001; 19:3635-3648.

54. Balch CM, Soong SJ, Bartolucci AA et al. Efficacy of an elective regional lymph node dissection of 1 to 4 mm thick melanomas for patients 60 years of age and younger. Ann Surg. 1996; 224:255-263.

55. Cascinelli N, Morabito A, Santinami M et al. Immediate or delayed dissection of regional nodes in patients with melanoma of the trunk: a randomised trial. WHO Melanoma Programme. Lancet. 1998; 351:793-796.

56. Drepper H, Kohler CO, Bastian B et al. Benefit of elective lymph node dissection in subgroups of melanoma patients. Results of a multicenter study of 3616 patients. Cancer. 1993; 72:741-749.

57. Sim FH, Nelson TE, Pritchard DJ. Malignant melanoma: Mayo Clinic experience. Mayo Clinic proceedings Mayo Clinic. 1997; 72:565-569.

58. Veronesi U, Adamus J, Bandiera DC et al. Delayed regional lymph node dissection in stage I melanoma of the skin of the lower extremities. Cancer. 1982; 49:2420-2430.

59. Sim FH, Taylor WF, Ivins JC et al. A prospective randomized study of the efficacy of routine elective lymphadenectomy in management of malignant melanoma. Preliminary results. Cancer. 1978; 41:948-956.

60. Balch CM, Soong S, Ross MI et al. Long-term results of a multi-institutional randomized trial comparing prognostic factors and surgical results for intermediate thickness melanomas (1.0 to 4.0 mm). Intergroup Melanoma Surgical Trial. Ann Surg Oncol. 2000; 7:87-97.

61. Morton DL, Wen DR, Wong JH et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg. 1992; 127:392-399.

62. Reintgen D, Cruse CW, Wells K et al. The orderly progression of melanoma nodal metastases. Ann Surg. 1994; 220:759-767.

63. Braithwaite LR. The flow of lymph from the ileocacecal angle and its possible bearing on the cause of duodenal and gastric ulcer. Br J Surg. 1923; 11:7-26.

64. Gray J.H. The relation of lymphatic vessels to the spread of cancer. Br J Surg. 1939; 24:462-495.

65. Weinberg J, Greaney EM. Identification of regional lymph nodes by means of a vital staining dye during surgery of gastric cancer. Surg Gynecol Obstet. 1950; 90:561-567.

66. Weinberg JA. Identification of regional lymph nodes in the treatment of bronchiogenic carcinoma. The Journal of thoracic surgery. 1951; 22:517-526.

67. Gould EA, Winship T, Philbin PH et al. Observations on a "sentinel node" in cancer of the parotid. Cancer. 1960; 13:77-78.

68. Busch FM, Sayegh ES, Chenault OW. Some uses of lymphangiography in the management of testicular tumors. J Urol. 1964; 93:490-493.

69. Sayegh E, Brooks T, Sacher E et al. Lymphangiography of the retroperitoneal lumph nodes through the inguinal route. J Urol. 1966; 95:102-107.

70. Cabanas RM. An approach for the treatment of penile carcinoma. Cancer. 1977; 39:456-466.

71. Thompson JF, McCarthy WH, Bosch CM et al. Sentinel lymph node status as an indicator of the presence of metastatic melanoma in regional lymph nodes. Melanoma Res. 1995; 5:255-260.

72. van der Veen H, Hoekstra OS, Paul MA et al. Gamma probe-guided sentinel node biopsy to select patients with melanoma for lymphadenectomy. Br J Surg. 1994; 81:1769-1770.

73. Bostick P, Essner R, Glass E et al. Comparison of blue dye and probe-assisted intraoperative lymphatic mapping in melanoma to identify sentinel nodes in 100 lymphatic basins. Arch Surg. 1999; 134:43-49.

74. Alex JC, Weaver DL, Fairbank JT et al. Gamma-probe-guided lymph node localization in malignant melanoma. Surg Oncol. 1993; 2:303-308.

75. Uren RF, Howman-Giles RB, Shaw HM et al. Lymphoscintigraphy in high-risk melanoma of the trunk: predicting draining node groups, defining lymphatic channels and locating the sentinel node. J Nucl Med. 1993; 34:1435-1440.

76. Pijpers R, Borgstein PJ, Teule GJ et al. Vital dye and radiolabelled colloids--complement or alternative? Recent Results Cancer Res. 2000; 157:130-137.

77. Pijpers R, Collet GJ, Meijer S et al. The impact of dynamic lymphoscintigraphy and gamma probe guidance on sentinel node biopsy in melanoma. Eur J Nucl Med. 1995; 22:1238-1241.

78. Pijpers R, Borgstein PJ, Meijer S et al. Sentinel node biopsy in melanoma patients: dynamic lymphoscintigraphy followed by intraoperative gamma probe and vital dye guidance. World J Surg. 1997; 21:788-792.

79. Clary BM, Mann B, Brady MS et al. Early recurrence after lymphatic mapping and sentinel node biopsy in patients with primary extremity melanoma: a comparison with elective lymph node dissection. Ann Surg Oncol. 2001; 8:328-337.

80. Essner R, Conforti A, Kelley MC et al. Efficacy of lymphatic mapping, sentinel lymphadenectomy, and selective complete lymph node dissection as a therapeutic procedure for early-stage melanoma. Ann Surg Oncol. 1999; 6:442-449.

81. Gadd MA, Cosimi AB, Yu J et al. Outcome of patients with melanoma and histologically negative sentinel lymph nodes. Arch Surg. 1999; 134:381-387.

82. Gershenwald JE, Colome MI, Lee JE et al. Patterns of recurrence following a negative sentinel lymph node biopsy in 243 patients with stage I or II melanoma. J Clin Oncol. 1998; 16:2253-2260.

83. Harlow SP, Krag DN, Ashikaga T et al. Gamma probe guided biopsy of the sentinel node in malignant melanoma: a multicentre study. Melanoma Res. 2001; 11:45-55.

84. Jansen L, Nieweg OE, Peterse JL et al. Reliability of sentinel lymph node biopsy for staging melanoma. Br J Surg. 2000; 87:484-489.

85. Statius Muller MG, van Leeuwen PA, de Lange-De Klerk ES et al. The sentinel lymph node status is an important factor for predicting clinical outcome in patients with Stage I or II cutaneous melanoma. Cancer. 2001; 91:2401-2408.

86. Doting MH, Hoekstra HJ, Plukker JT et al. Is sentinel node biopsy beneficial in melanoma patients? A report on 200 patients with cutaneous melanoma. Eur J Surg Oncol. 2002; 28:673-678.

87. Lee ML, Tomsu K, Von Eschen KB. Duration of survival for disseminated malignant melanoma: results of a meta-analysis. Melanoma Res. 2000; 10:81-92.

88. Soengas MS, Lowe SW. Apoptosis and melanoma chemoresistance. Oncogene. 2003; 22:3138-3151.

89. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature. 2007; 445:851-857.

90. Kalinsky K, Haluska FG. Novel inhibitors in the treatment of metastatic melanoma. Expert Rev Anticancer Ther. 2007; 7:715-724.

91. Lejeune FJ, Rimoldi D, Speiser D. New approaches in metastatic melanoma: biological and molecular targeted therapies. Expert Rev Anticancer Ther. 2007; 7:701-713.

Outline of the Thesis

2

Outline of the thesis

Although we have an increasingly good understanding of the genetics and biochemistry of melanoma, the mechanisms underlying their combinatorial interactions are still poorly defined and need to be fathomed to ultimately lead to more successful therapeutic strategies and evidence-based management of patients with melanoma. The overarching goal of this thesis, therefore, is to highlight some of the issues that have arisen over the past decade, as reviewed in Chapter 1 (General Introduction).

This thesis is divided into three parts. The first part describes molecular and cellular aspects of melanoma. The second part describes clinicopathological aspects of melanoma, especially studies concerning the sentinel lymph node. Part three is a discussion of the current knowledge of melanoma and future perspectives in molecular and clinical melanoma research.

Cancer cells interact with the microenvironment via complex autocrine and paracrine mechanisms. **Chapter 3** summarizes recent findings regarding the expression, structure and function of proteolytic enzymes at or near the cell surface in cell-cell and cell-stroma interactions during melanoma progression. Proteolytic enzymes in melanoma are involved in cell-surface proteolysis, growth factor activation and intercellular communication, and activate or release growth factors from the microenvironment or act directly on the microenvironment itself, thereby facilitating angiogenesis or tumor cell migration.

Cutaneous melanomas are notorious for their tendency to metastasize. Because the tumor microenvironment plays an important part in tumor development and progression, melanoma-stroma interactions and their structural and functional aspects are reviewed and discussed in **Chapter 4**. We emphasise fibrovascular patterns (both in uveal and cutaneous melanoma), cellular and extracellular composition of the stroma, and the molecules involved. Also, we discuss functional interactions, focusing on melanoma-fibroblast cross-talk by soluble factors and by direct cell-cell contact.

Metastatic melanoma is a radiation- and chemotherapy-refractory neoplasm for which no standard therapy currently exists. So far, genetic and molecular studies have revealed few non-random chromosomal abnormalities and infrequent mutational spectra. Consequently, the precise molecular determinants responsible for a resistance to apoptotic mechanisms in melanoma are yet to be delineated. **Chapter 5** details our understanding of the molecular mechanisms of apoptosis and drug resistance in melanoma.

One family of genes with potential importance in melanoma progression are the neurotrophins, namely nerve growth factor (NGF), brain derived growth factor (BDNF), neurotrphin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) and their cognate receptors: tyrosine kinase receptors encoded by the Trk family of related genes (Trk A, Trk B and Trk C), and the low affinity receptor, $p75^{NTR}$. $p75^{NTR}$ shows homology to a family of cell-surface receptors that includes the apoptosis-mediating Fas cell-surface antigen and the type I and II receptors for tumor necrosis factor (TNFR). The cytoplasmatic domains of the TNF receptor family members do not contain any discernible anzymatic activity, but may serve signalling functions. Point mutations in

the cytoplasmatic domain of the Fas antigen results in lymphoproliferative disorders in mice.
$p75^{NTR}$ is a cysteine-rich transmembrane glycoprotein which is frequently overexpressed in advanced stages of human melanoma. The biological consequences of this overexpression are unknown; however, it has been shown that $p75^{NTR}$ can enhance the invasive potential of melanoma cells in vitro. Presumably, defects in the highly conserved cytoplasmatic domain of $p75^{NTR}$ would also affect receptor-related function. The study outlined in **Chapter 6** therefore examined the transmembrane domain in the $p75^{NTR}$ gene for structural defects, and analysed $p75^{NTR}$ protein levels in normal melanocyte cultures and metastatic melanoma cell lines.

Key learnings from clinical and histological observations in melanoma are all too often overlooked. Induction of multiple eruptive dermal and atypical melanocytic naevi has frequently been reported in children with malignant haematological diseases and chemotherapy-induced immunosuppression. In **Chapter 7** we present the first report of an adult patient to develop multiple eruptive melanocytic skin lesions while undergoing chemotherapy with an oral 5-fluorouracil prodrug (capecitabine/Xeloda®) for metastasizing cancer.

Out of all melanoma patients with sentinel lymph node metastatic deposits, about 70% have metastases limited to the sentinel lymph node (SN), with no involvement of additional (non-sentinel) lymph nodes. In spite of that fact, all patients undergo completion lymph node dissection, which frequently is associated with considerable morbidity. Additionally, many of these patients receive potentially unnecessary adjuvant treatment, e.g. with interferon-alpha, which is also associated with potentially harmful side effects. In **Chapter 8**, a model is re-evaluated and refined in a multi-center study, which might predict the absence of additional lymph node metastases in patients with melanoma. Of all sentinel lymph node positive patients, histological slides were re-examined under the microscope. Using a sophisticated morphometry system, two-dimensional surface area of metastasis in the sentinel lymph node was measured and correlated to non-sentinel (additional) lymph node involvement.

As a sequel to Chapter 8, **Chapter 9** examines the repercussions of the aforementioned findings on the disease-free and overall survival of melanoma patients with positive sentinel lymph nodes. Even though there is mounting evidence that small melanoma metastases to the SN do not bear predictive value for non-SN involvement or prognostic value, there is at present no staging system that confers clinical consequences to the tumor burden found in melanoma SNs: for therapeutic decision making they are simply considered to be positive or negative. The objective of this study was therefore to further investigate the relationship between the regional SN tumor burden and the outcomes of recurrence and survival in patients with clinically localized cutaneous melanoma staged by SN biopsy and completion lymph node dissection (CLND) for identification of occult nodal metastases. Moreover, we analyze prognostic algorithms as defined by other groups which have been shown to detect SN-positive subgroups at low or zero risk for non-SN metastasis or improved survival.

A summary of this thesis as well as a discussion of the importance of our findings are presented in **Chapter 10.** Lastly, future prospects in clinical and molecular melanoma

research ad well as potential targets for drug development are reviewed in **Chapter 11**.

Cell-surface proteolysis, growth factor activation and intercellular communication in the progression of melanoma

Crit Rev Oncol Hematol 2002; 44:1-15

3

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Abstract

Normal skin architecture and melanocyte function is maintained by a dynamic interplay between the melanocytes themselves, the epithelial cells between which they are interspersed, and their microenvironment. The microenvironment consists of the extracellular matrix, fibroblasts, migratory immune cells, and neural elements supported by a vascular network, all within a milieu of cytokines, growth factors, and bioactive peptides as well as proteolytic enzymes. Cells interact with the microenvironment via complex autocrine and paracrine mechanisms. Proteolytic enzymes in melanoma may activate or release growth factors from the microenvironment or act directly on the microenvironment itself, thereby facilitating angiogenesis or tumor cell migration. This review summarizes recent findings regarding the expression, structure and function of proteolytic enzymes at or near the cell surface in cell–cell and cell–stroma interactions during melanoma progression. Cell-surface (membrane) peptidases are a multi-functional group of ectoenzymes that have been implicated in the control of growth and differentiation of many cellular systems. The potential, but yet speculative, role of other membrane-bound molecules, such as multifunctional surface proteins with adhesion and protease activity (ADAM gene family) or the ephrin/Eph receptor protein kinases in the pathogenesis of melanoma are discussed.

1. Introduction—or: why is cell-surface proteolysis important in tumorigenesis?

Normal skin homeostasis is maintained by dynamic interactions between the melanocytes and their microenvironment, such as keratinocytes, fibroblasts, endothelial and immunocompetent cells, and the extracellular matrix. Melanocytes adhere to keratinocytes, whereas communication between melanocytes and fibroblasts or endothelial cells occur through soluble factors. During the transformation and progression of melanocytes and melanoma cells, there are reciprocal interactions between the neoplastic cells and adjacent normal skin cells, such as dermal and epithelial cells (see [1 and 2] for review).

Cancer and melanoma research over the past decades has been largely focused on events occurring within the boundaries of the plasma membrane of the malignant cell. The dominant paradigm, wherein multiple genetic lesions, e.g. of the cyclinD/cdk4-$p16^{INK4A}$-pRb-pathway [3 and 4], provide both the impetus for and the possible Achilles heel of cancer, which in return can be targeted for gene therapy [5], is not sufficient to understand melanoma as a disease process. Furthermore, some of the genetic lesions frequently encountered in other solid tumors, e.g. alterations of the p53 tumor suppressor gene product, are apparently not of relevance in the evolution of melanoma [6 and 7]. Considering that 2% of the gene products of organisms, whose genome has been sequenced are proteases [8], many exciting discoveries about the functions of these molecules in physiological and neoplastic processes can be expected in the future. In the following review, we will use selected examples to illustrate the influence of cell-surface proteolysis and the resulting alteration of the pericellular microenvironment for the evolution of melanoma.

2. From slave to master: selected players in maintaining normal skin architecture

The basic properties of cellular behavior that define function are growth, morphology, polarity, adhesion, migration, and expression of tissue-specific proteins [9]. These properties constitute the cell phenotype, which is conferred by interaction between the expression of specific genes and the cells' responses to ECM, to neighbouring cells and to soluble effectors, such as growth factors and cytokines [5 and 10]. Normal melanocytes are tightly controlled by keratinocytes (Table 1). Keratinocytes, the 'masters', dictate when the melanocytes, the 'slaves', can grow and what cell-

surface molecules are expressed [11 and 12]. The keratinocytes need cell-cell contact to establish this control, which is mediated by E-cadherin. E-cadherin is found on normal melanocytes and to a lesser degree on nevi and little on melanomas (Fig. 2) [13]. The loss of E-cadherin expression has significant biological consequences in melanocytic cells. Melanoma cells have escaped from keratinocyte control by shutting off expression of E-cadherin and activating N-cadherin [14]. They can now leave the epidermis, invade the dermis and closely adhere to and communicate with fibroblasts, endothelial cells, and other stromal cells and components. The ‘run-away’ slave has become a powerful ‘master’, accepting growth factors from keratinocytes; it now directs the presence and functions of fibroblasts, endothelial and inflammatory cells in its microenvironment. The melanoma cells tell the fibroblasts to produce a scaffolding with matrix proteins, and to release growth factors, which melanomas cannot synthesize on their own, but which increase their growth, survival and invasive capacity [15]. The symbiosis has been reversed and the malignant melanoma cells are in the driver's seat.

Table 1. Interactions of keratinocytes with melanocytes and melanoma cells are E-cadherin-dependent

Characteristic	Melanocytes	Melanoma cells	
		No E-cadherin	With E-cadherin
Mel-CAM/MUC18	Negative	High	Negative
$\alpha v\beta 3$-Integrin	Negative	High	Negative
Invasiveness in skin reconstructs	Negative	High	Low
Attachment to keratinocytes	Yes	No	Yes
Growth regulation[a]	Yes	No	Yes
Gap junction[b]	Yes	No	Yes

The escape of melanoma cells from the epidermis can be experimentally reversed. Forced reexpression of E-cadherin in melanoma cells leads to growth retardation, inhibition of invasion and induction of apoptotic death in three-dimensional skin reconstructs, and decreased tumorigenicity in mice [14]. Thus, E-cadherin may act as an invasion suppressor in the melanoma system. Melanoma cells, even the most aggressive metastatic ones, can again come under the control of keratinocytes, if the expression of E-cadherin is re-established by gene transfer [16]. The N-cadherin gene is then downregulated and the melanoma cells no longer establish gap junctions with fibroblasts [14]. The keratinocytes are again in the driver's seat: They can adhere to the E-cadherin expressing melanoma cells and dictate whether these grow or not [16]. Within a few days, all melanoma cell surface molecules associated

with growth, invasion and metastasis are shut off. Important markers are the β3 integrin subunit that allows biologically early melanoma cells to invade into the dermis [17 and 18], and the cell-cell adhesion marker Mel-CAM/MUC18 [12 and 18]. We do not know the mechanisms, by which keratinocytes can transmit their signals, but these signal are strong enough to force the melanoma cells back into a subservient position.

3. Melanoma development is a multi-step process

Based on clinical and histopathological features, five steps of melanoma progression have been proposed (Fig. 1) [15 and 19]: common acquired and congenital nevi with structurally normal melanocytes, dysplastic nevus with structural and architectural atypia, early radial growth phase (RGP) primary melanoma, advanced vertical growth phase (VGP) primary melanoma with competence for metastasis, and metastatic melanoma. Despite a wealth of research resources (tissues, cell lines, and antibodies), the genetic and biochemical alterations responsible for the development and stepwise progression of melanoma still remain enigmatic. Cytogenetic analyses have failed to identify consistent gene deletions, mutations, translocations, or amplifications in sporadic cases [1 and 2].

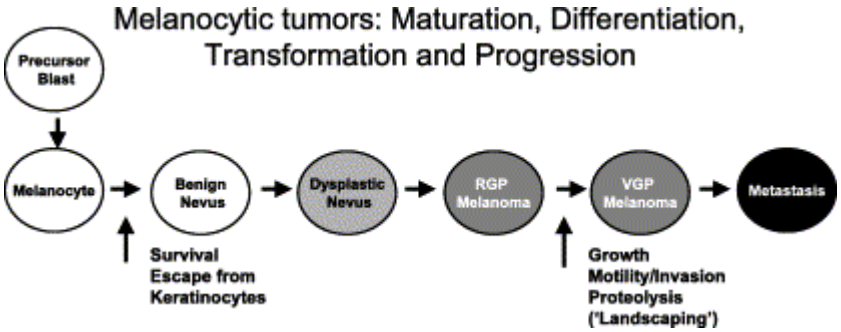

Fig. 1. Biological events leading to melanoma development and progression. The model, developed by Clark et al. [19], implies that melanoma commonly develops and progresses in a sequence of steps from nevic lesions, which can be histologically identified in approximately 35% of cases. However, melanoma may also develop directly from normal cells. The role of melanoblasts (immature melanocytes) in melanogenesis remains poorly defined. The progression from normal melanocyte to nevus may be initiated by loss of contact between melanocytes and keratinocytes, i.e. the melanocytes escape from keratinocyte (KC) control. Genetic changes, which are currently not defined, are expected at the transition from common acquired (benign) nevus to dysplastic nevus/RGP/in situ melanoma (left vertical arrow), allowing cells to persist. Additional genetic changes are expected in the progression from RGP/in situ melanoma to VGP (right vertical arrow). At the VGP (tumorigenic) step, increased growth, invasion and stromal 'landscaping' by proteolysis occurs.

Fig. 2 summarizes selected genetic and biological events leading to melanoma development and progression. The dynamic progression from a resting melanocyte to a common acquired nevus is very common and does not appear to accompany

genetic changes. Nevus cells isolated from common acquired nevi have a finite life span and generally do not carry cytogenetic abnormalities [20, 21 and 22].

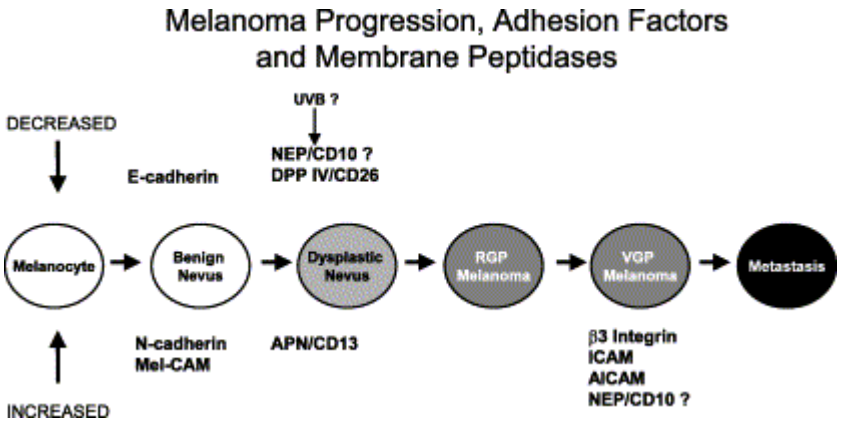

Fig. 2. Dynamic changes in expression of adhesion receptors, ECM proteins and proteolytic surface enzymes in melanoma progression. Decreased expression (downward arrow) is seen for some cadherins, CAMs, integrins, and cell-surface peptidases. A strong increase (upward arrow) is seen for a variety of adhesion-related molecules and cell-surface peptidases, first in nevi, then in VGP primary melanomas.

We postulate that melanocytes progress to a nevus by escaping from the normal contact-mediated controls of keratinocytes. Keratinocytes are the dominant cellular partner of melanocytes in the epidermis and control the growth, morphology, and antigenic phenotype of melanocytes [11 and 23] by establishing direct contact through the cell-cell adhesion receptor E-cadherin. This contact, in turn, facilitates formation of gap junctions through connexin 43 [14]. It remains unclear, whether signals for phenotypic control over melanocytes are relayed through E-cadherin, gap junctions or other accessory mechanisms. Nevertheless, E-cadherin downregulation coincides with melanoma progression. Reduced E-cadherin expression can be observed early in the nevus stage and the majority of melanomas are E-cadherin negative [13].

In contrast, expression of N-cadherin is upregulated in nevi and melanomas. Such a shift in cadherin profile confers new adhesive properties to the cells. Acquisition of N-cadherin may allow gap junctional communication of nevus and melanoma cells with N-cadherin-expressing fibroblasts and endothelial cells [15]. Genetic changes are anticipated when dysplastic nevi develop, but the nature of these changes is currently unknown. It is possible that mechanisms leading to persistence and proliferation of dysplastic nevi rest in the dysfunction of the physiological cascade of apoptosis. Progression from dysplasia to RGP primary melanoma is gradual and spontaneous, and may not require additional molecular changes [15]. The transition

from RGP to VGP is a biologically and clinically critical step, accompanying additional genetic abnormalities. However, the specifics are largely unknown. In sections of lesions and in cultured cells, we have described a variety of changes at the biological level, which explain RGP to VGP progression [24].

Unlike RGP melanomas, VGP cells are metastasis-competent [25] and easily adapted to growth in culture. In addition, VGP cells are less dependent on exogenous growth factors [26] and have growth characteristics similar to metastatic cells, such as anchorage-independent growth in soft agar and tumorigenesis in immunodeficient mice. VGP primary melanomas display numerous cytogenetic abnormalities, suggesting considerable genomic instability. No major additional genetic changes may be required for further progression to metastatic dissemination since most VGP melanomas can be readily adapted to a metastatic phenotype through selection in growth factor-free medium or by induction of invasion through artificial basement membranes [27]. This suggests that micro-environmental factors, such as cell–matrix and cell–cell signaling are critical for the metastatic phenotype.

4. Gatekeepers, caretakers and landscapers

The prevailing paradigm for the development of cancer is a multi-step process, during which a cell acquires multiple genetic mutations [5, 9 and 28]. The central question that has dominated the literature in the past years is: how many and what genetic changes are necessary for a cell to become malignant [5 and 9]? In a step towards functionally categorizing these genetic changes, Kinzler and Vogelstein have classified the genes involved, as those that monitor growth by suppressing proliferation, inducing apoptosis or promoting differentiation (‘gatekeepers’). These are assisted by genes that indirectly suppress neoplasia by ensuring the fidelity of the DNA code through effective repair of DNA damge or by regulating genomic stability (‘caretakers’) [29]. Recently they have also recognized enabling genes (‘landscapers’) [30], which might affect non-target cells by modulating the microenvironment, in which tumor cells grow, perhaps by direct/indirect regulation of extracellular matrix proteins, cell-surface markers, adhesion proteins, or secreted growth factors [31]. Others refer to the aforementioned by the well-accepted term of microenvironmental ‘effectors’ [5].

Malignant tumors are complex tissues, composed of many cell types, such as fibroblasts, endothelial and inflammatory cells, and cannot exist in isolation [9]. Thus,

normal cells within the neoplastic tissue are not idle bystanders, but active participants that shape the frequency and features of malignant tumors. Hence, the multi-step genetic modification theory often fails to acknowledge the significance of such forces in the development of neoplasia [5]. Biological events are now beginning to be understood in terms of specific proteolytic proteins affecting cell–cell contacts, cell adhesion and their dynamic reciprocal interaction.

5. Stroma and the pericellular microenvironment

The pericellular microenvironment ('stroma') of the normal melanocyte and its malignant counterpart, the melanoma cell, is remarkably complex and consists of cellular, molecular and mechanical components. The insoluble extracellular matrix (ECM) [32] is composed of proteins, glycoproteins, proteoglycans, and glycosaminoglycans in a complex arrangement that provides structure, generates biological signals, stores factors that generate biological signals, and exerts mechanical influences on the epidermis, including the melanocyte. Cells that influence a melanoma cell or its normal progenitor, the melanocyte, include keratinocytes, fibroblasts, adipose cells, endothelial and resident immune cells (in skin: Langerhans cells), each of which represents a heterogeneous population of cellular phenotypes [1 and 2]. In addition, the stroma has temporal and spatial complexity: it changes with time and tumor progression and is tissue-type specific. The specific molecules that are responsible for tumor-induced changes in the microenvironment and the reciprocal modifications of the tumor by the microenvironment are starting to be known, as are the intracellular pathways that result from these influences.

For example, Mel-CAM/MUC18 is an adhesion receptor that is involved in cell–cell interactions. Its expression is upregulated during melanoma development in a step-wise fashion and coincides with the separation of nevus cells from keratinocytes (Fig. 2) [12]. Mel-CAM binds to a currently unidentified ligand [18], and may play a major role in metastasis by mediating not only melanoma cell–cell interactions, but also melanoma-endothelial cell adhesion. Mel-CAM appears to act in concert with αvβ3, the vitronectin receptor, in promoting metastasis. As the cells progress from RGP to VGP, expression of αvβ3, α2β1, α3β1, α4β1, ICAM-1, and GD2 ganglioside is increased. The most notable marker is the beta3 subunit of αvβ3 integrin, which appears to be the most specific melanoma-associated marker distinguishing RGP

from VGP melanomas (Fig. 2) [33]. It is also a prime candidate for prognostic studies [34].

6. ECM and cell-surface proteolysis regulating cellular ecology

The cell-surface and the pericellular space are a dynamic microenvironment. Cell–cell and cell–ECM interactions provide cells with information essential for controlling morphogenesis, cell fate specification, gain or loss of tissue-specific functions, cell migration, tissue repair, and cell death [10, 32 and 35].

During cellular responses to developmental or pathological cues, ECM, cell surface proteins, and receptors are activated or removed by proteolysis [36 and 37]. Sequestration, presentation or activation of growth factors is also regulated by proteolysis [10 and 38]. One often overlooked aspect of pericellular proteolysis is its potential role in angiogenesis [39], immunity and host defense. Deficient proteolysis leads to disease processes, just as overproduction of proteinases does. Another level of complexity derives from the multiple cell types involved in protease expression within a tumor. In many types of carcinomas, matrix-degrading proteases or cell-surface peptidases are produced not by the epithelial cancer cells but by surrounding stromal and inflammatory cells [10 and 40].

Over the past decade, cell biology has firmly established in model systems that the complex interactions between epithelial cells and the microenvironment are critical for maintaining a normal, balanced homeostasis [5]. We will now discuss in more detail evidence illustrating the contribution of the microenvironment to normal melanocyte homeostasis. Disrupting this balance by altered cell surface proteolysis can induce aberrant cell proliferation, adhesion, function and migration that might promote malignant behavior of melanocytes.

7. Cell-surface peptidases: hydrolyzing bioactive peptides as a critical component of growth control

Cell-surface peptidases are a group of ubiquitously occurring ectoenzymes with a broad functional, pleiotropnic repertoire (Table 2, Fig. 3). They are integral membrane proteins of the plasma membrane, asymmetrically oriented with the catalytic site exposed at the external surface of the cell [41]. They are widely distributed in human tissues and the physiologic consequences of their activity vary according to their cellular location (reviewed in [42]).

Table 2. Melanoma-associated cell surface peptidases

Cell surface peptidase	Regulatory function	Known substrates	Role in melanoma
APN (Aminopeptidase N, CD13, EC 3.4.11.2), 140 000-Da glycoprotein	Reduces cellular responses to peptide hormones. Processes peptides at the intestinal and renal proximal tubular brush borders. Serves as coronavirus receptor. Limits the analgestic effects of opioid peptides	Opioid peptides, fMLP, enkephalins	Melanocytes: not expressed. Dysplastic nevi: 25%. Melanoma in situ: 44%. Invasive melanoma: 67%. Metastatic melanoma: 100%. Adhesion to extracellular matrix, hydrolyses ECM proteins, activator of type IV collagenase
NEP (Neutral endopeptidase, CD10, EC 3.4.24.11, enkephalinase, neprilysin), 100 000-Da glycoprotein	Reduces cellular responses to peptide hormones. Processes peptides at the intestinal and renal proximal tubular brush borders. Limits the analgesic effects of opoid peptides	Opioid peptides, fMLP, substance P, bombesin-like peptides, atrial natriuretic factor, endothelin, oxytocin,bradykinin, angiotensins I and II, α-MSH (α-melanocyte-stimulating hormone)	Melanocyte: expressed; downregulated upon UVB-irradiation. Melanoma: all stages 30–40%
DPP IV (Dipeptidyl peptidase IV, CD26, EC 3.4.14.5, adenosine deaminase-binding protein), 120 000-Da glycoprotein	Reduce cellular responses to peptide hormones. Processes peptides at the intestinal and renal proximal tubular brush borders. Adhesion molecule. Collagen ECM degradation	Substance P, casomorphin, kenzin, α chain of fibrin, growth hormone releasing hormone, RANTES (regulated on actiation, normal T-cell expressed and secreted), MCP-1/-2/-3 (monocyte chemotactic protein)	Melanocyte: consistently expressed. Dysplastic nevi: loss of expression. Primary and metastatic melanoma: absent

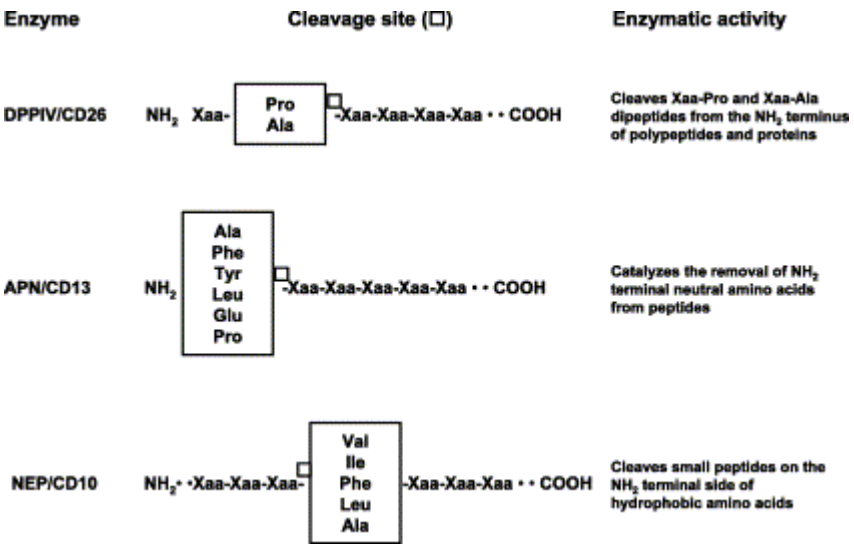

Fig. 3. Melanoma-associated cell-surface peptidases. Cleavage site and enzymatic activity (modified from [42]).

In protein metabolism, their functional importance is well documented, especially in peptide degradation and amino acid scavenging in the brush border membranes of renal and intestinal microvilli: peptidases hydrolyze peptides to facilitate absorption by enterocytes in the intestinal brush-border membrane (in this location 6–8% of the protein is aminopeptidase N [APN]), and recycle amino acids in the brush border of kidney proximal tubule cells [41]. They also perform more subtle physiological tasks. For example, in synaptic membranes, APN and neutral endopeptidase (NEP, enkephalinase) inactivate endorphins and enkephalins [43 and 44]. They cleave

bioactive peptides (Fig. 3), resulting in activation or inactivation, and function as receptors and as molecules participating in adhesion or signal transduction (reviewed in [42] and [45]). Hence, cell-surface peptidases might have a key role in the control of growth and differentiation of many cellular systems by modulating the activity of peptide growth factors and by regulating their access to adjacent cells [41] (Table 2). Whether the enzymatic activity is necessary for all of these different functions remains to be determined [45].

In hematopoiesis, the expression of cell-surface peptidases is a characteristic of several distinct developmental stages. The classification of leukemias and lymphomas is based in part on the expression of cell-surface antigens, which are also present on normal precursor hematopoietic cells. For example, molecules, such as CD10/NEP (common acute lymphoblastic leukemia antigen [CALLA]) and CD13/APN have been used for years in the characterization and typing of leukemia or lymphoma cells [46]. Subsequent analyses of cloned cDNAs identified three of these differentiation antigens, as well-known membrane peptidases with common structural and regulatory features (reviewed in [42]): Aminopeptidase N (APN, CD13, EC 3.4.11.2), neutral endopeptidase (NEP, CD10, CALLA, EC 3.4.24.11, enkephalinase, neprilysin), and dipeptidyl peptidase IV (DPPIV, CD26, EC 3.4.14.5). Cell-surface peptidases are also involved in the control of cell growth and differentiation by controlling the access of peptide growth factors to their receptors on the cell membrane [47, 48 and 49] and in the final steps of collagen degradation in the ECM [50] (Table 2). Therefore, control of bioactive peptides through either activation or inactivation by cell-surface peptides is a critical component of growth control. This idea has also direct implications for the development of neoplasia. Two basic mechanisms of cell-surface peptidase involvement in carcinogenesis can be predicted [46]: (1) loss of function, resulting in an inability of the transformed cell to inactivate a mitogenic peptide or activate an inhibitory peptide; and (2) gain of function, resulting in the activation of a mitogenic peptide or the inactivation of an inhibitory peptide. Consequently, abnormalities in expression pattern and/or catalytic function of cell-surface peptidases result in altered peptide activity, which contributes to neoplastic transformation and/or progression. Data, which implicate specific cell-surface peptidases in the pathogenesis of human cancers (reviewed in [46]), including melanoma, are beginning to emerge. We will now discuss which evidence to date indicates a role for cell-surface peptidases in the development of melanoma.

7.1. Dipeptidyl peptidase IV (DPP IV, CD26, EC 3.4.14.5)

Dipeptidyl peptidase IV (DPPIV) is the best characterized cell-surface peptidase in melanoma. It is a type II membrane glycoprotein with multiple properties, including serine protease activity and the ability to bind ECM components [51, 52 and 53]. DPPIV has also been called adenosine deaminase binding protein or adenosine deaminase complexing protein [54]. Chemokines are potential substrates for DPPIV [55], including RANTES (regulated on activation, normal T-cell expressed and secreted) and monocyte chemotactic proteins (MCP) 1, -2, and -3 [56, 57 and 58] (Table 2). DPPIV expression on T cells has been designated CD26 [42 and 51]. DPPIV is also expressed on epithelia and melanocytes [42, 51 and 52]. It has long been recognized that expression of DPPIV can be downregulated or altered on cancer cells [59 and 60]. Specifically, loss or alteration of membrane expression of DPPIV has been reported in prostate [40 and 61], colorectal [62 and 63], gastric [62], lung [60], and hepatocellular [60] carcinomas and melanomas [52]. In addition, DPPIV is involved in the fibronectin (FN)-mediated adherence of metastatic breast cancer cells to lung endothelium, in which case DPPIV is expressed on the endothelial cells and FN is expressed on the cell surfaces of the malignant cells [64]. DPPIV expression during malignant transformation has been best characterized in melanocytic cells. A series of work by Houghton and colleagues has shown that DPPIV is expressed in vitro and in vivo by normal melanocytes, but not by melanoma [52 and 65]. Loss of DPPIV expression probably occurs at an early stage of melanoma progression, when melanocytes transform into melanoma cells [52]. More specifically, DPPIV is expressed by cutaneous melanocytes and common nevi, but is not detected in vivo or in vitro on cells from primary or metastatic melanomas. In an in vitro system that sequentially transformed melanocytes in defined steps, loss of DPPIV expression occurred concomitantly with the emergence of growth factor independence [65 and 66]. More recently, the re-expression of DPPIV in human melanoma cells at levels comparable with those found in normal melanocytes has been shown to produce profound phenotypic changes [67]. These included abrogation of tumorigenicity, re-emergence of requirements for exogenous growth factors to maintain cell survival, and removal of a block in cell differentiation. Using a point mutation in the active serine protease domain of DPPIV, the authors also observed that serine peptidase activity was required for most effects but not for cell survival. Re-expression of DPPIV rescued expression of a second putative surface

peptidase, fibroblast activation protein (FAP/seprase; see below) [68 and 69], suggesting that expression of this second molecule contributes to effects on cell survival in malignant cells.

7.2. Aminopeptidase N (APN, CD13, EC 3.4.11.2)

The functional role of APN varies depending on its location. Other membrane peptidases, such as NEP or DPPIV often co-localize with APN and seem to cooperate in peptide degradation [42]. Furthermore, like DPPIV [53], APN has been considered an auxiliary adhesion molecule [45].

In contrast to DPPIV, APN is not expressed by normal melanocytes, but becomes increasingly prevalent as melanocytes transform to dysplastic nevocytes and malignant melanoma cells [70 and 71]. When melanoma cells form colonies, the majority of APN molecules relocate to sites of cell–cell contact; in those cells, APN seems to be tightly associated with ECM components [70]. APN has a direct role in melanoma cell invasion and ECM degradation [50]. Betastatin, a competitive inhibitor of APN function, as well as antibodies to APN, inhibit the penetration of melanoma cells through an artificial basement membrane in vitro without affecting cell adhesion or cell growth [50 and 70]. Thus, the expression of APN is thought to play a critical role as one member of a cascade of enzymes that hydrolyse extracellular matrix proteins [50 and 70]. APN may serve as an activator of type IV collagenase and other matrix proteins by cleaving N-terminal amino acids (Fig. 3), thereby allowing the acquisition of invasive and metastatic competence [50 and 72]. Interestingly, APN was shown to be the major receptor for the transmissible gastroenteritis virus (TGEV) [73] that causes severe gastroenteritis in piglets, and for the human coronavirus 229E [74] that causes upper respiratory infections. More recent data indicate that APN plays an important role in tumor vasculogenesis, identifying it as a critical regulator of angiogenesis [75 and 76].

7.3. Neutral endopeptidase (NEP, CD10, CALLA, EC 3.4.24.11, enkephalinase, neprilysin)

Neutral endopeptidase (EC 3.4.24.11), also termed neprilysin, enkephalinase or CD10 is a 90–110 kDa zinc-dependent metallopeptidase that cleaves peptide bonds on the amino side of hydrophobic amino acids (Fig. 3). It is identical to the common acute lymphoblastic leukemia antigen (CALLA) [77]. NEP inactivates a variety of

physiologically active peptides, including neurotensin, met-enkephalin, substance P, bombesin and endothelin-1, thereby reducing local concentrations of peptides available for receptor binding and signal transduction [78 and 79] (Table 2). NEP is normally expressed by a wide range of tissues and cells [78].

NEP is also expressed by one-third to one-half of primary and metastatic melanomas and the percentage of NEP-positive cells within a given lesion appears to increase with tumor progression [80]. Thus, unlike other solid tumor malignancies, melanoma does not fit with the paradigm that NEP is lost upon tumor progression, but that gain of NEP function may be advantageous. More recently, however, it has been reported that NEP is highly expressed by human melanocytes, and that its expression and catalytic activity are downregulated by UVB light. In addition, it has been shown that α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) are specific substrates for NEP and that specific inhibition of NEP increases the melanogenic activity of these peptides on human melanocytes [81]. Among keratinocyte-derived agents, the melanotropic hormones (α-MSH) and ACTH appear to be very potent stimulators of human pigmentation. These data indicate that NEP inactivation by UVB in melanocytes may enhance the proopiomelanocortin (POMC)-derived peptides paracrine loop, mediating UV-induced pigmentation.

Until now, the biological and regulatory effects of NEP were presumed to result only from its enzymatic function [49]. However, recent data suggest that NEP may possess other biological properties in addition to its ability to catalytically inactivate neuropeptide substrates. NEP protein expression by itself can effect signal transduction pathways, which, in turn, regulate cell growth [82 and 83] and apoptosis [84].

8. Seprase/fibroblast activating protein: yet another proteolytic enzyme in malignant tumors

A subfamily of membrane-bound nonclassical serine proteases, including seprase and DPPIV, are implicated in matrix degradation and invasiveness of migratory cells [53, 85, 86 and 87]. Seprase is a homodimeric 170-kDa integral membrane gelatinase, whose expression appears to correlate with levels of invasiveness manifested by the human melanoma cell line, LOX, in an in vitro ECM degradation/invasion assay [88]. The deduced amino acid sequence of its 97-kDa subunit (seprase-l), predicts a type II membrane topology with a short cytoplasmic tail

(six amino acids) followed by a transmembrane region (20 amino acids) and a large extracellular domain (734 amino acids) [89]. Seprase requires the dimerization of its inactive subunits for activity [89 and 90]. Comparisons of their deduced amino acid sequences indicate that seprase is essentially identical to human fibroblast activation protein (FAP), which is expressed on reactive stromal fibroblasts of various carcinomas and on fibroblasts of healing wounds [91 and 92]. Additionally, seprase exhibits a striking sequence homology (52%) to DPPIV, which increases to a 68% amino acid identity between their catalytic regions [89].

Seprase is selectively expressed by fibroblastic cells in areas of active tissue remodeling, such as the embryonic mesenchyme, areas of wound healing, the gravid uterus, and the reactive stroma of epithelial cancers (over 90% of breast, colorectal and lung carcinomas) [91 and 92]. It is also expressed in vivo in subsets of bone and soft tissue sarcomas [68 and 69]. This protease is generally absent from the stroma of benign epithelial tumors and normal adult tissues [69]. In vitro, seprase induction is observed in proliferating cultured fibroblasts and in melanocytes grown with basic fibroblast growth factor and phorbol ester [69]. Seprase is a dual-specificity enzyme that acts as a dipeptidyl peptidase and collagenase in vitro [93]. Seprase (−/−) mice are fertile, show no overt developmental defects, and have no general change in cancer susceptibility [94].

9. Ephrins and eph receptors: control of cell behavior by intercellular communication

The Eph receptors are the largest family of receptor tyrosine kinases and include at least 14 structurally related members. Initially isolated as orphan receptors (lacking known ligands), at least eight Eph ligands—ephrins—have recently been reported (Fig. 4) [95 and 96]. Recent advances have started to elucidate the developmental functions and biochemistry of Eph receptor tyrosine kinases and their membrane-bound ligands, ephrins. Unlike most ligands, ephrins do not function in a soluble form but must be membrane-bound to activate their receptor(s) [95] (Fig. 5).

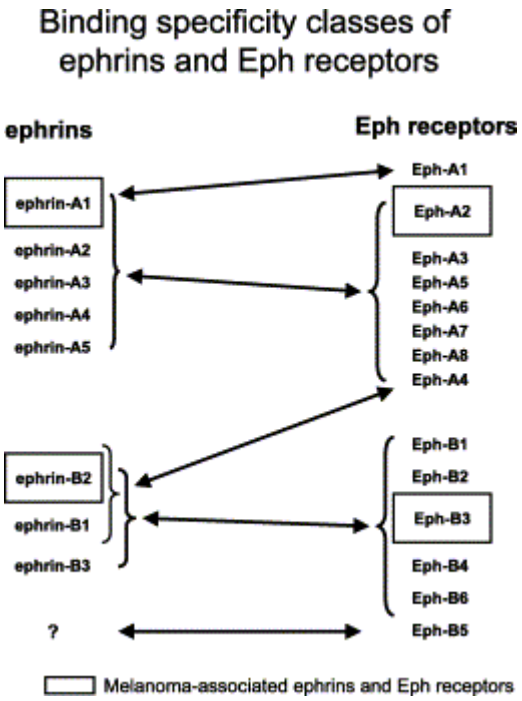

Fig. 4. Melanoma-associated ephrins and Eph receptors. The EphA class of receptors bind promiscuously with ephrin-A ligands; EphB receptors bind ephrin-B proteins. EphB5 does not bind to any known ephrin. The affinity of interactions differs between respective receptor–ligand combinations (modified from [101]).

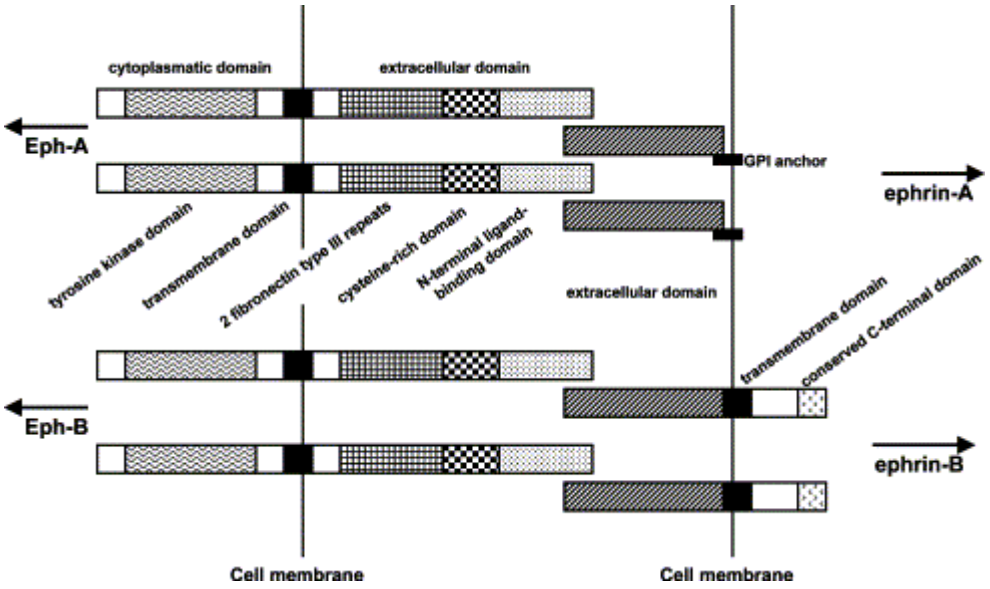

Fig. 5. Structure, interactions and signal transduction of Eph receptors and ephrins. Eph receptors share a number of features, as indicated. Ephrins have conserved residues in the extracellular domain and fall into two structural classes: proteins of the ephrin-A subclass are anchored in the plasma membrane through the covalent attachment of a glycosylphosphatidylinositol (GPI) group. Proteins of the ephrin-B subclass have a transmembrane domain and short cytoplasmatic region. Bidirectional signaling (arrows) can occur upon interaction of cells expressing Eph receptors and ephrins. Modified from [101 and 99].

Juxtacrine interactions between Eph (receptor) and ephrin (ligand) on opposing cells were initially implicated in patterning of the brain and somites, and in the process of neural cell guidance (reviewed in [97 and 98]). Eph receptor tyrosine kinases and

ephrins mediate contact-dependent cell interactions that regulate the repulsion and adhesion mechanisms involved in the guidance and assembly of cells, and thus the establishment, maintenance, and remodelling of patterns of cellular organization (reviewed in [95 and 99]). Eph receptors and ephrins can also trigger an adhesive response of endothelial cells and are required for remodelling of blood vessels (reviewed in [95 and 100]).

A number of studies have implicated Eph receptors in carcinogenesis based on their elevated expression and/or expression of aberrant transcripts in tumor cell lines [101]. Eph-B3 (Hek-2) and Eph-A2 (Eck) are ectopically expressed in over 90% of melanoma cell lines [102 and 103]. Cell lines from increasingly advanced melanomas express increasing amounts of Eph-A2 [104]. The first identified Eph ligand was ephrin-A1/B61, a ligand for Ephs including Eph-A2 [105 and 106]. Ephrin-A1 was found to stimulate proliferation of melanoma cells that overexpress Eph-A2, and therefore, proposed to be an autocrine growth factor [104]. Normal melanocytes do not respond to ephrin-A1 nor express the Eph-A2 receptor. Immunoreactive Eph-A2 was not detectable in most uncultured lesions [107]. However, the ligand ephrin-A1 is expressed by melanoma cells, both in cultured and in uncultured lesions, and correlates with progression [107].

Ephrin-B2 (Lerk-5), a ligand for Eph-B3, is also overexpressed in melanoma and correlates with tumorigenicity and metastatic potential [108]. Eph receptors and ephrins may promote angiogenesis within forming melanoma, or cell–cell repulsion and hence invasion as well as metastatic spread.

10. The ADAM family: multifunctional surface proteins with adhesion and protease activity

The ADAMs are a multifunctional gene family, some of which play a role in diverse biological processes, such as fertilization [109], myogenesis, neurogenesis [110], and the activation of growth factors/immune regulators such as TNF-α [111]. Moreover, the ADAMs have potential implications for tumor metastasis via cell adhesion and protease activities [112 and 113].

The term ‘ADAM’ stands for a disintegrin and metalloproteinase, which represent the two key structural domains in these molecules. ADAMs, which can process or remove the extracellular domains of cell-surface proteins, are critically placed for regulating signaling. These multifunctional surface proteinases are particularly

intriguing in that they contain both cell adhesion and proteolytic domains. The emerging properties of the ADAM gene family have been the subject of several recent reviews [36, 37, 110, 114 and 115]. Among the 29 known ADAM cDNAs to date, 17 have a metalloproteinase active site.

Cells have the ability to modify their surface to regulate various kinds of functions [37]. For example, the extracellular domain of >40 plasma membrane-anchored cytokines, growth factors, receptors, adhesion molecules, and enzymes can be cleaved and thereby released (shed) from the plasma membrane by various proteases (called sheddases or secretases) [116, 117 and 118]. These sheddases are themselves transmembrane proteins and, in several cases, are ADAMs. One of the best-studied cases of shedding is the release of tumor-necrosis factor α (TNF-α), a cytokine that is involved in the inflammatory response. The release of TNF-α from various cell types occurs in response to injury or infection and plays an important role in the adaptive immune response. TNF-α is synthesized as a 26 kDa membrane-anchored protein from which an active soluble 17 kDa extracellular domain is proteolytically released. This proteolytic release is catalyzed by TNF-α converting enzyme (TACE, or ADAM 17) [116 and 118].

Kuzbanian (ADAM 19) is also a sheddase that has been found to release a soluble form of delta, Notch ligand [119 and 120]. Notch is a surface receptor that regulates cell fate determination in various aspects of development, such as neurogenesis [110 and 117]. Metalloprotease disintegrin cysteine-rich 9 (MDC9, or ADAM 9) has been reported to shed the heparin-binding EGF-like receptor [113]. Both the membrane-anchored and soluble (shed) form of this growth factor are active, but the soluble, diffusible form can act on cells distant from the site of its release. ADAM 17/TACE, ADAM 10/Kuzbanian and ADAM 9/MDC9 are three ADAM metalloproteases for which a function has been reported and that act as sheddases (reviewed in [110]). The other 14 predicted proteases remain ‘orphan proteases’, lacking an identified endogenous substrate [37].

Potentially, cell-surface proteases are also involved in cleavage of growth factors such as TGF-α that are membrane-bound or enzymes and receptors that require activation. At present, no studies have yet comprehensively examined the expression or regulation of ADAMs in melanoma or, for that matter, most solid tumors. However, we anticipate that our understanding of the interplay at the cellular level between melanoma and stromal cells as well as the molecular processes underlying the

progression from RGP to VGP melanoma will improve dramatically by continued study of these proteins.

11. Summary and perspective

Our understanding of ECM proteolysis and cell-surface molecules in the progression of melanoma has expanded dramatically in recent years [121]. It is clear that the stroma is an integral part of the tumor and that it contributes to some of the most destructive characteristics of malignant cells [10, 38 and 122]. There is now voluminous evidence that melanoma cells are influenced by the surrounding microenvironment and vice versa [15]. Numerous studies support the concept that melanomagenesis is a multicellular process, in which destruction of the microenvironment is required for the conversion of normal melanocytes to aggressive melanoma cells with the potential for invasion and metastasis.

Understanding the molecular mechanisms by which membrane-bound proteinases are regulated and activated, the nature of their molecular and cellular targets, and how adhesion and proteolysis are integrated will provide exciting new areas for investigation over the coming years and could ultimately lead to novel therapeutical strategies for this aggressive neoplasm. The emerging appreciation of controlled, specific endoproteolytic cleavage of cell-surface receptors to modulate receptor activities and initiate novel signaling pathways also illustrates the complexity of the control mechanisms inherent in the processes of vasculogenesis and angiogenesis [39, 95 and 110]. The molecular mechanisms involved in the complex cross-talk between normal melanocytes as well as melanoma cells and their microenvironment hold great promise as targets for melanoma therapy.

Dissecting the molecular components of melanoma–stroma interactions requires model systems, in which a single variable can be manipulated and assessed. More recently, such powerful models have been emerging, e.g. melanoma in human skin reconstructs or orthotopic in human skin grafted to mice [123]; these will allow us to examine more accurately the pathways and events on the cell-surface and the pericellular space, which impinge on the microenvironment and drive the progression of melanoma to a fatal metastatic neoplasia.

12. Outstanding questions

What features of the microenvironment exactly promote melanoma? Are these melanoma specific?

What is the precise role of cell-surface peptidases and membrane-bound enzymes in regulating the pericellular microenvironment and what are their substrates?

Can changes in the expression of these proteolytic enzymes be used as clinico-pathologic markers for the diagnosis and prognosis of melanoma?

Can the microenvironment be targeted therapeutically to prevent invasive melanoma?

Can manipulating the expression of proteolytic enzymes reverse invasive or metastatic melanoma?

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References

1. A. Kamb and M. Herlyn , Malignant melanoma. In: C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle, Editors, *The Metabolc and Molecular Basis of Inherited Disease* (eighth ed.),, McGraw-Hill, New York (2001), pp. 967–977.

2. M. Herlyn and K. Satyamoorthy , Molecular biology of cutaneous melanoma. In: V.T. DeVita, Jr, S. Hellman and S.A. Rosenberg, Editors, *Cancer: Principles and Practice of Oncology* (sixth ed.),, Lippincott, Williams & Wilkins, Philadelphia (2001), pp. 2003–2012.

3. C.J. Sherr , The Pezcoller lecture: cancer cell cycles revisited. *Cancer Res.* 60 (2000), pp. 3689–3695.

4. M. Piepkorn , Melanoma genetics: an update with focus on the CDKN2A(p16)/ARF tumor suppressors. *J. Am. Acad. Dermatol.* 42 (2000), pp. 705–722.

5. C.C. Park, M.J. Bissell and M.H. Barcellos-Hoff , The influence of the microenvironment on the malignant phenotype. *Mol. Med. Today* 6 (2000), pp. 324–329.

6. A.P. Albino, M.J. Vidal, N.S. McNutt *et al.*, Mutation and expression of the p53 gene in human malignant melanoma. *Melanoma Res.* 4 (1994), pp. 35–45.

7. M. Castellano and G. Parmiani , Genes involved in melanoma: an overview of INK4a and other loci. *Melanoma Res.* 9 (1999), pp. 421–432.

8. A.J. Barret, N.D. Rawlngs and F.F. Woessner *Handbook of Proteolytic Enzymes*, Academic Press, San Diego (1998).

9. D. Hanahan and R.A. Weinberg , The hallmarks of cancer. *Cell* 100 (2000), pp. 57–70.

10. Z. Werb , ECM and cell surface proteolysis: regulating cellular ecology. *Cell* 91 (1997), pp. 439–442.

11. I.T. Valyi-Nagy, G. Hirka, P.J. Jensen, I.M. Shih, I. Juhasz and M. Herlyn , Undifferentiated keratinocytes control growth, morphology, and antigen expression of normal melanocytes through cell–cell contact. *Lab. Invest.* 69 (1993), pp. 152–159.

12. I.M. Shih, D.E. Elder, M.Y. Hsu and M. Herlyn , Regulation of Mel-CAM/MUC18 expression on melanocytes of different stages of tumor progression by normal keratinocytes. *Am. J. Pathol.* 145 (1994), pp. 837–845.

13. M.Y. Hsu, M.J. Wheelock, K.R. Johnson and M. Herlyn , Shifts in cadherin profiles between human normal melanocytes and melanomas. *J. Invest. Dermatol. Symp. Proc.* 1 (1996), pp. 188–194.

14. M.Y. Hsu, F.E. Meier, M. Nesbit *et al.*, E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. *Am. J. Pathol.* 156 (2000), pp. 1515–1525.

15. F. Meier, K. Satyamoorthy, M. Nesbit *et al.*, Molecular events in melanoma development and progression. *Front. Biosci.* 3 (1998), pp. D1005–1010.

16. M.Y. Hsu, D.T. Shih, F.E. Meier *et al.*, Adenoviral gene transfer of beta3 integrin subunit induces conversion from radial to vertical growth phase in primary human melanoma. *Am. J. Pathol.* 153 (1998), pp. 1435–1442.

17. F. Meier, M. Nesbit, M.Y. Hsu *et al.*, Human melanoma progression in skin reconstructs: biological significance of bFGF. *Am. J. Pathol.* 156 (2000), pp. 193–200.

18. I.M. Shih, D. Speicher, M.Y. Hsu, E. Levine and M. Herlyn , Melanoma cell–cell interactions are mediated through heterophilic Mel-CAM/ligand adhesion. *Cancer Res.* 57 (1997), pp. 3835–3840.

19. W.H. Clark, D.E. Elder, D. Guerry, M.N. Epstein, M.H. Greene and M. Van Horn , A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. *Hum. Pathol.* 15 (1984), pp. 1147–1165.

20. G.B. Balaban, M. Herlyn, W.H. Clark and P.C. Nowell , Karyotypic evolution in human malignant melanoma. *Cancer Genet. Cytogenet.* 19 (1986), pp. 113–122.

21. G. Balaban, M. Herlyn, D. Guerry *et al.*, Cytogenetics of human malignant melanoma and premalignant lesions. *Cancer Genet. Cytogenet.* 11 (1984), pp. 429–439.

22. M.L. Mancianti, T. Gyorfi, I.M. Shih *et al.*, Growth regulation of cultured human nevus cells. *J. Invest. Dermatol.* 100 (1993), pp. 281S–287S.

23. I.T. Valyi-Nagy, G.F. Murphy, M.L. Mancianti, D. Whitaker and M. Herlyn , Phenotypes and interactions of human melanocytes and keratinocytes in an epidermal reconstruction model. *Lab. Invest.* 62 (1990), pp. 314–324.

24. K. Satyamoorthy, E. DeJesus, A.J. Linnenbach *et al.*, Melanoma cell lines from different stages of progression and their biological and molecular analyses. *Melanoma Res.* 7 Suppl 2 (1997), pp. S35–S42.

25. D. Guerry, M. Synnestvedt, D.E. Elder and D. Schultz , Lessons from tumor progression: the invasive radial growth phase of melanoma is common, incapable of metastasis, and indolent. *J. Invest. Dermatol.* 100 (1993), pp. 342S–345S.

26. R. Kath, U. Rodeck, H.D. Menssen *et al.*, Tumor progression in the human melanocytic system. *Anticancer Res.* 9 (1989), pp. 865–872.

27. R. Kath, J.A. Jambrosic, L. Holland, U. Rodeck and M. Herlyn , Development of invasive and growth factor-independent cell variants from primary human melanomas. *Cancer Res.* 51 (1991), pp. 2205–2211.

28. R.A. Weinberg , Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. *Cancer Res.* 49 (1989), pp. 3713–3721.

29. K.W. Kinzler and B. Vogelstein , Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* 386 (1997), pp. 761–763.

30. K.W. Kinzler and B. Vogelstein , Landscaping the cancer terrain. *Science* 280 (1998), pp. 1036–1037.

31. K. Macleod , Tumor suppressor genes. *Curr. Opin. Genet. Dev.* 10 (2000), pp. 81–93.

32. N.J. Boudreau and P.L. Jones , Extracellular matrix and integrin signalling: the shape of things to come. *Biochem. J.* 339 (1999), pp. 481–488.

33. S.M. Albelda, S.A. Mette, D.E. Elder *et al.*, Integrin distribution in malignant melanoma: association of the beta 3 subunit with tumor progression. *Cancer Res.* 50 (1990), pp. 6757–6764.

34. T.J. Hieken, M. Farolan, S.G. Ronan, A. Shilkaitis, L. Wild and T.K. Das Gupta , Beta3 integrin expression in melanoma predicts subsequent metastasis. *J. Surg. Res.* 63 (1996), pp. 169–173.

35. C.B. Basbaum and Z. Werb , Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. *Curr. Opin. Cell. Biol.* 8 (1996), pp. 731–738.

36. G.P. Kaushal and S.V. Shah , The new kids on the block: ADAMTS, potentially multifunctional metalloproteinases of the ADAM family. *J. Clin. Invest.* 105 (2000), pp. 1335–1337.

37. P. Primakoff and D.G. Myles , The ADAM gene family: surface proteins with adhesion and protease activity. *Trends Genet.* 16 (2000), pp. 83–87.

38. G. Bergers and L.M. Coussens , Extrinsic regulators of epithelial tumor progression: metalloproteinases. *Curr. Opin. Genet. Dev.* 10 (2000), pp. 120–127.

39. W.G. Stetler-Stevenson , Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J. Clin. Invest.* 103 (1999), pp. 1237–1241.

40. T. Bogenrieder, C.L. Finstad, R.H. Freeman *et al.*, Expression and localization of aminopeptidase A, aminopeptidase N, and dipeptidyl peptidase IV in benign and malignant human prostate tissue. *Prostate* 33 (1997), pp. 225–232.

41. A.J. Kenny, S.L. Stevenson and A.J. Turner , Cell surface peptidases. In: A.J. Kenny and A.J. Turner, Editors, *Mammalian Ectoenzymes*, Elsevier, Amsterdam (1987), pp. 169–210.

42. M.A. Shipp and A.T. Look , Hematopoietic differentiation antigens that are membrane-associated enzymes: cutting is the key!. *Blood* 82 (1993), pp. 1052–1070.

43. R. Matsas, M. Rattray, A.J. Kenny and A.J. Turner , The metabolism of neuropeptides. Endopeptidase-24.11 in human synaptic membrane preparations hydrolyses substance P. *Biochem. J.* 228 (1985), pp. 487–492.

44. R. Matsas, A.J. Kenny and A.J. Turner , The metabolism of neuropeptides. The hydrolysis of peptides, including enkephalins, tachykinins and their analogues, by endopeptidase-24.11. *Biochem. J.* 223 (1984), pp. 433–440.

45. D. Riemann, A. Kehlen and J. Langner , CD13—not just a marker in leukemia typing. *Immunol. Today* 20 (1999), pp. 83–88.

46. D.M. Nanus, C.N. Papandreou and A.P. Albino , Expression of cell-surface peptidases in neoplastic cells. In: A.J. Kenny and C.M. Boustead, Editors, *Cell-Surface Peptidases in Health and Disease*, Bios Scientific Publishers, Oxford (1997), pp. 353–369.

47. M.A. Shipp, G.E. Tarr, C.Y. Chen *et al.*, CD10/neutral endopeptidase 24.11 hydrolyzes bombesin-like peptides and regulates the growth of small cell carcinomas of the lung. *Proc. Natl. Acad. Sci. USA* 88 (1991), pp. 10662–10666.

48. C.N. Papandreou, B. Usmani, Y. Geng *et al.*, Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. *Nat. Med.* 4 (1998), pp. 50–57.

49. M. Sumitomo, R. Shen, M. Walburg *et al.*, Neutral endopeptidase inhibits prostate cancer cell migration by blocking focal adhesion kinase signaling. *J. Clin. Invest.* 106 (2000), pp. 1399–1407.

50. I. Saiki, H. Fujii, J. Yoneda *et al.*, Role of aminopeptidase N (CD13) in tumor-cell invasion and extracellular matrix degradation. *Int. J. Cancer* 54 (1993), pp. 137–143.

51. C. Morimoto and S.F. Schlossman , The structure and function of CD26 in the T-cell immune response. *Immunol. Rev.* 161 (1998), pp. 55–70.

52. A.N. Houghton, A.P. Albino, C. Cordon-Cardo, L.J. Davis and M. Eisinger , Cell surface antigens of human melanocytes and melanoma. Expression of adenosine deaminase binding protein is extinguished with melanocyte transformation. *J. Exp. Med.* 167 (1988), pp. 197–212.

53. I. De Meester, S. Korom, J. Van Damme and S. Scharpe , CD26, let it cut or cut it down. *Immunol. Today* 20 (1999), pp. 367–375.

54. W.P. Schrader, C.A. West, A.D. Miczek and E.K. Norton , Characterization of the adenosine deaminase-adenosine deaminase complexing protein binding reaction. *J. Biol. Chem.* 265 (1990), pp. 19312–19318.

55. J. Van Damme, S. Struyf, A. Wuyts *et al.*, The role of CD26/DPP IV in chemokine processing. *Chem. Immunol.* 72 (1999), pp. 42–56.

56. T. Oravecz, M. Pall, G. Roderiquez *et al.*, Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage. *J. Exp. Med.* 186 (1997), pp. 1865–1872.

57. E. Van Coillie, P. Proost, I. Van Aelst *et al.*, Functional comparison of two human monocyte chemotactic protein-2 isoforms, role of the amino-terminal pyroglutamic acid and processing by CD26/dipeptidyl peptidase IV. *Biochemistry* 37 (1998), pp. 12672–12680.

58. P. Proost, I. De Meester, D. Schols *et al.*, Amino-terminal truncation of chemokines by CD26/dipeptidyl-peptidase IV. Conversion of RANTES into a potent inhibitor of monocyte chemotaxis and HIV-1-infection. *J. Biol. Chem.* 273 (1998), pp. 7222–7227.

59. E. Herbschleb-Voogt, J. Ten Kate and P. Meera Khan , Adenosine deaminase complexing protein (ADCP): a transformation sensitive protein with potentials of a cancer marker. *Anticancer Res.* 3 (1983), pp. 95–100.

60. J. Ten Kate, W.N. Dinjens, P. Meera Khan and F.T. Bosman , Adenosine deaminase complexing protein in cancer studies. *Anticancer Res.* 6 (1986), pp. 983–988.

61. W.N. Dinjens, J. Ten Kate, J.A. Kirch *et al.*, Adenosine deaminase complexing protein (ADCP) expression and metastatic potential in prostatic adenocarcinomas. *J. Pathol.* 160 (1990), pp. 195–201.

62. J. Sakamoto, T. Watanabe, S. Teramukai *et al.*, Distribution of adenosine deaminase binding protein in normal and malignant tissues of the gastrointestinal tract studied by monoclonal antibodies. *J. Surg. Oncol.* 52 (1993), pp. 124–134.

63. J. Ten Kate, H.F. van den Ingh, P.M. Khan and F.T. Bosman , Adenosine deaminase complexing protein (ADCP) immunoreactivity in colorectal adenocarcinoma. *Int. J. Cancer* 37 (1986), pp. 479–485.

64. H.C. Cheng, M. Abdel-Ghany, R.C. Elble and B.U. Pauli , Lung endothelial dipeptidyl peptidase IV promotes adhesion and metastasis of rat breast cancer cells via tumor cell surface-associated fibronectin. *J. Biol. Chem.* 273 (1998), pp. 24207–24215.

65. M.E. Morrison, S. Vijayasaradhi, D. Engelstein, A.P. Albino and A.N. Houghton , A marker for neoplastic progression of human melanocytes is a cell surface ectopeptidase. *J. Exp. Med.* 177 (1993), pp. 1135–1143.

66. A.P. Albino, G. Sozzi, D.M. Nanus, S.C. Jhanwar and A.N. Houghton , Malignant transformation of human melanocytes: induction of a complete melanoma phenotype and genotype. *Oncogene* 7 (1992), pp. 2315–2321.

67. U.V. Wesley, A.P. Albino, S. Tiwari and A.N. Houghton , A role for dipeptidyl peptidase IV in suppressing the malignant phenotype of melanocytic cells. *J. Exp. Med.* 190 (1999), pp. 311–322.

68. W.J. Rettig, S.L. Su, S.R. Fortunato *et al.*, Fibroblast activation protein: purification, epitope mapping and induction by growth factors. *Int. J. Cancer* 58 (1994), pp. 385–392.

69. W.J. Rettig, P. Garin-Chesa, J.H. Healey *et al.*, Regulation and heteromeric structure of the fibroblast activation protein in normal and transformed cells of mesenchymal and neuroectodermal origin. *Cancer Res.* 53 (1993), pp. 3327–3335.

70. A. Menrad, D. Speicher, J. Wacker and M. Herlyn , Biochemical and functional characterization of aminopeptidase N expressed by human melanoma cells. *Cancer Res.* 53 (1993), pp. 1450–1455.

71. D.E. Elder, U. Rodeck, J. Thurin *et al.*, Antigenic profile of tumor progression stages in human melanocytic nevi and melanomas. *Cancer Res.* 49 (1989), pp. 5091–5096.

72. H. Fujii, M. Nakajima, I. Saiki, J. Yoneda, I. Azuma and T. Tsuruo , Human melanoma invasion and metastasis enhancement by high expression of aminopeptidase N/CD13. *Clin. Exp. Metastasis* 13 (1995), pp. 337–344.

73. B. Delmas, J. Gelfi, R. L'Haridon *et al.*, Aminopeptidase N is a major receptor for the entero-pathogenic coronavirus TGEV. *Nature* 357 (1992), pp. 417–420.

74. C.L. Yeager, R.A. Ashmun, R.K. Williams *et al.*, Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* 357 (1992), pp. 420–422.

75. S.V. Bhagwat, J. Lahdenranta, R. Giordano, W. Arap, R. Pasqualini and L.H. Shapiro , CD13/APN is activated by angiogenic signals and is essential for capillary tube formation. *Blood* 97 (2001), pp. 652–659.

76. R. Pasqualini, E. Koivunen, R. Kain *et al.*, Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Res.* 60 (2000), pp. 722–727.

77. M.A. Shipp, J. Vijayaraghavan, E.V. Schmidt *et al.*, Common acute lymphoblastic leukemia antigen (CALLA) is active neutral endopeptidase 24.11 (‘enkephalinase’): direct evidence by cDNA transfection analysis. *Proc. Natl. Acad. Sci. USA* 86 (1989), pp. 297–301.

78. J. Kenny , Endopeptidase-24.11: putative substrates and possible roles. *Biochem. Soc. Trans.* 21 (1993), pp. 663–668.

79. B.P. Roques , Zinc metallopeptidases: active site structure and design of selective and mixed inhibitors: new approaches in the search for analgesics and anti-hypertensives. *Biochem. Soc. Trans.* 21 (1993), pp. 678–685.

80. S. Carrel, L. Zografos, M. Schreyer and D. Rimoldi , Expression of CALLA/CD10 on human melanoma cells. *Melanoma Res.* 3 (1993), pp. 319–323.

81. E. Aberdam, P. Auberger, J.P. Ortonne and R. Ballotti , Neprilysin, a novel target for ultraviolet B regulation of melanogenesis via melanocortins. *J. Invest. Dermatol.* 115 (2000), pp. 381–387.

82. P. Angelisova, K. Drbal, V. Horejsi and J. Cerny , Association of CD10/neutral endopeptidase 24.11 with membrane microdomains rich in glycosylphosphatidylinositol-anchored proteins and Lyn kinase. *Blood* 93 (1999), pp. 1437–1439.

83. R.K. Ganju, R.G. Shpektor, D.G. Brenner and M.A. Shipp , CD10/neutral endopeptidase 24.11 is phosphorylated by casein kinase II and coassociates with other phosphoproteins including the lyn src-related kinase. *Blood* 88 (1996), pp. 4159–4165.

84. G. Cutrona, N. Leanza, M. Ulivi *et al.*, Expression of CD10 by human T cells that undergo apoptosis both in vitro and in vivo. *Blood* 94 (1999), pp. 3067–3076.

85. T. Kelly, S. Kechelava, T.L. Rozypal, K.W. West and S. Korourian , Seprase, a membrane-bound protease, is overexpressed by invasive ductal carcinoma cells of human breast cancers. *Mod. Pathol.* 11 (1998), pp. 855–863.

86. T. Kelly, S.C. Mueller, Y. Yeh and W.T. Chen , Invadopodia promote proteolysis of a wide variety of extracellular matrix proteins. *J. Cell. Physiol.* 158 (1994), pp. 299–308.

87. A. Aoyama and W.T. Chen , A 170-kDa membrane-bound protease is associated with the expression of invasiveness by human malignant melanoma cells. *Proc. Natl. Acad. Sci. USA* 87 (1990), pp. 8296–8300.

88. W.L. Monsky, C.Y. Lin, A. Aoyama *et al.*, A potential marker protease of invasiveness, seprase, is localized on invadopodia of human malignant melanoma cells. *Cancer Res.* 54 (1994), pp. 5702–5710.

89. L.A. Goldstein, G. Ghersi, M.L. Pineiro-Sanchez *et al.*, Molecular cloning of seprase: a serine integral membrane protease from human melanoma. *Biochim. Biophys. Acta* 1361 (1997), pp. 11–19.

90. M.L. Pineiro-Sanchez, L.A. Goldstein, J. Dodt, L. Howard, Y. Yeh and W.T. Chen , Identification of the 170-kDa melanoma membrane-bound gelatinase (seprase) as a serine integral membrane protease. *J. Biol. Chem.* 272 (1997), pp. 7595–7601.

91. P. Garin-Chesa, L.J. Old and W.J. Rettig , Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. *Proc. Natl. Acad. Sci. USA* 87 (1990), pp. 7235–7239.

92. M.J. Scanlan, B.K. Raj, B. Calvo *et al.*, Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. *Proc. Natl. Acad. Sci. USA* 91 (1994), pp. 5657–5661.

93. J.E. Park, M.C. Lenter, R.N. Zimmermann, P. Garin-Chesa, L.J. Old and W.J. Rettig , Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J. Biol. Chem.* 274 (1999), pp. 36505–36512.

94. J. Niedermeyer, M. Kriz, F. Hilberg *et al.*, Targeted disruption of mouse fibroblast activation protein. *Mol. Cell. Biol.* 20 (2000), pp. 1089–1094.

95. N. Ilan and J.A. Madri , New paradigms of signaling in the vasculature: ephrins and metalloproteases. *Curr. Opin. Biotechnol.* 10 (1999), pp. 536–540.

96. N. Holder and R. Klein , Eph receptors and ephrins: effectors of morphogenesis. *Development* 126 (1999), pp. 2033–2044.

97. D.D. O'Leary and D.G. Wilkinson , Eph receptors and ephrins in neural development. *Curr. Opin. Neurobiol.* 9 (1999), pp. 65–73.

98. J.G. Flanagan and P. Vanderhaeghen , The ephrins and Eph receptors in neural development. *Annu. Rev. Neurosci.* 21 (1998), pp. 309–345.

99. G. Mellitzer, Q. Xu and D.G. Wilkinson , Control of cell behaviour by signalling through Eph receptors and ephrins. *Curr. Opin. Neurobiol.* 10 (2000), pp. 400–408.

100. N.W. Gale and G.D. Yancopoulos , Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes. Dev.* 13 (1999), pp. 1055–1066.

101. D.G. Wilkinson , Eph receptors and ephrins: regulators of guidance and assembly. *Int. Rev. Cytol.* 196 (2000), pp. 177–244.

102. D.J. Easty, S.E. Ganz, C.J. Farr, C. Lai, M. Herlyn and D.C. Bennett , Novel and known protein tyrosine kinases and their abnormal expression in human melanoma. *J. Invest. Dermatol.* 101 (1993), pp. 679–684.

103. D.J. Easty, M. Herlyn and D.C. Bennett , Abnormal protein tyrosine kinase gene expression during melanoma progression and metastasis. *Int. J. Cancer* 60 (1995), pp. 129–136.

104. D.J. Easty, B.A. Guthrie, K. Maung *et al.*, Protein B61 as a new growth factor: expression of B61 and up-regulation of its receptor epithelial cell kinase during melanoma progression. *Cancer Res.* 55 (1995), pp. 2528–2532.

105. T.D. Bartley, R.W. Hunt, A.A. Welcher *et al.*, B61 is a ligand for the ECK receptor protein-tyrosine kinase. *Nature* 368 (1994), pp. 558–560.

106. E. Magal, J.A. Holash, R.J. Toso, D. Chang, R.A. Lindberg and E.B. Pasquale , B61, a ligand for the Eck receptor protein-tyrosine kinase, exhibits neurotrophic activity in cultures of rat spinal cord neurons. *J. Neurosci. Res.* 43 (1996), pp. 735–744.

107. D.J. Easty, S.P. Hill, M.Y. Hsu *et al.*, Up-regulation of ephrin-A1 during melanoma progression. *Int. J. Cancer* 84 (1999), pp. 494–501.

108. T. Vogt, W. Stolz, J. Welsh *et al.*, Overexpression of Lerk-5/Eplg5 messenger RNA: a novel marker for increased tumorigenicity and metastatic potential in human malignant melanomas. *Clin. Cancer Res.* 4 (1998), pp. 791–797.

109. C.P. Blobel , Functional processing of fertilin: evidence for a critical role of proteolysis in sperm maturation and activation. *Rev. Reprod.* 5 (2000), pp. 75–83.

110. J. Schlondorff and C.P. Blobel , Metalloprotease-disintegrins: modular proteins capable of promoting cell–cell interactions and triggering signals by protein-ectodomain shedding. *J. Cell. Sci.* 112 (1999), pp. 3603–3617.

111. C.P. Blobel , Metalloprotease-disintegrins: links to cell adhesion and cleavage of TNF alpha and Notch. *Cell* 90 (1997), pp. 589–592.

112. K. Iba, R. Albrechtsen, B.J. Gilpin, F. Loechel and U.M. Wewer , Cysteine-rich domain of human ADAM 12 (meltrin alpha) supports tumor cell adhesion. *Am. J. Pathol.* 154 (1999), pp. 1489–1501.

113. Y. Izumi, M. Hirata, H. Hasuwa *et al.*, A metalloprotease-disintegrin, MDC9/meltrin-gamma/ADAM9 and PKCdelta are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. *EMBO J.* 17 (1998), pp. 7260–7272.

114. C.P. Blobel , Remarkable roles of proteolysis on and beyond the cell surface. *Curr. Opin. Cell. Biol.* 12 (2000), pp. 606–612.

115. R.A. Black and J.M. White , ADAMs: focus on the protease domain. *Curr. Opin. Cell. Biol.* 10 (1998), pp. 654–659.

116. R.A. Black, C.T. Rauch, C.J. Kozlosky *et al.*, A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 385 (1997), pp. 729–733.

117. A.J. Turner and N.M. Hooper , Role for ADAM-family proteinases as membrane protein secretases. *Biochem. Soc. Trans.* 27 (1999), pp. 255–259.

118. N.M. Hooper, E.H. Karran and A.J. Turner , Membrane protein secretases. *Biochem. J.* 321 (1997), pp. 265–279.

119. D. Pan and G.M. Rubin , Kuzbanian controls proteolytic processing of notch and mediates lateral inhibition during drosophila and vertebrate neurogenesis. *Cell* 90 (1997), pp. 271–280.

120. H. Qi, M.D. Rand, X. Wu *et al.*, Processing of the notch ligand delta by the metalloprotease kuzbanian. *Science* 283 (1999), pp. 91–94.

121. U.B. Hofmann, J.R. Westphal, G.N. Van Muijen and D.J. Ruiter , Matrix metalloproteinases in human melanoma. *J. Invest. Dermatol.* 115 (2000), pp. 337–344.

122. N. Boudreau and M.J. Bissell , Extracellular matrix signaling: integration of form and function in normal and malignant cells. *Curr. Opin. Cell. Biol.* 10 (1998), pp. 640–646.

123. K. Satyamoorthy, F. Meier, M.Y. Hsu, C. Berking and M. Herlyn , Human xenografts, human skin and skin reconstructs for studies in melanoma development and progression. *Cancer Metastasis. Rev.* 18 (1999), pp. 401–405.

Melanoma–stroma interactions: structural and functional aspects

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4

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Abstract

Cutaneous melanomas are notorious for their tendency to metastasise. Because the tumour microenvironment plays an important part in tumour development and progression, we review the structural and functional aspects of interactions between melanoma and the stroma. We emphasise fibrovascular patterns (both in uveal and cutaneous melanoma), cellular and extracellular composition of the stroma, and the molecules involved. Also, we discuss functional interactions, focusing on melanoma-fibroblast cross-talk by soluble factors and by direct cell-cell contact. On the basis of recent findings we propose that involvement of fibroblasts in melanoma-stromagenesis occurs through different stages: recruitment, activation, and conversion to myofibroblasts, or differentiation to fibrocytes. We reason that this involvement is topographically linked to different areas in and around the tumour, and hypothesise that stromal activation, as seen in tumor ulceration or immunological regression in melanoma, stimulates tumour progression.

Introduction

Solid tumours consist of a mixture of neoplastic and non-neoplastic cells, in variable proportions. A solid tumour should therefore be regarded as an intricate, yet poorly organised tissue, the function of which is maintained by a dynamic interplay between neoplastic and host cells[1, 2 and 3]. Tumour progression, including metastasis, is only possible through close interaction of both cell types. The overall architecture of a malignant solid tumour is generally a compact mass, often with a necrotic centre, and a peripheral vital part (Figure 1). It contains a complex mixture of neoplastic cells and tumour stroma, distributed in irregular strands or septa. Strands of stroma run into the outer border of the tumour, which is referred to as the invasive front (Figure 2)[4]. Directly surrounding this border is a poorly defined area in which reactive changes such as oedema and inflammation occur. We call this the 'peritumoral zone', to distinguish it from the adjacent uninvolved tissue. The tumour stroma consists of microvessels, supporting connective tissue, and infiltrating inflammatory cells (Table 1). The assumed functions of the tumour stroma involve physical support and transportation of fluids and cells. Although some researchers[3] equate the tumour stroma with the tumour microenvironment, we suggest that these terms should have different definitions. The term 'tumour stroma' implies topographical and structural aspects and is restricted to non-neoplastic cells. In contrast, 'tumour microenvironment' implies the total functional and structural constellation of neoplastic and non-neoplastic cells and extracellular components, with emphasis on their functional interactions. Therefore, the microenvironment not only includes the structural components of the tumour stroma (Table 1), but also cytokines, chemokines, and growth factors[2] that may be derived from either the neoplastic or the non-neoplastic cells. The powerful pathophysiological effects caused by these molecules make the tumour microenvironment an important focus for research, both in the laboratory and in the clinic.

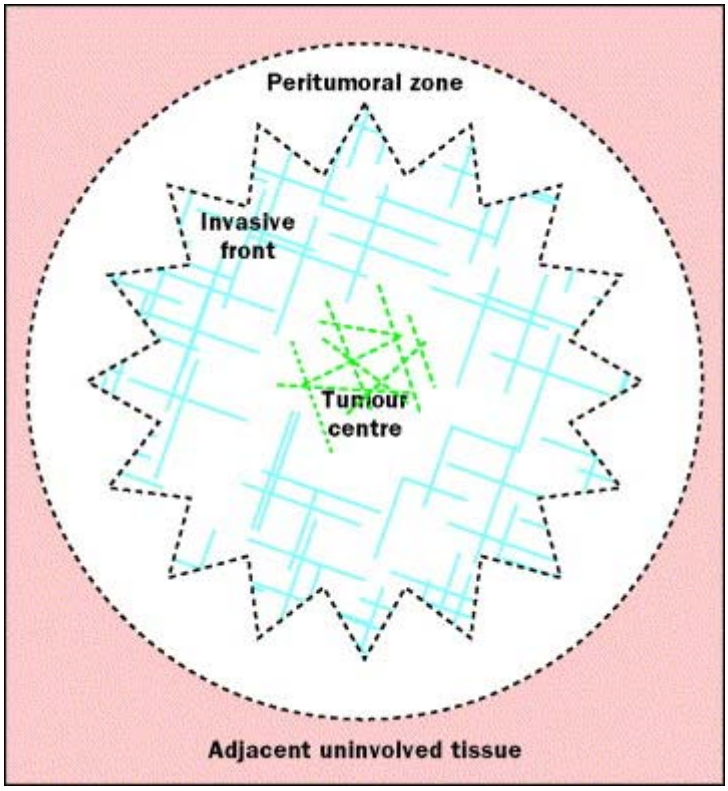

Figure 1. Overall architecture of a solid malignant tumour, shown as a star shaped mass. There is often an area of necrosis in the tumour centre. The remaining vital tumour tissue consists of a complex mixture of neoplastic cells and tumour stroma (blue crosses), which reaches the invasive front (blue contour of star) of the tumour. A poorly defined peritumoral zone is shown between the tumour and the adjacent uninvolved tissue.

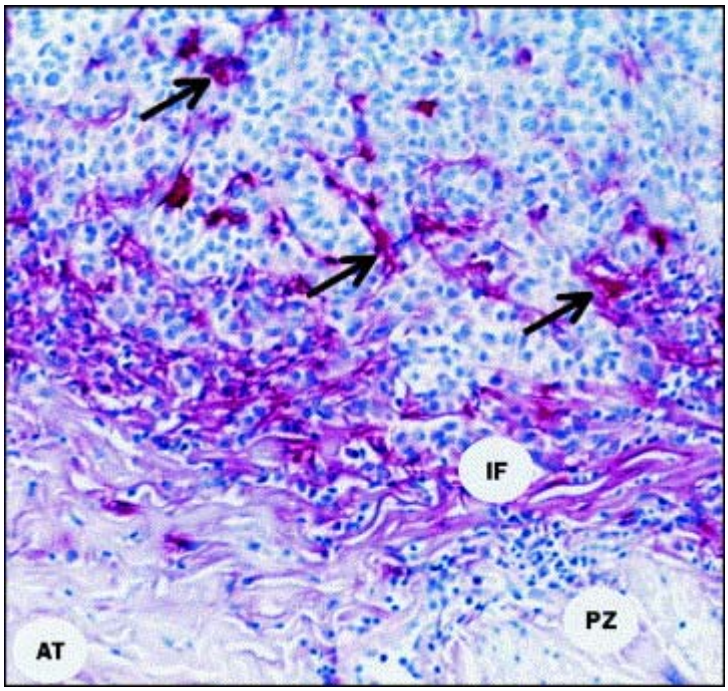

Figure 2. Primary cutaneous melanoma immunohistochemically stained for the receptor of urokinase plasminogen activation components, showing immunoreactivity of the tumour stroma (arrows), including the invasive front (IF). PZ, peritumoral zone; AT, adjacent uninvolved tissue (x 100, with haematoxylin counterstaining). Adapted from reference 68.

Table 1. Composition of tumour stroma

Microvessels	Fibroblast-like cells
Blood vessels*	Fibroblasts
Endothelial cells	Myofibroblasts
Pericytes	Fibrocytes
Basal lamina: laminins,collagen IV, proteoglycans	Smooth-muscle cells
Inflammatory cells	**Extracellular matrix**
Macrophages	Fibrin
Lymphocytes	Fibronectin
Neutrophils	Collagens I, III, VI , XV, XVIII
Eosinophils	Laminin 7
Mast cells	Tenascin
Dendritic cells	Matrix proteoglycans
	Hyaluronan
	Osteopontin

*Lymph vessels may also be present.

Cutaneous melanoma, the incidence of which is rising, is a highly malignant tumour type and is notorious for its tendency to give rise to metastases[5]. Extensive research has revealed that growth factors, adhesion molecules, proteases, and other related components, play an important part in the pathogenesis of melanoma metastasis[6, 7 and 8]. Although these molecular studies originally focused on melanoma cells, it has become increasingly evident that the stromal cells are also involved in key metastatic processes, including proliferation, matrix degradation, and migration[4 and 9]. Morphological features of the stroma in melanocytic lesion[10] and image analyses have been described[11]. Additional studies have helped to identify growth factors that affect the stromc[12] and how these factors are distributed in cutaneous melanoma[13]. Fibroblast activating protein (FAP), a factor associated with fibroblast activation in melanoma and other tumours, has also been identifie[14]. Several studies have investigated angiogenesis in cutaneous melanoma.[15, 16 and 17] In uveal melanoma, a relatively rare tumour, the structure and function of the tumour stroma has led to intriguing observations[18, 19 and 20] that may provide greater insights into the stroma's role in other tumours. Consideration of tumour stromal properties in general, together with specific findings in melanoma, raises important questions. Which cell types play a dominant role in melanoma-stroma interactions? Which molecules are involved in these interactions? How are these molecules regulated? How do melanoma-stroma interactions influence the biological behaviour of the tumour? And, finally, what are the pathobiological and clinical implications? To address some of these issues, we

will focus on the structural stromal patterns in uveal and cutaneous melanoma, the functional interactions between melanoma cells and stromal components, the relevant pathobiological implications, and topics for future research.

Structure of melanoma stroma

Uveal melanoma

An impression of the contour and internal structure of uveal melanoma in patients can be obtained using ultrasonography or magnetic resonance imaging[20]. Extrascleral extension can also be visualised[21]. The limited resolution of these techniques does not allow detailed information to be obtained about the tumour stroma, although Silverman and colleagues[22] suggest that non-vascular extracellular matrix components associated with microvessels may be the structures identified by ultrasonographic examination. Conventional histology, including histochemistry, immunohistochemistry, electron microscopy, and confocal microscopy of the enucleation specimen, can provide detailed information about stromal structure[18, 19 and 23]. The stromal distribution in uveal melanoma is heterogeneous between different tumours, but also within the tumour. Using a modification of the periodic acid-Schiff reaction, Folberg and co-workers[17] identified so-called 'vascular patterns', some of which were associated with an unfavourable prognosis[18 and 19]. On the basis of a reassessment by Foss and colleagues[19], and more recently Clarijs and colleagues[24], we conclude that the vascular patterns in uveal melanoma represent fibrovascular stromal septa and not microvessels *per se*. The Folberg patterns are illustrated in Figure 3. Foss and colleagues[19] suggested that the presence of 'arcs' and 'loops' may represent disordered and rapidly growing subclones of melanoma cells. This interpretation, and the strong association of these patterns with the presence of epitheloid melanoma cells (known to have an adverse effect on patient outcome), may explain the unfavourable prognosis. These patterns have been associated with expression of markers that may in turn indicate tumour aggressiveness and prognosis[25]. The fibrovascular septa consist of collagen I and VI[26], and laminin, accompanied by fibroblasts and macrophages[24]. In contrast, true microvessels are lined by vascular endothelial cells that express factor VIII-related antigen, CD31, CD34, *Ulex europeus* lectin, and the PAL-E antigen[18, 19 and 24]. They are occasionally covered by pericytes and lined by a basal lamina (to which the endothelial cells are attached) that contains laminin and collagen IV. Clarijs and colleagues[24] and others[27]

were unable to detect the 'vascular channels' described by Maniotis and co-workers[25] as erythrocyte-filled spaces lined by melanoma cells communicating directly with endothelium-lined microvessels. Instead, they suggested that the fibrovascular septa may act as an extracellular matrix meshwork that provides a medium for diffusion of soluble factors[24]. Similar growth patterns can also be found in human uveal melanoma xenografts in rabbit choroid[28 and 29], making this model a suitable tool for further investigation of stromal structure.

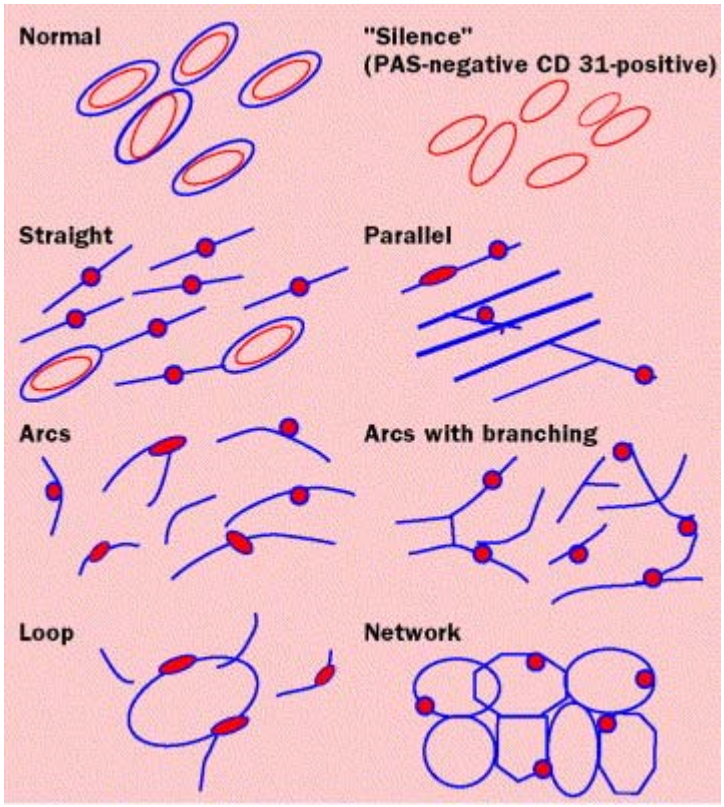

Figure 3. Fibrovascular stromal patterns in uveal melanoma visualised by means of histochemical staining. 'Normal' represents pre-existing blood vessels; 'silence' does not represent a fibrovascular stroma, but does reveal tiny microvessels that can only be detected using immunohistochemistry; 'straight' and 'parallel' have straight septa, without or with cross-links, respectively; 'arcs' and 'arcs with branching' show irregular curved septa, without and with arborisation, respectively; and 'loops' and 'network' show single or compound closed septa, respectively. Representation of findings from references 18 and 19.

Cutaneous melanoma

Cutaneous melanoma cells may have varied morphological and immunophenotypical characteristics[30, 31 and 32] even within a given lesion. These variations include architectural patterns and stromal changes. The stroma can show myxoid (stellate fibroblast-like cells with predominant deposition of proteoglycans) or desmoplastic (fibroblasts and fibrocytes with considerable deposition of fibrillated extracellular matrix components) changes and, rarely, excessive fibroblastic or endothelial-cell

proliferation[3]. Warso and colleagues[33] identified fibrovascular stromal patterns which were similar to those in uveal melanoma, in cutaneous melanoma. They also described the presence of circular packets of melanoma cells in the superficial dermis, surrounded by a broad rim of PAS-positive material. Because of the broader cellular and architectural heterogeneity in cutaneous melanoma, as compared with uveal melanoma, one might expect differences between the stromal patterns. Moreover, in cutaneous melanoma the overall architecture is dominated by the radial (RGP) or vertical growth phases (VGP)[6 and 34]. These growth phases and associated stromal patterns are represented in Figure 4, which shows nodular, micronodular, and mixed nodular/micronodular VGP compatible with the 'loop' and 'network' patterns illustrated in Figure 3. A desmoplastic pattern is also shown. Patients with primary melanomas containing 'parallel with cross-linking' or 'network' patterns had substantially shorter disease-free survival[32]. However, further studies are needed to validate this finding. Architectural variations in and among cutaneous melanoma lesions consist of nesting, trabeculation, whorling, and fasciculation[32]. Accompanying variations of the melanoma cells include epithelioid and spindle-cell features; the latter may have a fibroblast-like appearance and may be accompanied by collagen deposition. A melanoma lesion may even be predominantly composed of spindle cells. This type of tumour, called desmoplastic melanoma, is relatively rare[32] and seems to be the result of epithelial-mesenchymal transition of the melanoma cells. As in uveal and cutaneous melanoma lesions, nested and micronodular growth patterns can be found in xenografts of human melanoma cells; these are accentuated by VEGF (vascular endothelial growth factor) transfection[35].

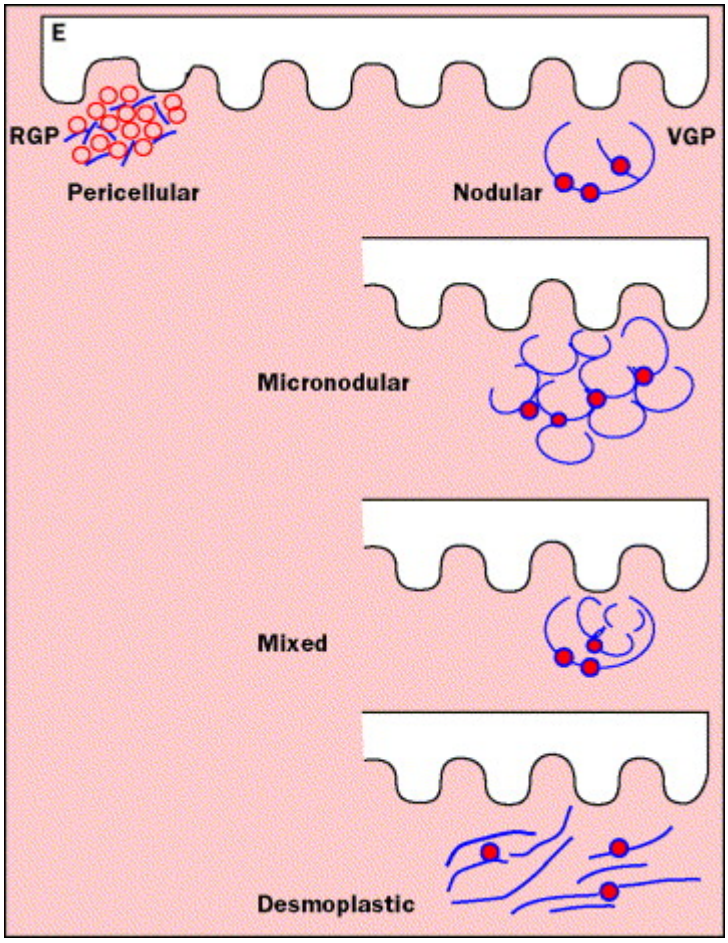

Figure 4. Fibrovascular stromal patterns in cutaneous melanoma visualised by means of histochemical staining. In radial growth phase (RGP) melanoma, the microinvasive cells in the papillary dermis show a pericellular deposition of fibrovascular stroma in a reticular pattern. Vertical growth phase (VGP) melanoma may show four different patterns: nodular, micronodular or nested, mixed nodular/micronodular, and desmoplastic. E, epidermis.

Function of the melanoma stroma

Molecular players and functional interactions

Melanoma cells interact with their microenvironment through the release of soluble substances and by direct cell-cell contact[6, 12, 36 and 37]. The most important growth factors and cytokines involved in melanoma-stroma cross-talk are illustrated in Figure 5. Autocrine growth factors such as bFGF (basic fibroblast growth factor) produced by melanoma cells stimulate proliferation of the originator cell itself and paracrine growth factors, such as PDGF (platelet-derived growth factor) and VEGF, modulate the microenvironment promoting tumour growth and invasion[12 and 37]. Paracrine effects include fibroblast proliferation and fibroplasia (desmoplasia), angiogenesis, and inflammation. We have previously proposed[6 and 36] that the production of growth factors by melanoma cells is the driving force for progression from RGP to VGP melanoma, because they control both tumour growth and stroma formation. The four

melanoma-derived growth factors with stroma-inducing potential are bFGF, PDGF, VEGF, and TGFβ (transforming growth factor β). bFGF, TGFβ, and PDGF are constitutively expressed by VGP primary melanoma and melanoma metastasis cells. bFGF seems to be a stimulation factor for melanocytic cells of all stages because, when it is overexpressed in normal melanocytes, it allows growth in serum-free media in the absence of bFGF and insulin/IGF1 (insulin-like growth factor). It is unclear whether melanoma-derived bFGF has paracrine functions, as well as stimulating stromal fibroblasts and endothelial cells, because the lack of a signal sequence does not allow protein secretion by conventional mechanisms; it may be released from cells in other ways. bFGF seems to act in functional synergy with other melanoma-derived growth factors[6]. One of the bFGF isoforms, an 18 kDa protein, has great potential as a paracrine growth factor for stimulating fibroblasts and endothelial cells[38 and 39]. bFGF is also highly expressed in desmoplastic melanoma, but since various fibrogenic cytokines are expressed in this type of tumour, its specific role in causing melanoma-associated fibrosis is unclear.

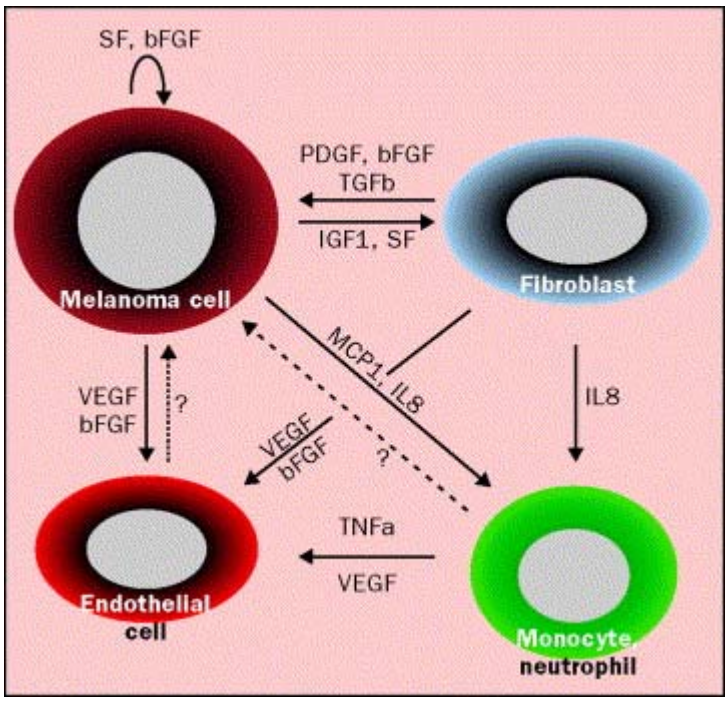

Figure 5. Melanoma-stroma cell interactions through growth factors, cytokines, and chemokines. Emphasis is placed on the interaction between melanoma cells and fibroblasts, endothelial cells and monocytes/neutrophils. Note that interactions also take place between different types of stromal cells. bFGF, basic fibroblast growth factor; IGF1, insulin-like growth factor 1; IL8, interleukin 8; MCP1, monocyte chemoattractant protein 1; PDGF, platelet-derived growth factor; SF, scatter factor; TGFβ, transforming growth factor β; TNFα, tumour necrosis factor α; VEGF, vascular endothelial-cell growth factor; ?, not known.

PDGF acts as a ‘maintenance and survival factor’ in melanoma progression; it helps the tumour to organise the stroma, including extracellular matrix production. PDGFB

is secreted by melanoma cells exclusively for paracrine stimulation, because unlike fibroblasts, melanoma cells lack the PDGF receptor[40]. Those melanoma cells that are already tumorigenic grow more rapidly and are more viable when transduced with PDGFB[40], but RGP melanomas cannot be induced to tumorigenicity when overexpressing PDGFB, suggesting that PDGF acts in synergy with other growth factors. The ability of PDGF to induce angiogenesis on its own is limited. Instead, PDGF induces VEGF which, in turn, orchestrates angiogenesis[41]. In the progression of RGP to VGP melanoma, VEGF seems to be important as an 'expansion and invasion factor' because of its strong angiogenic properties[42]. It stimulates not only endothelial-cell growth, but also migration, invasion, and tube formation[42]. Whether VEGFis constitutively expressed by melanoma cells *in situ* is unclear. It has been detected in most VGP melanoma and melanoma metastases[43 and 44], which is consistent with the importance of VEGF in advanced melanoma. Because VEGF expression is inducible by several factors such as PDGF, bFGF, interleukin 1, and TNFα (tumour necrosis factor α), or by hypoxic conditions[45], it is conceivable that expression may be temporarily stimulated during expansion of the tumour mass. Melanoma cells have several cell-membrane molecules in common with stromal cells[6]. Such shared molecules are not only growth factors, but also proteinases, adhesion molecules, and cytokines. For instance, members of the matrix metalloproteinase (MMP) family, ie MMP2, MT1MMP, TIMP1, and TIMP3 are expressed by melanoma cells, fibroblasts, and macrophages, especially in VGP melanoma[46]. The regulation of MMP expression and activity and the localisation of the active molecules require several factors. For instance, the cytokine interleukin 8 may activate MMP2[47]. In addition, interleukin 1β and TNFα can upregulate MMP9 expression. Furthermore, perturbation of integrins such as α_v,β_3 and α_2,β_1 on melanoma cells results in upregulation of MMP2, MT1MMP, and TIMP2. The integrin α_v,β_3 and another adhesion molecule, CD44, are involved in positioning activated MMP molecules on the cell surface of invasive tumour cells[46 and 48]. Therefore, both the presence of active MMP and its cellular localisation are important in tumour invasion. The presence of MMP molecules on tumour and stromal cells suggests a similar and parallel contribution of both cells in the invasive process. This relation also seems true for FAP, also known as seprase, a serine integral membrane proteinase with gelatinase activity, which is expressed by human melanoma cells, certain carcinoma cells, and activated fibroblasts in the tumour stroma[14].

Functional interactions between melanoma and stromal cells may also occur through direct cell-to-cell contact. Cell-to-cell crosstalk mediated by cadherins and connexins results in coordinated regulation of cell growth, differentiation, apoptosis, and migration[49]. Thus, melanoma cells in the papillary dermis form N-cadherin-mediated adhesion and connexin-mediated gap junctions with N-cadherin-expressing fibroblasts, microvascular endothelial cells, and adjacent melanoma cells. Thus, abnormal expression of adhesion receptors and disregulated intercellular communication may drive tumour development and progression[49].

Role of the fibroblast

Fibroblasts organise tumour stroma formation by production of extracellular matrix components and are responsible for many of its functions. Here, we focus on interactions between melanoma cells and fibroblasts. Fibroblasts are a rich source of growth factors, such as IGF1, SF (scatter factor), bFGF, and TGFβ, but only after they have been activated[6 and 12]. Tumour growth cannot be sustained unless the tumour cells attract and stimulate fibroblasts, which are the main source of extracellular matrix. The involvement of fibroblasts in tumour stromagenesis requires a dynamic process of attraction, proliferation, and differentiation. On the basis of recent insights into tumour stromagenesis[50], the functional repertoire of fibroblasts and myofibroblasts[51 and 52] and observations in carcinomas[53], we propose that the involvement of fibroblasts in melanoma growth and progression probably occurs through several stages: (a) recruitment of resident fibroblasts or circulating mesenchymal precursor or stem cells (MSC) derived from the bone marrow, (b) activation and proliferation of recruited fibroblasts, (c) conversion to myofibroblasts, or (d) differentiation to fibrocytes that secrete fibrillated extracellular matrix components (Figure 6). Little is known about the dynamics of fibroblast involvement and the molecules that regulate it. The growth factors produced by melanoma cells and fibroblasts (Figure 5) may have different roles in this part of the process of tumour progression. It is important to remember that the extracellular matrix may release several growth factors as it is broken down by proteinases[53 and 55].

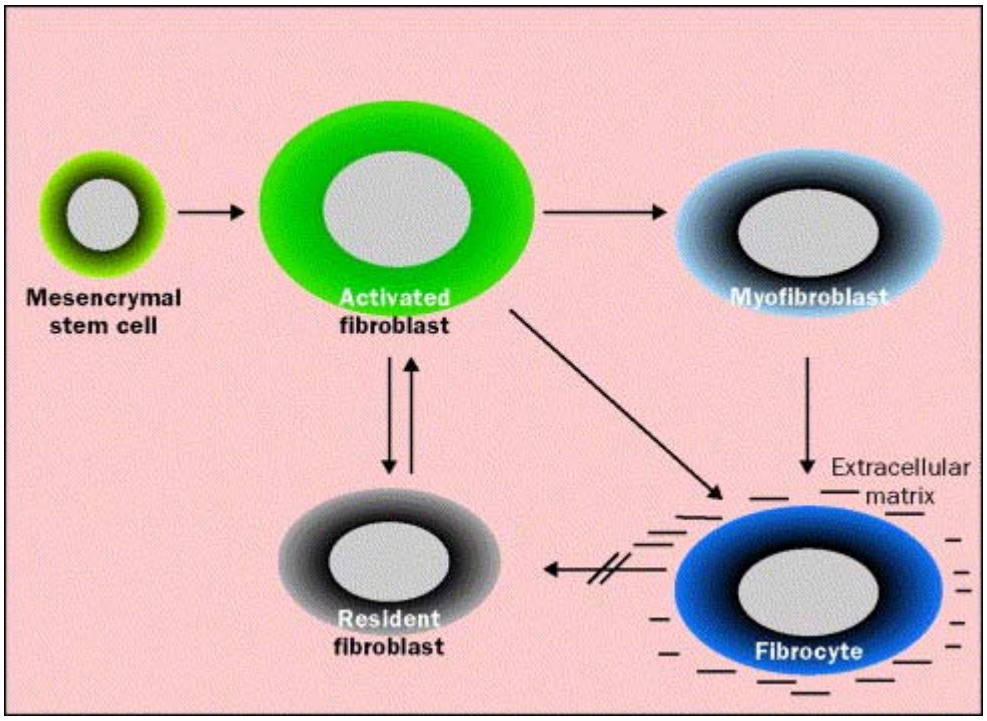

Figure 6. Fibroblast recruitment, activation, and differentiation. Fibroblasts in the tumour stroma can be attracted from resident fibroblasts or from bone-marrow-derived, circulating, mesenchymal, stem cells. Upon activation, the fibroblasts proliferate and are converted to myofibroblasts, or differentiate to become fibrocytes. The latter is accompanied by production of fibrillated extracellular matrix components. Differentiated fibrocytes do not contribute to the maintenance of resident fibroblasts. Representation of findings from references 18 and 19.

For investigation of fibroblast involvement at the molecular level, gene expression profiles would be highly informative, but only limited specific data has been reported, eg on FAP[14]. However, the molecular response *in vitro* of resting fibroblasts to serum has been investigated using cDNA microarray technology[56]. Remarkably, the expression of about 500 genes was found to change substantially upon activation by serum. The transcriptional response of fibroblasts happened extremely quickly, within 15 minutes. More importantly, the expression of several genes that have roles in the patho-physiology of wound healing occurred, which emphasises the central role of fibroblasts in wound repair: interpreting, amplifying, modifying, and broadcasting signals controlling inflammation and angiogenesis (Table 2)[56]. These data are also highly relevant to the tumour stroma, especially in the context of the paradigm coined by Dvorak[57] that tumours are “wounds that do not heal”.

Table 2. Molecular changes following fibroblast activation[56]

Gene category	Number of genes	Examples
Signal transduction	24	KIT, MKP1
Immediate-early transcription factors	17	MYC, C-FOS
Other transcription factors	14	ERF-2
Cell cycle/proliferation	28	PCNA, Cyclin D1
Coagulation/haemostasis	10	Endothelin 1, PAI1
Inflammation	8	IL1β, IL6
Angiogenesis	12	VEGF, VEGFR, IL8
Tissue remodelling	19	TIMP3, PAI1
Cytoskeletal reorganisation	7	Vimentin
Re-epithelialisation	6	Endothelin 1, FGF2
Others	24	KIT, IPP isomerase

Pathobiological implications

So, is it possible to develop an integrated view on melanoma-stroma interactions that affect the biology of tumours? Here we consider some of the key biological aspects: tumour topography, the hierarchy between tumour cells and the stroma, and the pathological mechanisms by which the stroma drives tumour development and progression.

Tumour topography: structural and functional zones? A hypothesis

As summarised in Table 3, we hypothesise that resident or non-activated fibroblasts would be situated in pre-existent adjacent tissue; activated fibroblasts in the peritumoural zone; myofibroblasts in the outer rim of the tumour -especially in the invading edge; and differentiated fibrocytes mainly in the inner tumour compartment, where fibrovascular septa or more compact areas of fibrillated extracellular matrix are produced. This hypothesis is based on a translation of the different fibroblast stages (Figure 6) and their presumed functional role in the tumour microenvironment[51 and 52] into the overall architecture of a malignant solid tumour (Figure 1). The four fibroblast phenotypes may have different biological roles in tumour growth and progression. The resident fibroblast has a passive role, waiting for activating signals to come to it. Other resident mesenchymal cells, in addition to fibroblasts and circulating bone marrow-derived MSC's, are candidates for recruitment in wound healing and tumour stroma formation[50, 58 and 59]. Activated fibroblasts in the peritumoral zone are already mobilised and prepared for cell division and wound healing, but not yet subverted by

the tumour cells. In a benign tumour, fibroblasts would probably proceed further in the formation of a fibrous capsule to demarcate the pathological process. However, myofibroblasts at the invading edge of a malignant tumour are subverted to collaborate with the tumour. Differentiated fibrocytes are involved in a dynamic balance between growth promoting and growth limiting forces. The fibrovascular stromal patterns in uveal melanoma, and possibly in other tumour types, may promote tumour growth by providing a scaffold for tumour cells, blood microvasculature, and macrophages, and allowing transport of interstitial fluid carrying nutrients[24]. A less organised stromal architecture with an abundance of fibrillary extracellular matrix probably limits tumour growth, as is often encountered in the inner compartment of carcinomas[53]. This extracellular matrix barrier may hinder the penetration of immune competent cells, creating a state of immune privilege[60]. In conclusion, the complexity of melanoma-stroma interactions can be reduced by considering structurally and functionally defined zones in and around the tumour.

Table 3. Presumed properties of fibroblasts involved in tumour stromagenesis

Cell type	Presumed role	Phenotypic hallmark	Proposed localisation
Resident fibroblast	Tissue stem cell	Not known	Adjacent uninvolved tissue
Activated fibroblast	Preparation for cell division and wound healing	Multiple gene activation*	Peritumoral zone
Myofibroblast	Collaboration with tumour cells	Smooth muscle actin	Invasive front and tumour periphery
Differentiated fibrocyte	Providing of scaffold for tumour cells	Abundant pericellular fibrillated ECM	Tumour centre

*See Table 2

Tumour cell hierarchy: who is the boss?

Since fibroblasts and extracellular matrix produced by fibroblasts influence growth of melanoma cells, can the genes that encode components involved in tumour stroma formation be viewed as merely ‘landscapers’, as suggested by Kinzler and Vogelstein?[61]. Considering the central role of fibroblasts at the invasive front, we share the view of Dano and colleagues[55], that a tumour is comparable to an army: the neoplastic cells being the commanding officers and the stroma the executing soldiers. Boudreau and Bissell[62] stress that the extracellular matrix molecules and their receptors must integrate form and function to achieve a differentiated phenotype

for a cell. Malignant transformation is seen as the result of mutated genes in tumour cells and aberrant interactions with the microenvironment. Elenbaas and Weinberg[53] note the relevance of heterotypic signaling in the tumour stroma between non-neoplastic cells for tumour growth and progression. This seems of particular importance in melanoma, as its stroma often is cellular and quite diverse in composition. In melanoma, various stromal pathways are activated (Figure 5), including fibroplasia, angiogenesis, inflammation and immune response. We believe that tumour progression in melanoma can be augmented by prominent stromal activation, the prime examples of which being tumour ulceration[63] and immunological regression[5]. This is in line with Balkwill and Mantovani's[64] suggestion that the inflammatory cells and cytokines found in tumours are more likely to contribute to tumour growth, progression, and immunosuppression than they are to mount an effective host antitumour response. Their metaphor that “if genetic damage is the match that lights the fire of cancer, some types of inflammation may provide the fuel that feeds the flames” makes sense to us. In addition, it implies a scenario of uncontrollable tumour growth and progression in which the neoplastic cells lose their commanding position, leaving the army of tumour cells in a state of anarchy. This theory is compatible with a dynamic shift in the hierarchy between the neoplastic cells and the stroma, implying an increasingly commanding role of the stromal cells. To cope with this demanding challenge, the stromal cells must have remarkable plasticity in their molecular and cellular repertoire[2 and 62]. This holistic view of the pathomechanisms of tumour progression makes it clear that increasing genetic alterations of the malignant tumour cells are not the only important determinant. It may also explain why progressing malignant tumours need to mobilise bone-marrow-derived stromal precursor cells to support growth, and why they lose most of the roots of the original tissue they arise in, making them likely to metastasise. The pathomechanisms of tumour progression in melanoma are probably different to most other solid malignant tumours because the phenotype of the tumour cells is extremely heterogeneous and often includes a spindle-cell variant, which may express molecular and functional features of stromal cells[32]. This is why melanoma was called melanosarcoma by early histopathologists. Likewise, with increasing progression, sarcomatous and even blastomatous dedifferentiation may occur in melanoma, which could give rise to so called ‘vasculogenic mimicry’ or ‘transendothelial differentiation’[25].

Clinical implications

Do the molecular players and pathomechanisms discussed above provide new instruments or strategies for clinical application? Indeed they do, and here we briefly discuss assessment of prognosis and use of biological therapy.

With the exception of fluorescence imaging in uveal melanoma, no diagnostic applications have so far been identified[20, 21 and 22]. Conventional assessment of prognosis is mainly based on careful tumour staging. This is also true for melanoma[63]. These assessments could be improved by refinement of the staging procedure[63] and by identification of molecular markers in individual tumour lesions that play a part in tumour progression[34]. Almost all such markers are molecules derived from neoplastic cells with the exception of proteases, related components[65], and some growth factors and cytokines which are, entirely or in part, derived from stromal cells[66 and 67]. In several types of carcinoma, the concentration of serine protease plasminogen activation components uPA, uPAR, and PAI1 in the tumour (detected by ELISA) were shown to be prognostically unfavourable, whereas tPA had a favourable effect[65]. These components were shown by immunohistochemistry and mRNA *in situ* hybridisation to be partly derived from the tumour stroma, especially from fibroblast-like cells and macrophages[55 and 65]. An overview of such prognostic markers in primary cutaneous melanoma is given in Table 4[33,46,66,68,69]. Molecules derived from the tumour stroma may exert paracrine effects on the local tumour cells, and they may be shed into the circulation either directly into the microvessels of the fibrovascular septa or indirectly via interstitial fluid transported in the extracellular matrix meshwork of the fibrovascular septa[24]. The molecules shed into the circulation may become detectable in the serum of cancer patients, as was shown for components of the plasminogen activation system in several types of carcinoma[65] and for the growth factors and cytokines, VEGF, bFGF, and interleukin 8[66 and 67] in melanoma. These studies strongly suggest that molecules derived (in part) from the tumour stroma may have prognostic relevance. Therefore, as proposed elsewhere[70], staging of cancer patients based on examination of serum samples should not be restricted to molecular detection of the neoplastic cells, but should also include determinants of the tumour microenvironment to increase the prognostic information.

Table 4. Prognostic markers in cutaneous melanoma in part expressed by the tumour stroma

Group	Marker	Expression or pattern	Prognosis	ref
Proteinase	tPA	> 50% of tumour cells	Favourable	68
	MMP2	> 33% of tumour cells	Unfavourable	69
	Cathepsin B,D	Present	Unfavourable	46
Cytokine	Interleukin 8	Present	Unfavourable	66
Fibro-vascular septum	Collagens (PAS, Azan)	Parallel with cross links or network	Unfavourable	33

Regarding therapeutic implications, both obstacles and opportunities are encountered. It was shown that intravenously injected monoclonal antibodies that recognise melanoma-associated antigens in human melanoma xenografts achieved highest concentrations in tumour stroma, particularly at the tumour-stroma interface[71]. This distribution, which is similar to that of bovine serum albumin[35], would imply a barrier in the deliverance of circulating reagents to the tumour cells. High interstitial pressure in tumours may severely hamper drug delivery[72]. Still, molecules expressed both by tumour cells and stromal cells may be suitable targets for immunotherapeutic or biotherapeutic approaches[73], an example of the latter being bFGF. Wang and Becker[74] showed that antisense targeting of bFGF and FGFR1 in human melanoma xenografts blocks intratumoral angiogenesis and tumour growth. They therefore suggest that inhibition of bFGF/FGFR1-mediated signalling may open a new avenue for the treatment of advanced-stage melanoma. Also, Davol and colleagues[75] showed that combining suramin, which blocks autocrine and paracrine growth factors, and a chimeric toxin directed to bFGFR increases therapeutic efficacy against human melanoma xenografts.

Future research

Key issues to address in future research efforts are: (a) what is the participation of bone marrow-derived precursor cells to melanoma stromagenesis? (b) which molecules play a role in the different stages of fibroblast involvement in the tumour stroma? (c) can we visualise the peritumoral zone as an indication of paracrine activity of a tumour? (d) how do the fibrovascular septa promote tumour growth and progression? (e) which growth factors or cytokines are responsible for augmentation of malignancy in melanoma eg in ulceration? (f) what are the pathomechanisms of these pathways of melanoma progression? and (g) which other molecular substrates can be identified that could serve as potential target for diagnostic, prognostic or therapeutic purpose? These issues are crucial goals for future molecular, cell biology,

and translational research, not only in melanoma, but also in other solid malignant tumours.

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References

1 D Hanahan and RA Weinberg, The hallmarks of cancer, *Cell* **100** (2000), pp. 57–70.

2 CG Park, MJ Bissell and MH Barcellos-Hoff, The influence of the microenvironment on the malignant phenotype, *Mol Med Today* **6** (2000), pp. 324–329.

3 LJ McCawley and LM Matrisian, Tumor progression: defining the soil round the tumor seed, *Curr Biol* **11** (2001), pp. R25–R27.

4 LA Liotta, P Steeg and WG Stetler-Stevenson, Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation, *Cell* **4** (1991), pp. 327–336.

5 MT Lotze, RM Dallal and JM Kirkwood *et al.*, Cutaneous melanoma (6th ed.). In: De Vita, VT Jr, S Hellman and S Rosenberg, Editors, *Cancer, principles and practice of oncology*, Lippincott-Raven, New York (2001), pp. 2012–2069.

6 M Herlyn and K Satyamoorthy, Molecular Biology of Cutaneous Melanoma (6th ed.). In: De Vita, VT Jr, S Hellman and S Rosenberg, Editors, *Cancer, principles and practice of Oncology*, Lippincott-Raven, New York (2001), pp. 2003–2012.

7 EA Clark, RG Todd and ES Lander *et al.*, Genomic analysis of metastasis reveals an essential role for RhoC, *Nature* **406** (2000), pp. 532–535.

8 M Bittner, P Meltzer and Y Chen *et al.*, Molecular classification of cutaneous malignant melanoma by gene expression profiling, *Nature* **406** (2000), pp. 536–540.

9 IJ Fidler, Modulation of the organ microenvironment for the treatment of cancer metastasis, *J Natl Cancer Inst* **84** (1995), pp. 1588–1592.

10 WH Clark, AF Hood and RM Jampel, Atypical melanocytic nevi of the genital type with a discussion of reciprocal parenchymal-stromal interactions in the biology of neoplasia, *Hum Pathol* **29** (1998) (suppl1), pp. S1–S24.

11 J Smolle, R Hofmann-Wellenhof and R Fink-Puches, Melanoma and stroma: an interaction of biological and prognostic importance, *Semin Cutan Med Surg* **15** (1996), pp. 326–336.

12 R Halaban, Growth factors and melanomas, *Semin Oncol* **23** (1996), pp. 673–681.

13 JA Reed, NS McNutt and AP Albino, Differential expression of basic fibroblast growth factor (bFGF) in melanocytic lesions demonstrated by *in situ* hybridization, *Am J Pathol* **144** (1994), pp. 329–336.

14 MJ Scanlan, BK Raj and B Calvo *et al.*, Molecular cloning of fibroblast activation protein α, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers, *Proc Natl Acad Sci USA* **91** (1994), pp. 5657–5661.

15 RL Barnhill, K Fandrey and MA Levy *et al.*, Angiogenesis and tumor progression of melanoma. Quantification of vascularity in melanocytic nevi and cutaneous malignant melanoma, *Lab Invest* **67** (1992), pp. 331–337.

16 JR Westphal, RG van 't Hullenaar and JA van der Laak *et al.*, Vascular density in melanoma xenografts correlates with vascular permeability factor expression but not with metastatic potential, *Br J Cancer* **76** (1997), pp. 561–570.

17 O Straume, HB Salvesen and LA Akslen, Angiogenesis is prognostically important in vertical growth phase melanomas, *Int J Oncol* **15** (1999), pp. 595–599.

18 R Folberg, J Pe'er and LM Gruman *et al.*, The morphological characteristics of tumor blood vessels as a marker of tumor progression in primary human uveal melanoma: a matched case-control study, *Hum Pathol* **23** (1992), pp. 1298–1305.

19 AJ Foss, RA Alexander and JL Hungerford *et al.*, Reassessment of the PAS patterns in uveal melanoma, *Br J Ophtalmol* **82** (1997), pp. 240–246.

20 JA Shields, CL Shields and P De Potter *et al.*, Diagnosis and Treatment of Uveal Melanoma, *Semin Oncol* **23** (1996), pp. 763–767.

21 N Hosten, N Bornefeld and R Wassmuth *et al.*, Uveal Melanoma: Detection of Extraocular Growth with MR Imaging and US, *Radiology* **202** (1997), pp. 61–67.

22 RH Silverman, R Folberg and HC Boldt *et al.*, Correlation of ultrasound parameter imaging with microcirculatory patterns in uveal melanomas, *Ultrasound Med Biol* **23** (1997), pp. 573–581.

23 V Rummelt, R Folberg and C Rummelt *et al.*, Microcirculation architecture of melanocytic nevi and malignant melanomas of the ciliary body and choroid. A comparative histopathologic and ultrastructural study, *Ophtalmology* **101** (1994), pp. 718–727.

24 R Clarijs, I Otte-Höller and D Ruiter *et al.*, Presence of a fluid-conducting meshwork in xenografted cutaneous and primary human uveal melanoma, *Invest Ophtalmol Vis Sci* (2002) (in press).

25 AJ Maniotis, R Folberg and A Hess *et al.*, Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry, *Am J Pathol* **155** (2000), pp. 739–752.

26 KJ Daniels, HC Boldt and JA Martin *et al.*, Expression of type VI collagen in uveal melanoma: its role in pattern formation and tumor progression, *Lab Invest* **75** (1996), pp. 55–66.

27 DM McDonald, L Munn and RK Jain, Vasculogenic mimicry: how convincing, how novel, and how significant?, *Am J Pathol* **156** (2000), pp. 383–388.

28 AJ Mueller, R Folberg and WR Freeman *et al.*, Evaluation of the Human Choroidal Melanoma Rabbit Model for Studying Microcirculation Patterns with Confocal ICG and Histology, *Exp Eye Res* **68** (1999), pp. 671–678.

29 P Bonicel, J Michelot and F Bacin *et al.*, Establishment of IPC 227 cells as human xenografts in rabbits: a model of uveal melanoma, *Melanoma Res* **10** (2000), pp. 445–450.

30 RE Nakhleh, MR Wick and A Rocamora *et al.*, Morphologic diversity in malignant melanomas, *Am J Clin Pathol* **93** (1990), pp. 803–805.
31 WH Clark, MA Tucker and AM Goldstein, Parenchymal-stromal interactions in neoplasia. Theoretical considerations and observations in melanocytic neoplasia, *Acta Oncol* **34** (1995), pp. 749–757.

32 SS Banerjee and M Harris, Morphological and immunophenotypic variations in malignant melanoma, *Histopathology* **36** (2000), pp. 387–402.

33 MA Warso, AJ Maniotis and X Chen *et al.*, Prognostic significance of periodic acid-schiff-positive patterns in primary cutaneous melanoma, *Clin Cancer Res* **7** (2001), pp. 473–477.

34 D Elder, Tumor progression, early diagnosis and prognosis of melanoma, *Acta Oncol* **38** (1999), pp. 535–547.

35 AJ Potgens, MC Van Altena and NH Lubsen *et al.*, Analysis of the tumor vasculature and metastatic behavior of xenografts of human melanoma cell lines transfected with vascular permeability factor, *Am J Pathol* **148** (1996), pp. 1203–1217.

36 M Herlyn and IM Shih, Interactions of melanocytes and melanoma cells with the microenvironment, *Pigment Cell Res* **7** (1994), pp. 81–88.

37 E Lazar-Molnar, H Hegyesi and S Toth *et al.*, Autocrine and paracrine regulation by cytokines and growth factors in melanoma, *Cytokine* **12** (2000), pp. 547–554.

38 F Czubayko, EDE Liaudet-Coopman and A Aigner *et al.*, A secreted FGF-binding protein can serve as the angiogenic switch in human cancer, *Nat Med* **3** (1997), pp. 1137–1140.

39 C Berking, R Takemoto and K Satyamoorthy *et al.*, Basic fibroblast growth factor and ultraviolet B transform melanocytes in human skin, *Am J Pathol* **158** (2001), pp. 943–953.

40 K Forssberg, I Valvi-Nagy and CH Heldin *et al.*, Platelet-derived growth factor (PDGF) in oncogenesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB, *Proc Natl Acad Sci USA* **90** (1993), pp. 393–397.

41 N Sato, JG Beitz and J Kato *et al.*, Platelet-derived growth factor indirectly stimulates angiogenesis in vitro, *Am J Pathol* **142** (1993), pp. 1119–1130.

42 HF Dvorak, LF Brown and M Detmar *et al.*, Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis, *Am J Pathol* **146** (1995), pp. 1029–1039.

43 H Erhard, FJ Rietveld and van Altena *et al.*, Transition of horizontal to vertical growth phase melanoma is accompanied by induction of vascular endothelial growth factor expression and angiogenesis, *Melanoma Res* **suppl 2** (1997), pp. S19–S26.

44 T Vlaykova, P Laurila and T Muhonen *et al.*, Prognostic value of tumour vascularity in metastatic melanoma and association of blood vessel density with vascular endothelial growth factor expression, *Melanoma Res* **9** (1999), pp. 59–68.

45 P Carmeliet and RK Jain, Angiogenesis in cancer and other diseases, *Nature* **407** (2000), pp. 249–257.

46 UB Hofmann, JR Westphal and GN Van Muijen *et al.*, Matrix metalloproteinases in human melanoma, *J Invest Dermatol* **115** (2000), pp. 337–344.

47 M Bar-Eli, Role of interleukin-8 in tumor growth and metastasis of human melanoma, *Pathobiology* **67** (1999), pp. 12–18.

48 K Takahashi, H Eto and KK Tanabe, Involvement of CD 44 in matrix metalloproteinase-2 regulation in human melanoma cells, *Int J Cancer* **80** (1999), pp. 387–395.

49 G Li and M Herlyn, Dynamics of intercellular communication during melanoma development, *Mol Med Today* **4** (2000), pp. 163–169.

50 S Rafii, Circulating endothelial precursors: mystery, reality, and promise, *J Clin Invest* **105** (2000), pp. 17–19.

51 G Gabbiani, The cellular derivation and the life span of the myofibroblast, *Pathol Res Pract* **192** (1996), pp. 708–711.

52 DW Powell, RC Miffin and J Valentich *et al.*, Myofibroblasts I. Paracrine cells important in health and disease, *Am J Physiol* **277** (1999), pp. C1–C19 (Cell physiol **46**).

53 B Elenbaas and RA Weinberg, Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation, *Exp Cell Res* **264** (2001), pp. 169–184.

55 K Danø, J Romer and BS Nielsen *et al.*, Cancer invasion and tissue remodeling - cooperation of protease systems and cell types, *APMIS* **107** (1999), pp. 120–127.

56 V Iyer, MB Eisen and DT Ross *et al.*, The transcriptional program in the response of human fibroblasts to serum, *Science* **283** (1999), pp. 83–87.

57 HF Dvorak, Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing, *New Engl J Med* **315** (1986), pp. 1650–1659.

58 T Asahara, T Murohara and T Sullivan *et al.*, Isolation of putative progenitor endothelial cells for angiogenesis, *Science* **275** (1997), pp. 964–967.

59 M Studeny, F Marini and I Fidler *et al.*, Bone marrow mesenchymal stem cells serve as precursors for stromal fibroblasts in solid tumors, *Proc Am Assoc Cancer Res* **42** (2001) (suppl), p. 73.

60 E Tartour and WH Fridman, Cytokines and cancer, *Int Rev Immunol* **16** (1998), pp. 683–704.

61 KW Kinzler and B Vogelstein, Landscaping the cancer terrain, *Science* **280** (1998), pp. 1036–1037.

62 N Boudreau and M Bissell, Extracellular matrix signaling: integration of form and function in normal and malignant cells, *Curr Op Cell Biol* **10** (1998), pp. 640–646.

63 CM Balch, AC Buzaid and MB Atkins *et al.*, A new American joint committee on cancer staging system for cutaneous melanoma, *Cancer* **88** (2000), pp. 1484–1491.

64 F Balkwill and A Mantovani, Inflammation and cancer: back to Virchow?, *Lancet* **357** (2001), pp. 539–555.

65 PA Andreasen, L Kjoller and L Christensen *et al.*, The urokinase-type plasminogen activator system in cancer metastasis: a review, *Int J Cancer* **72** (1997), pp. 1–22.

66 W Nuernberg, D Tobias and F Otto *et al.*, Expression of interleukin-8 detected by *in situ* hybridization correlates with worse prognosis in primary cutaneous melanoma, *J Pathol* **189** (1999), pp. 546–551.

67 S Ugurel, G Rappel and W Tilgen *et al.*, Increased serum concentration of angiogenic factors in malignant melanoma patients correlates with tumor progression and survival, *J Clin Oncol* **19** (2001), pp. 577–583.

68 CM Ferrier, S Suciu and WL van Geloof *et al.*, High tPA-expression in primary melanoma of the limb correlates with good prognosis, *Br J Cancer* **83** (2000), pp. 1351–1359.

69 A Vaisanen, M Kalloinen and PJ Taskinen *et al.*, Prognostic value of MMP2 immunoreactive protein (72 kD type IV collagenase) in primary skin melanoma, *J Pathol* **186** (1998), pp. 51–58.

70 DJ Ruiter, JHJM Van Krieken and GNP van Muijen *et al.*, Tumour metastasis: is tissue an issue?, *Lancet Oncol* **2** (2001), pp. 109–112.

71 K Lin, JA Nagy and H Xu *et al.*, Compartmental distribution of tumor-specific monoclonal antibodies in human melanoma xenografts, *Cancer Res* **54** (1994), pp. 2269–2277.

72 RK Jain, Barriers to drug delivery in solid tumors, *Sci Am* **271** (1994), pp. 58–65.

73 LA Liotta and EC Kohn, The microenvironment of the tumour-host interface, *Nature* **411** (2001), pp. 375–379.

74 Y Wang and D Becker, Antisense targeting of basic fibroblast growth factor and fibroblast growth factor receptor-1 in human melanomas blocks intratumoral angiogenesis and tumor growth, *Nat Med* **3** (1997), pp. 887–893.

75 PA Davol, S Garza and AR Frachelton Jr., Combining suramin and a chimeric toxin directed to basic fibroblast growth factor receptors increased therapeutic efficacy against human melanoma in an animal model, *Cancer* **86** (1999), pp. 1733–1741.

No longer a molecular black box – new clues to apoptosis and drug resistance in melanoma

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Abstract

Disseminated melanoma is a radiation- and chemotherapy-refractory neoplasm for which no standard therapy currently exists. So far, genetic and molecular studies have revealed few non-random chromosomal abnormalities and infrequent mutational spectra. Consequently, the precise molecular determinants responsible for melanoma progression are yet to be delineated. Recent studies, however, have identified defects at multiple levels of the apoptosis program in melanoma, which provided new clues to drug resistance of this highly aggressive neoplasm. The process of apoptosis provides a conceptual framework to link melanoma genetics with the outcome of melanoma therapy. Hence, the genes and proteins that control apoptosis provide exciting new targets for rationally designed anti-melanoma therapeutic strategies.

Introduction

Normal skin homeostasis is maintained by dynamic interactions between melanocytes and their microenvironment, such as keratinocytes, fibroblasts, endothelial and immunocompetent cells, and the extracellular matrix. During transformation of melanocytes and progression of melanoma cells, there are reciprocal interactions between the neoplastic and adjacent normal stromal cells [1]. Thus, normal skin cells within the neoplastic tissue are not idle bystanders, but are active participants that shape the aggressive features of melanoma. Genetic and molecular analyses have failed to identify consistent gene deletions, mutations, translocations or amplifications in sporadic melanoma [1]. Thus, the genetic and biochemical alterations responsible for the development and stepwise progression of melanoma remain enigmatic. However, by considering the microenvironment and tumor-cell interactions, biological events are now beginning to be understood in terms of specific proteins that affect the escape from apoptosis and drug resistance(Fig. 1).

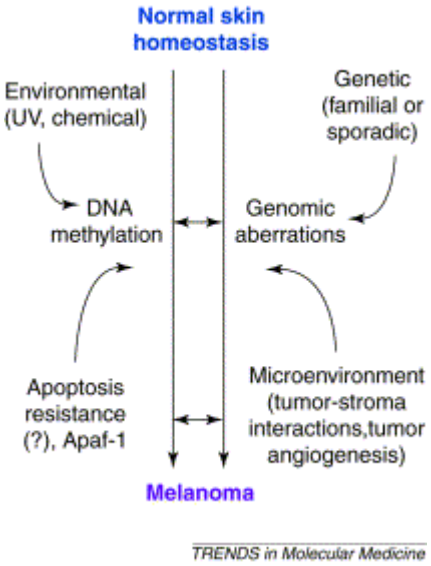

Fig. 1. A global view of melanomagenesis. This model suggests that genetic (such as mutations and deletions of crucial genes) and epigenetic (DNA-methylation leading to gene silencing) alterations, as well as the acquired capability of evading apoptosis progressively disturb normal skin homeostatis, which leads to melanoma development.

Selective components of the apoptotic machinery

Acquired resistance toward apoptosis is a hallmark of cancer [2]. The concept that apoptosis might influence the malignant phenotype was first raised in 1972 (Ref. [3]). However, the importance of apoptosis in the pathogenesis of cancer remained under-appreciated for almost two decades. A comprehensive overview of apoptosis is inscrutably complex and beyond the scope of this discussion [4 and 5]. As a general

summary, most apoptotic pathways involve a sensor that detects a death-inducing signal, a signal-transduction network and execution machinery that actively carries out the process of cell death. DNA damage can induce apoptosis through a central sensor, p53, although p53-independent pathways exist (Fig. 2). Many of the signals that elicit apoptosis converge on the mitochondria, which respond to pro-apoptotic signals by releasing cytochrome *c* (Ref. [6]). Cytochrome *c* can interact in a multi-protein complex with apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9, leading to caspase-9 activation and initiation of a protease cascade [7]. One of the most important modulators is Bcl-2, owing to its ability to affect cytochrome *c* release from mitochondria [8] and to modulate the Apaf-1–caspase-9 interaction [7 and 9]. Bcl-2 is the founding member of an expanding protein family [10] that includes other anti-apoptotic molecules such as Bcl-x_L and pro-apoptotic members such as Bax. However, neither Bcl-2 nor Bcl-x_L has been shown to be completely cytoprotective against Apaf-1–caspase-9-mediated cell death [11].

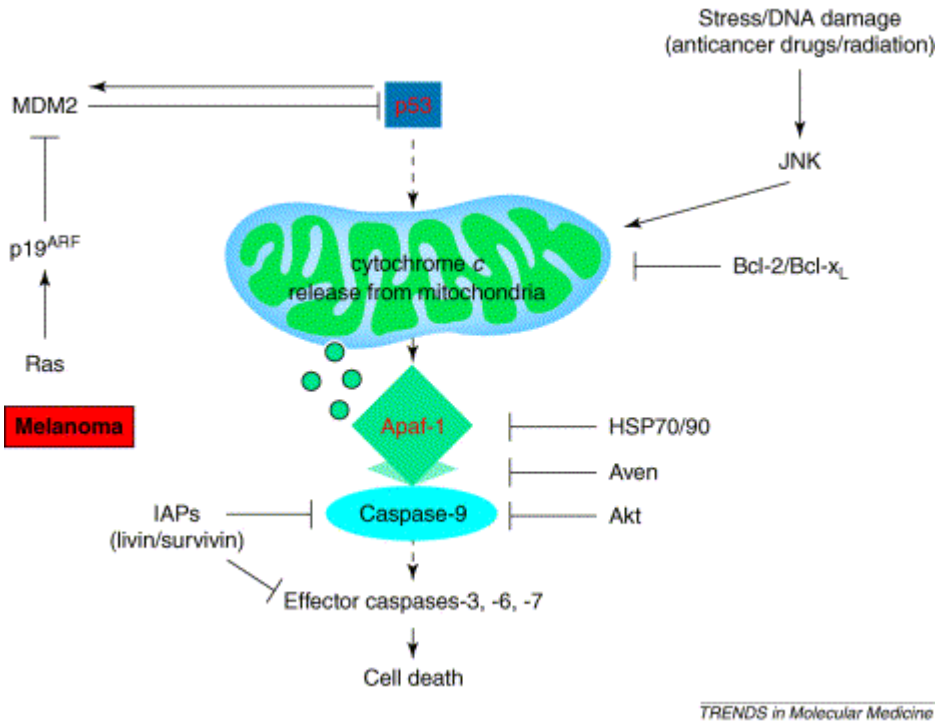

Fig. 2. Interrelationship between selected key molecules in the apoptotic program in melanoma. The basic premise is that p53, although wild-type, is unable to induce apoptosis owing to silencing of Apaf-1. The apoptotic p53 pathway includes cytochrome *c* release from mitochondria, activation of Apaf-1, its complex formation with caspase-9, and the 'effector' caspase family. Inhibition of Apaf-1-mediated apoptosis is inhibited by heat shock proteins, Aven and IAPs (inhibitor of apoptosis proteins). Post-transcriptional modifications such as phosphorylation by Akt also influence the apoptotic effects of caspase-9. In addition, cytochrome *c* release from the mitochondria is inhibited by anti-apoptotic proteins such as Bcl-2 and Bcl-x_L. However, the apoptotic network is much too complicated to be accurately described by simple linear pathways. Key defective molecules in melanoma are shown in red.

A series of enzymes known as caspases is considered the engine of apoptotic cell death. Caspases are cysteine proteases that are expressed as inactive pro-enzymes. The 'signaling' caspase-9 associates with Apaf-1, and oligomerization of this complex in the presence of cytochrome *c* can activate the downstream 'effector' caspase cascade. Thus, essential downstream components of p53-induced cell death include caspase-9 and its adapter Apaf-1 (Ref. [12]). It is now becoming increasingly clear that mutations in many cancer-related genes can disrupt the apoptotic machinery, and compelling evidence indicates that apoptotic activity is important in tumor suppression.

Melanoma, apoptosis and drug resistance

p53 was the first tumor suppressor gene linked to apoptosis. Mutations in *p53* occur in the majority of human tumors and are often associated with advanced tumor stage and poor patient prognosis [13]. Moreover, several upstream and downstream components of the p53 pathway (e.g. MDM2, $p19^{ARF}$ and Bax) are altered in human tumors [13] (Fig. 2). What triggers apoptosis during tumor development? A variety of signals appears to be important. Extracellular triggers include radiation, growth or survival factor depletion and loss of cell–matrix interactions and cell–cell adherence-based survival signals [14]. In the skin, excessive exposure to ultra-violet (UV) radiation induces apoptosis, which presumably serves to eliminate heavily damaged cells. UV irradiation induces apoptosis, and loss of p53 function leads to the survival of these damaged cells thereby initiating tumor development [15]. Hence, loss of apoptotic function can impact tumor initiation, progression and metastasis. Mutations in *p53* were found to be associated with poor prognosis, *de novo* or acquired resistance and relapse in a broad field of solid and haematologic malignancies [4 and 5]. Melanoma is a radiation- and chemotherapy-refractory neoplasm and there is no standard therapy for patients with disseminated disease: the commonly used anti-cancer drugs do not alter the prognosis, which is invariably a fatal outcome [16]. However, contrary to other malignancies, *p53* mutations are very rare in melanoma, with a frequency of <5%. Tumor-derived p53 does localize in the nucleus in melanoma, but its transcriptional activity is weak, and overexpression of wild-type p53 does not induce immediate cell death. Another intriguing factor is that when wild-type p53 is overexpressed in cells that carry mutant p53, these cells

undergo apoptosis [17]. This suggests that p53 is kept in an inactive state, either by other factors (such as Apaf-1) or through post-transcriptional mechanism(s), thereby functionally inactivating its pathways. Thus far, studies have failed to correlate *p53* mutations with reduced toxicity to anti-cancer agents in melanoma.

A (first) molecular clue to melanoma chemoresistance: Apaf-1

Recent findings by Scott Lowe and colleagues [18] now provide exciting and promising new insights into mechanisms of how melanomas become apoptosis-, and thus chemotherapy-resistant, despite a fully functional p53. Melanoma cells of different progression stages can avoid apoptosis by inactivating a downstream component of the apoptotic pathway, namely Apaf-1, thus disabling the p53 apoptotic program. What makes the results of Soengas *et al.*[18] even more interesting is that they demonstrated (1) a deletion of one of the alleles in 42% of the specimens tested, and (2) a reversible 'switching off' of the remaining allele by methylation, rather than a permanent deactivation by deletion or mutation. The methylation of *Apaf-1* could experimentally be reversed by a methylation inhibitor (5-aza-2dC) and thus its pro-apoptotic function restored. Pre-treatment assessment of Apaf-1 status could therefore improve the therapeutic management of patients with melanoma.

More targets – survivin and TRAIL

Recently, defects at other levels of the apoptotic network in melanoma have been discovered, adding further dimensions of complexity. The newly described apoptosis inhibitor survivin is overexpressed in many human malignancies, and appears to play a crucial part in both regulation of the apoptotic program and cell-cycle progression [19, 20 and 21]. Survivin has been reported to bind to and inhibit the active forms of the effector caspases-3 and -7 (Ref. [20]). Strong survivin expression is a common feature of invasive and metastatic melanoma [21], and targeting of survivin in a melanoma cell line using antisense or a dominant-negative mutant is sufficient to cause apoptosis [21]. More recently, livin – also, like survivin a member of the inhibitor of apoptosis protein (IAP) family – was identified [22]. Livin inhibits caspase activity and binds to caspase-3, -7 and -9 (Ref. [22]). Expression of survivin could not rescue cells from apoptosis triggered by livin antisense and *vice versa*, suggesting that livin and survivin are not redundant, but are distinct and play important roles in regulating apoptosis [22].

Tumor necrosis factor- (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily of cytokines, which, like TNF-α, Fas ligand (FasL) and CD40 ligand (CD40L) can induce apoptotic cell death in a variety of cell types [23]. TRAIL appears to be particularly important because it can induce apoptosis in a variety of cultured malignant cells but not normal tissues [24 and 25]. TRAIL induces apoptosis of melanoma cells by recruitment of mitochondrial pathways that are dependent on caspase-8 (Refs [26], [27]). Induction of apoptosis by TRAIL appears to be mediated by interaction with two death receptors (DR) on cells referred to as TRAIL-R1–DR4 and -R2–DR5 (see Ref. [23] for alternative nomenclature). These receptors were found to be widely expressed on normal tissues, but the latter are believed to be protected from apoptosis by two additional inhibitory (decoy) receptors, TRAIL-R3–DcR1 and TRAIL-R4–DcR2 (Ref. [23]). A series of studies showed that TRAIL is able to induce apoptosis in the majority of melanoma cell lines tested [28 and 29]. Moreover, a correlation between expression of TRAIL death receptors (TRAIL-R1 or -R2) on melanoma and their susceptibility to TRAIL-induced apoptosis, but no correlation with TRAIL decoy receptor (TRAIL-R3 or -R4) expression was found [25, 29 and 30]. In this context, Griffith and colleagues showed recently the potential use for an adenovirus-encoding TRAIL in the gene therapy of melanoma [27].

Apoptosis and novel strategies for melanoma therapy

These observations could have profound repercussions on the future treatment of invasive and metastasizing melanoma. Because apoptotic programs can be manipulated experimentally to produce massive changes in cell death, the genes and proteins controlling apoptosis are attractive, yet still speculative drug targets. Many established, empirically derived cytotoxic drugs already target apoptosis, albeit indirectly and non-discriminately, in that they are also mutagenic and toxic to normal tissues. By contrast, agents that induce apoptosis directly might provide less opportunity for acquired drug resistance, decrease mutagenesis and reduce toxicity. Awealth of evidence suggests that such strategies are feasible [4 and 5]. More recently, powerful models have been emerging, for example, artificial skin reconstructs and orthotopic skin grafts [31], which will allow us to dissect more accurately the pathways and events of the melanoma apoptotic machinery. Although there is much more to learn, we anticipate that, in the near future, this information will produce new, rational strategies to exploit apoptosis for therapeutic benefit.

References

1. M. Herlyn and K. Satyamoorthy , Molecular biology of cutaneous melanoma. In: V.T. DeVita, Jr *et al.Cancer: Principles and Practice of Oncology*, Lippincott Williams & Wilkins (2001), pp. 2003–2012.

2. D. Hanahan and R.A. Weinberg , The hallmarks of cancer. *Cell* 100 (2000), pp. 57–70.

3. J.F. Kerr *et al.*, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26 (1972), pp. 239–257.

4. C.A. Schmitt and S.W. Lowe , Apoptosis and therapy. *J. Pathol.* 187 (1999), pp. 127–137.

5. S.W. Lowe and A.W. Lin , Apoptosis in cancer. *Carcinogenesis* 21 (2000), pp. 485–495.

6. V.P. Skulachev , Cytochrome *c* in the apoptotic and antioxidant cascades. *FEBS Lett.* 423 (1998), pp. 275–280.

7. P. Li *et al.*, Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91 (1997), pp. 479–489.

8. X. Luo *et al.*, Bid, a Bcl-2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 94 (1998), pp. 481–490.

9. Y. Hu *et al.*, Bcl-xL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc. Natl. Acad. Sci. USA* 95 (1998), pp. 4386–4391.

10. J.M. Adams and S. Cory , Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem. Sci.* 26 (2001), pp. 61–66.

11. D.D. Newmeyer *et al.*, Bcl-xL does not inhibit the function of Apaf-1. *Cell Death Differ.* 7 (2000), pp. 402–407.

12. M.S. Soengas *et al.*, Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science* 284 (1999), pp. 156–159.

13. R.R. Wallace-Brodeur and S.W. Lowe , Clinical implications of *p53* mutations. *Cell Mol. Life Sci.* 55 (1999), pp. 64–75.

14. F.G. Giancotti and E. Ruoslahti , Integrin signaling. *Science* 285 (1999), pp. 1028–1032.

15. A. Ziegler *et al.*, Sunburn and p53 in the onset of skin cancer. *Nature* 372 (1994), pp. 773–776.

16. D. Guerry *et al.*, Lessons from tumor progression: the invasive radial growth phase of melanoma is common, incapable of metastasis, and indolent. *J. Invest. Dermatol.* 100 (1993), pp. 342S–345S.

17. K. Satyamoorthy *et al.*, Aberrant regulation and function of wild-type p53 in radioresistant melanoma cells. *Cell Growth Differ.* 11 (2000), pp. 467–474.

18. M.S. Soengas *et al.*, Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature* 409 (2001), pp. 207–211.

19. G. Ambrosini *et al.*, A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med.* 3 (1997), pp. 917–921.

20. I. Tamm *et al.*, IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res.* 58 (1998), pp. 5315–5320.

21. D. Grossman *et al.*, Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. *J. Invest. Dermatol.* 113 (1999), pp. 1076–1081.

22. G.M. Kasof and B.C. Gomes , Livin, a novel inhibitor-of apoptosis (IAP) family member. *J. Biol. Chem.* 9 (2000), p. 9.

23. A. Ashkenazi and V.M. Dixit , Apoptosis control by death and decoy receptors. *Curr. Opin. Cell Biol.* 11 (1999), pp. 255–260.

24. S.R. Wiley *et al.*, Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 3 (1995), pp. 673–682.

25. X.D. Zhang *et al.*, Relation of TNF-related apoptosis-inducing ligand (TRAIL) receptor and FLICE-inhibitory protein expression to TRAIL-induced apoptosis of melanoma. *Cancer Res.* 59 (1999), pp. 2747–2753.

26. W.D. Thomas *et al.*, TNF-related apoptosis-inducing ligand-induced apoptosis of melanoma is associated with changes in mitochondrial membrane potential and perinuclear clustering of mitochondria. *J.Immunol.* 165 (2000), pp. 5612–5620.

27. T.S. Griffith *et al.*, Adenoviral-mediated transfer of the TNF-related apoptosis-inducing ligand/Apo-2 ligand gene induces tumor cell apoptosis. *J. Immunol.* 165 (2000), pp. 2886–2894.

28. W.D. Thomas and P. Hersey , TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. *J.Immunol.* 161 (1998), pp. 2195–2200.

29. X.D. Zhang *et al.*, Differential localization and regulation of death and decoy receptors for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. *J. Immunol.* 164 (2000), pp. 3961–3970.

30. T.S. Griffith *et al.*, Functional analysis of TRAIL receptors using monoclonal antibodies. *J.Immunol.* 162 (1999), pp. 2597–2605.

31. K. Satyamoorthy *et al.*, Human xenografts, human skin and skin reconstructs for studies in melanoma development and progression. *Cancer Metastasis Rev.* 18 (1999), pp. 401–405.

Mutation and expression of the low affinity nerve growth factor receptor in human malignant melanoma

Melanoma Res

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Mutation and expression of the low affinity neurotrophin receptor in human malignant melanoma

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The low affinity p75 neurotrophin receptor ($p75^{NTR}$) is a cysteine-rich transmembrane glycoprotein which is frequently overexpressed in advanced stages of human melanoma. The biological consequences of this overexpression are unknown; however, it has recently been shown that $p75^{NTR}$ can enhance the invasive potential of melanoma cells *in vitro*. In the present study we examined cell lines established from normal human melanocytes and metastatic melanomas for expression of $p75^{NTR}$ mRNA and protein. The results showed that, compared with normal melanocytes, levels of $p75^{NTR}$-specific protein were high in seven melanoma lines, markedly decreased in two melanoma lines and comparable in two melanoma lines. The conserved transmembrane domain of $p75^{NTR}$ was analysed for point mutations by single strand conformation polymorphism analysis and direct DNA sequencing. Identical point mutations were detected in the transmembrane domain of $p75^{NTR}$ in the two melanoma lines with reduced $p75^{NTR}$ protein expression, which resulted in the substitution of the uncharged amino acid Gly for the negatively-charged Asp.

Key words: Melanocyte, melanoma, neurotrophin, p75 receptor.

Introduction

The incidence of malignant melanoma of the skin is undergoing a dramatic increase in persons with light-coloured skin in all parts of the world.[1,2] At a molecular level, the stepwise evolution from normal diploid melanocytes to primary and metastatic melanoma involves a poorly understood series of genotypic and phenotypic alterations.[3] A number of studies have documented a large array of accumulated alterations in the expression and function of oncogenes, tumour suppressor genes, growth factors, cytokines, transcription factors, tyrosine kinases, proteolytic enzymes, angiogenic factors, chemotactic factors, integrins and adhesion molecules during the transition from normal melanocyte to metastatic cell (as reviewed elsewhere[3–6]). It is speculated that these alterations play important roles in the pathogenesis of melanoma by perturbing a diverse set of biological functions that includes DNA integrity, signal transduction, cell cycle control and differentiation.

One family of genes with potential importance in melanoma progression are the neurotrophins [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5)] and their cognate receptors: tyrosine kinase receptors encoded by the Trk family of related genes (Trk A, Trk B and Trk C), and the low affinity receptor, $p75^{NTR}$.[7] Expression of $p75^{NTR}$ has been shown to be increased in invasive malignant melanomas in comparison to normal melanocytes.[8,9] The significance of this observation was unclear until a recent study showed that NGF can stimulate a dose-dependent enhancement of invasion by melanoma cells through reconstituted basement membranes, and that this effect is mediated through $p75^{NTR}$ independent of Trk A.[10] Although the ability of $p75^{NTR}$ to signal transduce in response to NGF ligand independently of Trk has been disputed,[11] it has now been demonstrated that $P75^{NTR}$ signals use the sphingomyelin cycle.[12] Thus, elevated levels of $p75^{NTR}$ expression

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in melanomas, serving as a receptor for members of the neurotrophin family, may promote the invasive phenotype.[10]

The low affinity receptor $p75^{NTR}$ shows homology to a family of cell surface receptors that includes the apoptosis-mediating Fas cell surface antigen and the type I and II receptors for tumour necrosis factor (TNFR).[13] Point mutations in the cytoplasmic domain of the Fas antigen results in lymphoproliferative disorders in mice.[14] Presumably, defects in this highly conserved region in $p75^{NTR}$ would also affect receptor-related function. The present study examined the transmembrane domain in the $p75^{NTR}$ gene for structural defects, and analysed $p75^{NTR}$ protein levels in normal melanocyte cultures and metastatic melanoma cell lines.

Materials and methods

Cell lines

Human metastatic melanoma cell lines (SK-MEL) and foreskin melanocyte cell lines (FSM) were derived and maintained as previously described.[15,16]

Western blot analysis

Cells in logarithmic phase of cell growth were rinsed in phosphate-buffered saline (PBS) and then lysed in RIPA buffer (10 mM Tris, pH 7.4, 5 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% aprotinin, 2.5 μg/ml leupeptin, 2.5 μg/ml antipain, 250 μg/ml PMSF, 1 mm Na_3VO_4).[17] Lysates were cleared by centrifugation, protein concentrations measured with the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA) and 50 μg of total protein from each line fractionated on 10% polyacrylamide gels containing sodium dodecyl sulphate (SDS-PAGE) in Tris/glycine/SDS buffer. Following electrophoresis, proteins were transferred to nitrocellulose (BA-S, Schleicher & Schuell, Keene, NH, USA) in a Hoefer Scientific transfer apparatus as described previously.[18] Representative gels were stained with Coomassie blue to confirm equal amounts of protein in each lane. Western blots were blocked for 2–6 h at room temperature with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST). Blots were transferred to heat-sealed bags, rotated overnight at 4°C in 1.5 μg/ml of anti-$p75^{NTR}$ antibody,[19] prepared in 5% milk/TBST/0.01% sodium azide, washed 3 × 10 min in TBST at room temperature, reblocked for 30 min in 5% milk/TBST and incubated for 1 h at room temperature in 1 : 3000 dilution of secondary antibody (horseradish peroxidase-coupled sheep anti-murine IgG; Amersham Corp., Arlington Heights, IL, USA) in 5% milk/TBST. After washing three times in TBST, blots were incubated with enhanced chemiluminescent (ECL) detection reagents as described by the manufacturer (Amersham). $p75^{NTR}$ protein was detected by autoradiography by exposure of blots to Kodak XAR film for 2–15 min.

Polymerase chain reaction (PCR)

Isolated RNA was reverse transcribed into cDNA using MuLV Resverse Transcriptase (Gibco-BRL) as descibed elsewhere.[18] PCR amplification was performed as described previously.[16,20] Briefly, cDNA was heated to 95°C for 10 min, cooled on ice for 5 min, and then 1 μl added to a 25 μl reaction mixture containing 2.5 μl of 10 × PCR reaction buffer containing 100 mM $(NH_4)_2SO_4$, 0.2 M Tris, pH 8.5, 20 mM $MgCl_2$, 1000 μg/ml BSA, 1% Triton X-100 (Stratagene, La Jolla, CA, USA), 9.6 μM each of dATP, dCTP, dGTP and dTTP (Pharmacia), 150 ng of each priming oligomer, 1.0 unit pfu polymerase (Stratagene) and water. Reaction mixes were prepared for multiple samples and aliquoted. A negative control consisting of an aliquot without cDNA was included in each amplification. Amplification was performed using MJR Thermal Cycler (MJR Inc., Watertown, MA, USA) for 35–50 cycles. A cycle profile consisted of 1 min at 95°C for denaturation, 1 min at 55–65°C for annealing and 1 min at 72°C for primer extension. Optimal annealing temperatures were defined for each primer pair. Electrophoresis of 10 μl of reaction mixture on a 1% SeaKem agarose (FMC Bioproducts, Rockland, ME, USA) gel containing ethidium bromide was performed to evaluate amplification and size of fragments generated.

Oligonucleotide primers were synthesized using a DNA Synthesizer 380A (Applied Biosystems, Foster City, CA, USA). Gene sequences used to construct oligonucleotide primers were from published sources. Primers were designed so that amplified cDNA would include at least two exons. To evaluate efficiency of cDNA preparation, PCR was performed using 5′ amd 3′ β-actin primers as described.[16] The primer sequences for $p75^{NTR}$ were:[21]

5′-AGCCTGAGGCACCTCCAGAA-3′ (nt 754)
5′-TGGCTCCTTGCTTGTTCTGC-3′ (nt 969)
5′-GCCTACATAGCCTTCAAGAG-3′ (nt 915)
5′-AGCGACAGTGGCATCTCCGT-3′ (nt 1020)
5′-GGCAGGCCTCATGGGTAAAG-3′ (nt 1257)
5′-CTACCAGCCCGAGCACATAG-3′ (nt 1214)
5′-TGGACTCACTGCACAGACTC-3′ (nt 1377)
5′-ATTCCGACGAGGCCAACCAC-3′ (nt 574)

Single strand conformation polymorphism (SSCP) analysis

SSCP analysis was performed as described previously.[22,23] cDNA was used as template for amplification using pfu heat-stable DNA polymerase,[24] and PCR performed as described elsewhere.[20,25] Ten microlitres from each PCR reaction was heated at 90°C for 5 min, chilled in wet ice and loaded in an Mutation Detection Enhancement (HydroLink MDE Gel; J.T. Baker, Inc., Phillipsburg, NJ, USA) and electrophoresed at 6 W, constant power, overnight. After electrophoresis, the gel was dried for 17 h at 80°C and exposed to Kodak XAR film.

Sequencing of PCR products

Pfu-amplified products corresponding to the transmembrane domain of $p75^{NTR}$ (produced by using the 574/969 primer pair), were purified as described[26] and cloned into the PCR-script vector as per the manufacturers' recommendations (Stratagene). Plasmid DNA containing an insert was obtained from 20–30 colonies and pooled in order to obtain a consensus sequence. DNA sequencing was done using a dsDNA Cycle-Sequencing kit (Gibco BRL, Gaithersburg, MD, USA) as described.[27]

Results

Expression of $p75^{NTR}$ in melanomas

Expression of $p75^{NTR}$ protein was examined by Western blot in 11 metastatic melanoma cell lines and three normal melanocyte cultures. In seven of 11 melanoma cell lines, $p75^{NTR}$ was over-expressed 3–6-fold (as determined by densitometric analysis) when compared with three normal melanocyte cultures; in two of 11 cell lines $p75^{NTR}$ was expressed at levels comparable to that seen in melanocytes; and in two of 11 $p75^{NTR}$ was markedly decreased. Figure 1 shows representative data. A Northern blot analysis (not shown) indicated that the level of $p75^{NTR}$ protein in all cell lines correlated well with mRNA levels, indicating that the differences in $p75^{NTR}$ protein levels in melanoma cells are most likely due to altered transcriptional control, and not to translational or post-translational mechanisms.

Detection of mutations in $p75^{NTR}$ by SSCP analysis

In order to determine the existence of structural defects in the $p75^{NTR}$ gene, the conserved transmembrane domain was examined for point mutations by SSCP analysis and direct DNA sequencing in the 11 metastatic melanoma cell lines discussed above and the three normal melanocyte cultures shown in Figure 1. Aberrant mobility shifts of DNA fragments spanning nucleotides 754–969 and comprising the transmembrane domain were detected in three of the 11 melanoma lines (SK-MEL-94, SK-MEL-110 and SK-MEL-147). SK-MEL-94 and SK-MEL-147 are shown in Figure 2. The other melanoma cell lines examined exhibited a SSCP pattern for the transmembrane region identical to that observed in normal melanocyte DNA.

Detection of mutations in $p75^{NTR}$ by direct sequencing

The presence of nucleotide alterations in the transmembrane region domain of the $p75^{NTR}$ gene in SK-MEL-94 and SK-MEL-110 was confirmed by direct cDNA sequencing of $p75^{NTR}$ from these cell lines and from DNA extracted from noncultured autologous normal skin tissue specimens. Figure 2 shows that SK-MEL-94 and SK-MEL-110 melanoma cells contained an identical missense mutation at bp 802, which resulted in a nonconservative amino acid substitution of Gly → Asp. To confirm that the detected mutations at 802 bp were not the result of polymerase infidelity or cross-contamination, both the sense and antisense strands of at least three independent PCR clones for both SK-MEL-94 and SK-MEl-110 were sequenced using pfu DNA polymerase. Direct sequencing of DNA extracted from noncultured normal skin specimens from the two patients whose melanomas were

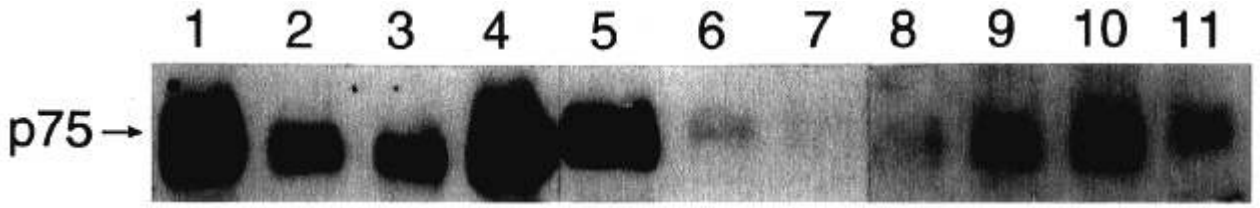

Figure 1. Expression of $p75^{NTR}$ protein in melanocytic cells: total cell lysate (75 μg) from metastatic melanoma (SK-MEL) and normal melanocyte (FSM) cell lines was analysed by Western blot. Lanes: 1, SK-MEL-93/DX3; 2, SK-MEL-119; 3, MeWo; 4, SK-MEL-147; 5, Sk-MEL-146; 6, SK-MEL-94; 8, SK-MEL-110; 9, FSM-274; 10, FSM-323; 11, FSM-329. As compared with normal melanocytes (lanes 9–11), levels of $p75^{NTR}$ protein are increased (lanes 1–5), comparable (lane 6) or decreased (lanes 7 and 8).

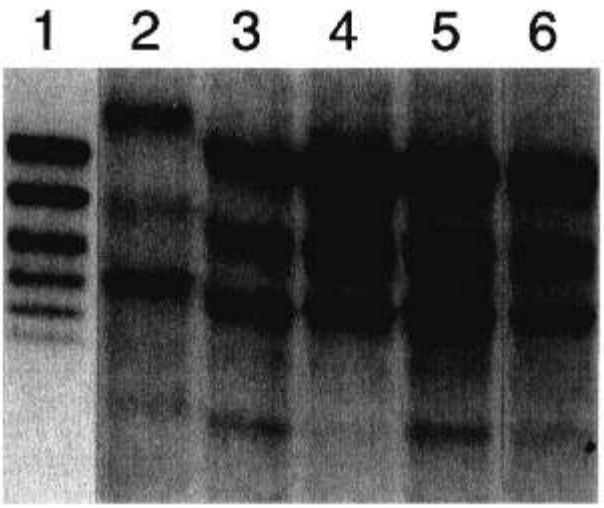

Figure 2. Single strand conformation poylmorphism analysis of $p75^{NTR}$ transmembrane domain : autoradiogram of polyacrylamide gel electrophoresis of ^{32}P-labelled DNA fragments containing the transmembrane domain of the human $p75^{NTR}$ gene. cDNAs were isolated from melanomas and amplified using exon-specific primers. Lanes: 1, SK-MEL-147; 2, SK-MEL-94; 3, SK-MEL-28; 4, SK-MEL-93/DX3; 5, SK-MEL-119; 6, normal cultured prostate cell culture control.

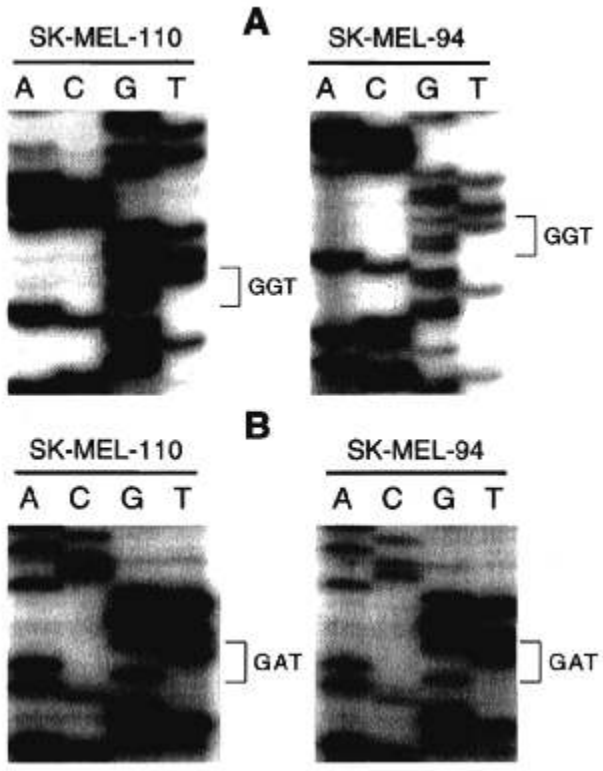

Figure 3. DNA sequence analysis of $P75^{NTR}$ transmembrane domain. **A** Sequence of DNA extracted from autologous normal skin of patients from whose melanoma SK-MEL-110 and SK-MEL-94 were derived. **B** Sequence of DNA extracted from SK-MEL-110 and SK-MEL-94 showing exon 4 mutation at nt 802.

used to derive SK-MEL-110 and SK-MEL-94 confirmed that the mutations at bp 802 were not the result of an unidentified polymorphism (see Figure 3). Direct sequencing of SK-MEL-147 cDNA revealed that the abnormal SSCP pattern observed resulted from a silent mutation at bp 908 that resulted in no amino acid substitution. Normal DNA from this patient was not available for analysis.

Discussion

The exact role of $p75^{NTR}$ expression in melanoma cells is unclear. High levels of $p75^{NTR}$ protein have been correlated with advanced stages of melanoma progression (i.e. primary invasive and metastatic melanoma) by immunohistochemical staining,[8,9] suggesting a role for this receptor and/or its ligand NGF in the progression of melanoma. Another study has showed that the degree of extracellular matrix invasion by cultured melanoma cells directly correlated with the levels of $p75^{NTR}$ expression and with NGF-induced secretion of collagenase and heparanase.[10] Thus, a model has been proposed for NGF-stimulated invasion of melanoma cells which is signalled through $p75^{NTR}$, and which may occur independent of Trk via the sphingomyelin and ceramide signal transduction pathway.[10,12]

Structural and functional similarities between $p75^{NTR}$ and apoptosis-inducing receptors Fas (apo-1) and TNFR I/II have been noted.[28] Point mutations in the cytoplasmic domain of the Fas antigen results in lymphoproliferative disorders in mice by blocking the natural apoptotic pathway of B lymphocytes.[14] In addition, partial nucleotide deletions of the transmembrane domain of the Fas antigen results in the expression of a soluble Fas receptor that protects cells from Fas-mediated apoptosis.[29] In this regard, studies have also implicated $p75^{NTR}$ in the apoptotic pathway: in selective neuronal cells $p75^{NTR}$ unbound to its ligand NGF can enhance apoptosis.[28]

The cytoplasmic domains of the TNF receptor family members do not contain any discernible enzymatic activity, but may serve signalling functions. For $p75^{NTR}$, the transmembrane and cytoplasmic domains are highly conserved between species. More than 90% homology exists in this region between chicken, rat and human $p75^{NTR}$,[30] which is greater than the homology that exists in the extacellular domain of the molecule. The highly conserved nature of the membrane–spanning and intracellular domain of the receptor suggests a functional role for these domains in neurotrophin signalling.

We report here for the first time the detection of point mutations in the transmembrane domain of $p75^{NTR}$ in human melanomas. These mutations occurred in a conserved region near the transmembrane domain at codon

802. The effect resulting from this mutation is unclear. It is possible that a conformational change in the structure of the transmembrane secondary to substitution of an uncharged amino acid (Gly) for a negatively charged one (Asp) could alter the affinity of the $p75^{NTR}$ for its ligand, resulting in an inhibition of apoptotic function. Alternatively, a mutated cytoplasmic domain could result in aberrant signal transduction via sphingomyelin and ceramide. Since p75 binds to all neurotrophins, a potential role may be to provide additional specificity to the interactions of neurotrophins with individual trk family members. The expression of high levels of p75 appears to be an important factor for enhancing the ability of $p140^{trk}$ to respond to NGF at low concentrations of ligand.[7,31,32] It is also plausible that mutations in the transmembrane and cytoplasmic domain may lead to alterations in the interaction with other membrane associated proteins or cytoskeletal elements which influence signal transduction. The conserved nature of these sequences, together with experimental finds that this domain is crucial for ligand binding;[33] cell survival in fibroblast cells,[34] and signal transduction,[12,35] indicate that a change in the p75 amino acid sequence may influence neurotrophin function. These effects may be different, depending upon the co-expression of trk family members and the cell type that $p75^{NTR}$ is found to be expressed.

Western blot analysis showed that the melanoma cell lines with p75 transmembrane mutations also had decreased $p75^{NTR}$ protein levels. The low levels of $p75^{NTR}$ protein appear to be due to decreased transcription since Northern blot analysis confirmed a concomitant decrease in mRNA levels in these melanomas. Whether the detected mutations affect RNA stability or processing is unknown and under investigation. Previous studies with a truncated p75 construct in PC12 cells indicated that the levels of the wild type p75 receptor was influenced by overexpression of the mutant receptor.[36] A defect in $p75^{NTR}$, therefore, could potentially affect the biosynthesis of this receptor. Moreover, since some melanomas produce neurotrophins, it is possible that these stimulate the growth of melanoma cells via an autocrine loop involving the p75 receptor.[37] Further functional studies are required to define the effects of mutations on $p75^{NTR}$ function and the biology of melanomas.

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References

1. Elwood JB, Koh HK. Etiology, epidemiology, risk factors, and public health issues of melanoma. *Curr Opin Oncol* 1994; **6**: 179–187.
2. Armstrong BK, Kricker A. Cutaneous melanoma. *Cancer Surv* 1994; **19**: 219–240.
3. Albino AP, Fountain JW. The molecular genetics of malignant melanoma. In: Nathanson L, ed. *Cancer Treatment and Research: Basic and Clinical Aspects of Malignant Melanoma*. Boston: Martinus Nijhoff Publishers, 1993; 210–255.
4. Halaban R, Moellmann G. Proliferation and malignant transformation of melanocytes. *Crit Rev Oncog* 1991; **2**: 247–258.
5. Mattei S, Colombo MP, *et al.* Expression of cytokine/growth factors and their receptors in human melanoma and melanocytes. *Int J Cancer* 1994; **56**: 853–857.
6. Rodeck U. Growth factor independence and growth regulatory pathways in human melanoma development. *Cancer Metastasis Rev* 1993; **12**: 219–226.
7. Chao MV. The p75 neurotrophin receptor. *J Neurobiol* 1994; **25**: 1373–1385.
8. Ross AH, Grob P, Bothwell M, *et al.* Characterization of nerve growth factor receptor in neural crest tumors using monoclonal antibodies. *Proc Natl Acad Sci USA* 1984; **81**: 6681–6685.
9. Herlyn M, Thurin J, Balaban G, *et al.* Characteristics of cultured human melanocytes isolated from different stages of tumor progression. *Cancer Res* 1985; **45**: 5670–5676.
10. Herrmann JL, Menter DG, Hamada J-I, *et al.* Mediation of NGF-stimulated extracellular matrix invasion by the human melanoma low-affinity p75 neurotrophin receptor: melanoma p75 functions independently of trkA. *Mol Biol Cell* 1993; **4**: 1205–1216.
11. Barbacid M. Nerve growth factor: a tale of two receptors. *Oncogene* 1993; **8**: 2033–2042.
12. Dobrowsky RT, Werner MH, Castellino AM, *et al.* Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. *Science* 1994; **265**: 1596–1599.
13. Tartaglia LA, Goeddel DV. Two TNF receptors. *Immunol Today* 1992; **13**: 151–153.
14. Takahashi T, Tanaka M, Brannan CI, *et al.* Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* 1994; **76**: 969–976.
15. Houghton AN, Eisinger M, Albino AP, *et al.* Surface antigens of melanocytes and melanomas. Markers of melanocyte differentiation and melanoma subsets. *J Exp Med* 1982; **156**: 1755–1766.
16. Albino AP, Davis BM, Nanus DM. Induction of growth factor RNA expression in human malignant melanoma: markers of transformation. *Cancer Res* 1991; **51**: 4815–4820.
17. Sudol M, Hanafusa H. Cellular proteins homologous to the viral yes gene product. *Mol Cell Biol* 1986; **6**: 2839–2846.
18. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989.
19. Huber LJ, Chao MV. Mesenchymal and neuronal expression of the p75 neurotrophin receptor occur from different mechanisms. *Dev Biol* 1995; **167**: 227–238.
20. Saiki RK, Scharf S, Faloona F, *et al.* Enzymatic amplification of beta-globin benomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985; **230**: 1350–1354.

21. Sehgal A, Patil N, Chao MV. A constitutive promoter directs expression of the nerve growth factor receptor gene. *Mol Cell Biol* 1988; **8**: 3160–3167.
22. Orita M, Suzuki Y, Sekiya T, *et al.* Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 1989; **5**: 874–879.
23. Albino AP, Vidal M, McNutt NS, *et al.* Mutation and expression of the p53 gene in human malignant melanoma. *Melanoma Res* 1994; **4**: 35–45.
24. Saiki RK, Gelfand DH, Stoffel S, *et al.* Primer-directed enzymatic amplication of DNA with a thermostable DNA polymerase. *Science* 1988; **239**: 487–491.
25. Albino AP, Nanus DM, Mentle IR, *et al.* Analysis of ras oncogenes in malignang melanoma and precursor lesions: correlation of point mutations with diferentiation phenotype. *Oncogene* 1989; **4**: 1363–1374.
26. Dicker AP, Volkenandt M, Albino AP. Mutational analysis of human NRAS genes in malignant melanoma: rapid methods for oligonucleotide hybridization and manual and automated direct sequencing of products generated by the polymerase chain reaction. *Genes Chromosomes Cancer* 1990; **1**: 257–269.
27. Murray V. Improved double-stranded DNA sequencing using the linear polymerase chain reaction. *Nuc Acids Res* 1989; **17**: 8889.
28. Rabizadeh S, Oh J, Zhong L-T, *et al.* Induction of apoptosis by the low-affinity NGF receptor. *Science* 1993; **261**: 345–348.
29. Cheng J, Zhou T, Liu C, *et al.* Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science* 1994; **263**: 1759–1762.
30. Large TH, Weskamp G, Helder JC, *et al.* Structure and developmental expression of the nerve growth factor receptor in the chicken central nervous system. *Neuron* 1989; **2**: 1123–1134.
31. Barker PA, Shooter EM. Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. *Neuron* 1994; **13**: 203–215.
32. Verdi JM, Birren SJ, Ibanez CF, *et al.* p75-lngfr regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. *Neuron* 1994; **12**: 733–745.
33. Hempstead BL, Patil N, Thiel B, *et al.* Deletion of cytoplasmic sequences of the nerve growth factor receptor leads to a loss of high affinity ligand binding. *J Biol Chem* 1990; **265**: 9595–9598.
34. Hantzopoulos PA, Suri C, Glass DJ, *et al.* The low affinity NGF receptor, p75, can collaborate with each of the trks to potentiate functional responses to the neurotrophins. *Cell* 1994; **13**: 187–201.
35. Yan H, Chao MV. Disruption of cysteine-rich repeats of the p75 nerve growth factor receptor leads to loss of ligand binding. *J Biol Chem* 1991; **266**: 12099–12104.
36. Benedetti M, Levi A, Chao MV. Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. *Proc Natl Acad Sci USA* 1993; **90**: 7859–7863.
37. Sherwin SA, Sliski AH, Todaro GJ. Human melanoma cells have both nerve growth factor and nerve growth factor-specific receptors on their cell surfaces. *Proc Natl Acad Sci USA* 1979; **76**: 1288–1292.

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Eruptive Multiple Lentigo-Maligna-Like Lesions in a Patient Undergoing Chemotherapy with an Oral 5-Fluorouracil Prodrug for Metastasizing Colorectal Carcinoma: A Lesson for the Pathogenesis of Malignant Melanoma?

7

***Dermatology* 2002; 205:174-175**

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Abstract

Induction of multiple eruptive dermal and atypical melanocytic naevi has frequently been reported in children with malignant haematological diseases and chemotherapy-induced immunosuppression. This is the first report of an adult patient to develop multiple eruptive melanocytic skin lesions while undergoing chemotherapy with an oral 5-fluorouracil prodrug for metastasizing cancer. Our observation adds further evidence to the link between systemic (iatrogenic or intrinsic) immunosuppression and the induction of melanocyte proliferation and transformation.

Introduction

Induction of multiple eruptive dermal and atypical melanocytic naevi has frequently been reported in children with malignant haematological diseases and chemotherapy-induced immunosuppression [1, 2]. Atypical eruptive melanocytic naevi preceding malignant melanoma have also been described in immunocompromised transplant patients [3]. In contrast, the induction of melanocytic skin lesions in adult cancer patients undergoing chemotherapy for metastatic disease has not yet been related. However, melanoma occurring as a second cancer has been recognized for patients that had had previous chemotherapy or radiation for another cancer [4, 5].

Case Report

A 56-year-old male patient was first diagnosed in January 1998 as having colorectal carcinoma metastasizing to the liver, abdominal lymph nodes and the peritoneum (pT_4, pN_3, G_3, M_1, R_1). While undergoing chemotherapy with capecitabine (N^4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine; Ro 09-1978) [6], an oral 5-fluorouracil (5-FU) prodrug, he developed about a dozen eruptive pigmented lesions. The lesions had suddenly appeared during the first cycles of treatment, administered twice daily at 2,500 mg/m^2/day as intermittent therapy (2 weeks treatment, 1 week rest), resulting in a cumulative dose of 910 g. They were found predominantly on the ear helices and the neck (Fig. 1), dermatoscopically resembling lentigo maligna (LM) and atypical melanocytic naevi. During the following summer, the patient also observed a tendency to tan more easily than in the past.

Fig. 1. Multiple LM-like skin lesions on the free rim of the ear helix in a 56-year-old patient undergoing chemotherapy with capecitabine for metastasizing colon carcinoma.

Histopathological examination of 6 separate skin biopsies over the course of 1 year consistently revealed a proliferation of pigmented atypical melanocytic cells arranged in solitary units and small nests within an atrophic epidermis and a marked solar elastosis (Fig. 2), features necessary for the diagnosis of LM [7]. Additionally, in 4 specimens, nests of inconspicuous melanocytes could be detected in the upper dermis. Melanocytes showed positivity for S-100 and, partially, for HMB-45 staining. In the dermis, fibrosis and a perivascular lymphohistiocytic infiltrate with abundant melanophages could be detected.

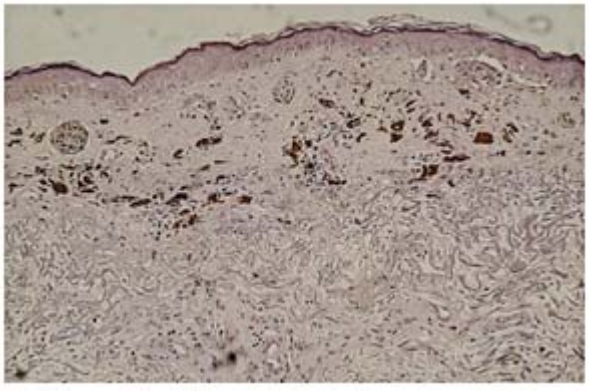

Fig. 2. Biopsy specimen revealing typical features of LM: proliferation of strongly pigmented atypical melanocytic cells within an atrophic epidermis and a marked solar elastosis as well as dermal fibrosis and a perivascular lymphohistiocytic infiltrate with abundant melanophages. Haematoxylin-eosin. ×40.

Routine laboratory testing showed normal values, except GOT (ASAT) 20 U/l, GPT (ALAT) 28 U/l and bilirubin 1.65 mg/dl. Tumour-associated parameters CEA and CA 19-9 were within the normal range.

Regular CT scans showed that the patient had stable disease over 24 months, with a Karnofsky performance status of 100%. After 16 cycles (cumulative dose 1,120 g), chemotherapy was discontinued, and subsequently the remaining pigmented skin lesions cleared within several months.

Discussion

Capecitabine, an orally administered prodrug of 5-FU, is activated by a cascade of three enzymes, resulting in the preferential conversion to 5-FU at the tumour site [6]. It was developed in an attempt to avoid the problem of gastro-intestinal, neurological and myelosuppressive toxicity of fluoropyrimidines and has undergone multicentric phase III clinical studies. A well-known side-effect of 5-FU on the skin is the 'hand-foot syndrome' or palmar-plantar erythrodysaesthesia [8], but the development of melanocytic naevi has not yet been specific.

Greene et al. [3] postulated that in systemic immunosuppression, destruction of tumour-specific lymphocytes by cytotoxic or immunosuppressive agents may lead to the formation of atypical naevi or malignant melanoma. Hughes et al. [2] believe that the increased naevus count in children having undergone chemotherapy was related to the immunosuppressive effects of treatment rather than to the underlying disease, because patients who had not yet completed chemotherapy had significantly fewer naevi. Despite typical histopathological features of LM, clinically the multiplicity of the eruptive lesions in our patient indicates rather an activation of melanocytes in sun-exposed areas and therefore these lesions might be regarded as clinical and histopathological simulators of LM.

Our observation is of importance for two reasons: firstly, it underlines the existence of a histological simulator of LM, as there has been found for in situ superficially spreading melanoma after experimental ultraviolet irradiation of melanocytic naevi [9]; secondly, an adult patient to develop eruptive melanocytic skin lesions while undergoing chemotherapy for metastatic cancer with 5-FU has previously not been described. Thus, this adds further evidence to the link between systemic (iatrogenic or intrinsic) immunosuppression and the induction of melanocyte proliferation and transformation.

References

1 Richert S, Bloom EJ, Flynn K, Seraly MP: Widespread eruptive dermal and atypical melanocytic nevi in association with chronic myelocytic leukemia: A case report and review of the literature. J Am Acad Dermatol 1996;35:326-329.

2 Hughes B, Cunliffe W, Bailey C: Excess benign melanocytic naevi in children after chemotherapy for malignancy in childhood. Br Med J 1989;299:88-91.

3 Greene MH, Young TL, Clark WH: Malignant melanoma in renal transplant recipients. Lancet 1981;i:1196-1199.

4 Corpron CA, Black CT, Ross MI, Herzog CS, Ried HL, Lally KP, Andrassy RJ: Melanoma as a second malignant neoplasm after childhood cancer. Am J Surg 1996;171:459-462.

5 Chua HC, Mortimer PS: Malignant melanoma occurring as a second cancer following primary germ cell tumour in males: Report of three cases. Br J Dermatol 1992;127:448-449.

6 Bajetta E, Carnaghi C, Somma L, Stampino CG: A pilot safety study of capecitabine, a new oral fluoropyrimidine, in patients with advanced neoplastic disease. Tumori 1996;82:450-452.

7 Cohen LM: Lentigo maligna and lentigo maligna melanoma. J Am Acad Dermatol 1995;33:923-936.

8 Leichman CG, Leichman L, Spears CP, Rosen PJ, Jeffers S, Groshen S: Prolonged continuous infusion of fluorouracil with weekly bolus leukoverin: A phase II study in patients with disseminated colorectal cancer. J Natl Cancer Inst 1993;85:41-44.

9 Tronnier M, Smolle, Wolff HH: Ultraviolet irradiation induces acute changes in melanocytic nevi. J Invest Dermatol 1995;104:475-478.

No non-sentinel node involvement in melanoma patients with limited Breslow thickness and low sentinel node tumor load

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ORIGINAL ARTICLE

No non-sentinel node involvement in melanoma patients with limited Breslow thickness and low sentinel node tumor load

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Key words: melanoma – sentinel lymph node – lymph node staging – S-classification –lymph node metastases

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ABSTRACT

Background: Most melanoma patients with a positive sentinel node (SN) undergo completion lymph node dissection and frequently experience associated morbidity. However, only 10-30% of SN-positive patients have further lymph node metastases. The aim of the present study was to predict the absence of non-SN metastases in a multicenter study of patients with a positive SN based on primary melanoma features and SN tumor load.

Methods: Of 70 SN positive patients, 18 had non-SN metastases. Penetrative depth of metastatic cells into the SN and SN tumor load were assessed by morphometry.

Results: None of the 19 patients (27%) with a Breslow thickness <2.3 mm and a SN tumor load <0.2 mm^2 had non-SN metastases. Similarly, none of the 22 patients (31.4%) with a Breslow thickness <2.3 mm and SN penetrative depth <600 μm had non-SN metastases. Lastly, none of the 19 patients (27%) with a Breslow thickness <2.3 mm and a diameter of the largest SN deposit <500 μm had non-SN metastases.

Conclusions: A combination of limited Breslow thickness and low SN tumor load predicts absence of non-SN metastases in melanoma patients with a positive SN with high accuracy. We propose that this subgroup may be spared completion lymph node dissection.

INTRODUCTION

Sentinel lymph node (SN) biopsy, commonly followed by completion lymph node dissection (CLND) when SNs bear metastases, has become the standard for management of intermediate and high risk-melanoma with clinically negative lymph nodes (i.e., AJCC stage I/II) [1, 2]. SN biopsy provides a reliable, low-morbidity approach to accurate nodal staging with low false-negative rates for patients with melanoma (reviewed in [3]). Moreover, it yields important prognostic information, it can identify patients that might benefit from early nodal dissection [4], and it can define prognostically more homogeneous patient populations for entry into clinical trials [5]

Before the emergence of the SN biopsy, elective lymph node dissection was often considered for patients with intermediate thickness melanoma and clinically negative lymph nodes, although four out of five prospective randomized trials have failed to demonstrate a survival advantage of routine elective lymph node dissection [6]. Some authors therefore hypothesize that a subset most likely to benefit might be those patients with clinically occult micrometastases in the regional nodes, as suggested by another prospective trial [7].

SN biopsy (with CLND only for those with a positive SN) spares approximately 80% of these patients from the morbidity of an elective lymph node dissection, because only 15% to 34% of patients undergoing SN biopsy will have metastases within the SN [1, 4, 8, 9]. Presence of SN metastases is the strongest prognosticator for melanoma [10]. However, several studies have also demonstrated that only 10-30% of patients with a positive SN will have further (non-SN) metastases at CLND [1, 8-15]. In view of the

morbidity associated with CLND, various groups have tried to predict non-SN involvement on the basis of histologic features of the primary tumor [9, 11]. Although the likelihood of non-SN involvement rises with increasing Breslow thickness of the primary melanoma, even tumors less than 2mm in thickness have an 11% risk of having non-SN metastases [11]. Furthermore, previous studies have used SN tumor penetrative depth, tumor volume or metastatic area as well as the microanatomic location of metastases within SN to predict non-SN status [9, 14-21]. Other investigators have proposed a classification system based on depth of metastatic cell invasion and number of tumor-involved 1-mm slices [14, 22] or a size/ulceration score [23]. Others have described reduced density of interdigitating dendritic cells in the SN (as a measure of nodal immune downregulation) as predictor of non-SN status and clinical outcome [24].

Clearly, if non-SN status could be predicted more accurately, the majority of patients with a positive SN might not need a CLND. The above mentioned studies that identified promising factors need however further confirmation to become generally accepted in clinical practice. Hence, this study was designed to determine if negative non-SN status in SN positive melanoma patients can be reliably predicted based on primary melanoma and SN tumor load characteristics, so that these patients could safely be spared CLND and its associated morbidity.

MATERIALS AND METHODS

Pathologic examination of SN and non-SN

Standard SN biopsy was performed as previously described [16, 17] in patients presenting with clinical stage I or II malignant melanoma, as defined by the American Joint Committee on Cancer (AJCC) [25]. SNs were identified using a standard technique of preoperative dynamic lymphoscintigraphy using radiolabeled colloid, followed by intraoperative injection of vital blue dye and use of a hand-held gamma probe (triple technique). SN specimens were processed as previously reported [16, 26]. In brief, after the SN procedure, harvested SN(s) were fixed in neutral buffered formaldehyde, lamellated according to size, and completely embedded in paraffin. SNs smaller than .5 cm were processed and paraffin-embedded intact. Those between .5 and 1.0 cm were halved, and SNs larger than 1.0 cm were lamellated into pieces approximately .5 cm in size. One initial 4-µm-thick H&E-stained section was made per block. When negative, four additional step ribbons were cut at 250-µm intervals. Of each ribbon, one section was stained with H&E, and two were used for S100 and HMB-45 immunohistochemistry. Non-SNs in the CLND specimen were routinely evaluated by H&E staining of the halved lymph nodes and, occasionally, sections immunostained for S100 protein and HMB-45. For the purposes of this study, all SNs were re-evaluated by a pathologist experienced in SNs and a dermatopathologist (P.J. D., M.C.D.). If necessary, further sections were cut and stained so that all patients were assessed using the same sectioning and processing protocol.

The SN metastatic area was determined and its maximum depth from the capsule was measured, as described previously, using an interactive video morphometry system (Q-PRODIT; Leica, Cambridge, UK) [16]. In brief, the SN section containing the largest visible amount of metastatic tumor cells was selected. The surface areas of the individual tumor deposits in this single section were measured, and the SN metastatic area was calculated by summing up these areas. In case of two positive SNs, the one with the largest tumor load was used. The SN metastatic areas were measured by two independent observers, of which one observer measured the areas twice with an interval of approximately 8 weeks. The intraobserver and interobserver reproducibility was good, with Spearman's correlation coefficients of .99 and .98, respectively.

Furthermore, in every SN found to contain metastatic tumor cells, the configuration of the metastatic cells, the number of foci, and their distribution within the lymph node were examined. The metastatic deposit within each sentinel node was classified as subcapsular, combined subcapsular and parenchymal, parenchymal, multifocal, or extensive as described by Dewar et al. [19]. When more than one positive SN had been removed from a single lymph node basin, the most extensive and/or deepest metastatic deposit overall was used to categorize that basin.

S-Staging

Based on SN characteristics, patients were divided into 3 different categories according the S classification (old and new versions) by Starz and co-workers. The

old S classification consists of three categories (S1, S2 and S3) based on the number of positive sections (*n*) and the maximum distance from the interior margin of the biggest metastatic group to the capsule of the SN (*d*). Criteria for these respective categories are as follows: $n \leq 1$ and d ≤1 mm for S1, $n > 2$ and d ≤1 mm for S2 and $n > 2$ and d > 1 mm for S3 [14]. The new S classification exhibits simpler criteria: d ≤0.3 mm for SI, d > 0.3 mm and ≤1 mm for SII and d >1 mm for SIII [27]. The size of the SN tumor burden according to Van Akkooi [18] was also recorded: sub-micrometastases (clusters of more than 10 cells, but <0.1 mm), tumor burden 0.1 mm–1 mm and tumor burden >1 mm. If multiple lesions were present within a SN, the largest lesion was recorded.

Statistical Analysis

Statistical analysis was performed as described previously using SPSS for Windows (SPSS Inc., Chicago, IL) [17] with special emphasis on variables predicting absence of non-SN metastases. Briefly, categorical variables were analyzed by using the X^2 test. Nonparametric data were analyzed by using the Mann-Whitney *U*-test. Pearson's correlation analysis was performed for Breslow thickness and area of metastases in the SN. All variables that were associated with the presence of non-SN metastases by univariate analysis were included in a multivariate logistic regression analysis. Two-sided *P* values <.05 were considered significant.

RESULTS

Patient, tumor, and SN characteristics

Between 1999 and 2006, 350 consecutive patients underwent a SN biopsy for melanoma at the 4 participating institutions. Of these, 70 (20%) patients with cutaneous melanoma whose SNs contained metastatic malignant melanoma and subsequently underwent CLND were identified from the databases of the University Medical Center Utrecht (n=18), Radboud University Nijmegen Medical Centre (n=21), St. Antonius Hospital Nieuwegein (n=6), The Netherlands, or the Städtisches Krankenhaus München-Schwabing (n=25), Germany.

Baseline characteristics of all reviewed patients are depicted in Table 1. The average age was 51 years (range 19–79 years). The mean Breslow thickness was 2.66 mm (range 0.7–6.8 mm). More than half of the tumors were on the trunk and 18/70 (26%) were ulcerated.

On average, 1.6 lymph nodes (range 1-6) were removed at each SLND, and 12 lymph nodes (range 1-56) were removed at the CLND. Sixty percent of patients had one identifiable SN, 29% two SNs, and 11% three or more SNs. Eighty-six percent of patients showed one positive SN, 13% two positive SNs, and 1% three positive SNs. Eighteen patients (26%) had positive non-SN at CLND. The distribution of SN characteristics, the S classification [14, 27] and the location according to Dewar [19] are shown in Table 2.

Features predictive of non-SN metastases

Patient age and sex, Clark level, ulceration, and Breslow thickness as a single feature were not related to either depth of invasion of the SN metastasis or non-SN involvement. Within the SNs, the morphological distribution of metastatic tumor cells varied from tiny clusters of melanoma cells lying in the subcapsular sinus to extensive infiltration of the entire lymph node, replacing nearly all of the normal lymphoid tissue. The total surface area of the tumor foci within a lymph node seemed to correlate with their morphological distribution: very small deposits were always found in the subcapsular or marginal sinuses, whereas larger masses tended to involve the adjacent parenchyma or even extended beyond the capsule. The number of sentinel nodes classified into each microanatomic location is listed in Table 2, with 34 (48.6%) in the subcapsular category. There was no significant correlation between the microanatomic location and non-SN involvement. Five patients with only a subcapsular deposit had subsequent non-SN metastases. The number of positive SNs did also not correlate with non-SN involvement. Age and Breslow thickness were not significantly different here, but penetrative depth of the metastatic deposit ($p=0.023$), total metastatic tumor load (0.014) and largest diameter of metastatic SN deposits ($p=0.021$) did show significant differences (Table 3). For penetrative depth of the metastatic deposit, patients with values below 189 μm (12/70 patients, 17.1%) never had non-SN metastases. For total metastatic tumor load, patients with values below 1467 μm^2 (2/70 patients, 2.9%) never had non-SN metastases. For largest diameter of metastatic SN deposits, patients with values

below 43μm (3/70 patients, 4.3%) never had non-SN metastases. In linear regression analysis, the SN size and depth variables correlated with each other, but not with age or Breslow thickness.

In bivariate analysis, combinations of Breslow thickness on the one hand and depth of SN invasion, diameter of the largest SN metastatic deposit and total SN metastatic area on the other identified subgroups of patients without non-SN deposits (Figures 1-3). No positive non-SNs were found in patients with both a Breslow thickness <2.3 mm and an SN depth of invasion of the metastatic deposit < 600 μm (22/70 patients, 31.4%), in patients with both a Breslow thickness <2.3 mm and a total SN metastatic area < 0.2 mm^2 (19/70 patients, 27%), or in patients with both a Breslow thickness <2.3 mm and diameter of the largest SN metastatic deposit < 500 μm (19/70 patients, 27%). In logistic regression, the SN metastatic area was the only independent factor predicting the presence of non-SN metastases.

DISCUSSION

SLND has become a routine procedure in the management of cutaneous malignant melanoma. The applied triple-technique procedure has been shown to be reliable, with a relatively low false-negative rate [17, 28]. The about 80% of patients with a negative SN [9, 10, 29, 30] are spared a CLND because the chance of finding non-SN metastases is very low at the cost of serious side effects for many, so they are followed up during visits at the outpatient department. If the SN contains however metastatic melanoma cells, a CLND follows at a later date since about 20% of these patients have further metastases in the non-SNs [9, 10, 29, 30] and removing them improves prognosis [31]. However, a significant subset of patients of SN positive patients appear to have no further metastases, so they are unlikely to benefit from CLND, while they may suffer from side effects of the procedure like paresthesias, wound infection, seroma formation, and lymphedema. It is therefore important to identify SN positive patients that are unlikely to have positive non-SNs and thus will not benefit from CLND.

In melanoma, primary tumor and SN characteristics; Breslow thickness and ulceration of the primary tumor, the number of positive SNs, SN metastatic tumor burden and tumor penetrative depth within the SN have all been identified as predictors of non-SN positivity [11, 15, 20, 30, 32, 33]

Yet, no single factor can yet reliably predict absence of non-SN positivity. The aim of this study was therefore to analyze the SN metastatic load in combination with primary tumor features as predictors of metastatic involvement of non-SNs, using

simple bivariate models described previously to predict the absence of lymphatic tumor spread beyond the SN.

We found that none of the patients with a Breslow thickness <2.3 mm and SN depth of invasion <600 μm had positive non-SN (Fig. 1). Our finding that primary tumor thickness may be useful in identifying a group at low risk for positive non-SNs agrees with the findings of Gershenwald et al. [15] as well as Lee et al. [20] who reported a higher incidence of positive non-SNs in patients with a tumor thickness of >2 mm and ≥3 mm, respectively.

Previously, Starz et al [14] proposed a classification based on the depth of the metastasis from the capsule and the number of 1-mm slices containing melanoma. Although a correlation between the classification by Starz et al and non-SN involvement exists, a subsequent study on a larger group of patients has shown that it does not predict non-SN metastasis with complete reliability, given that a small group of patients with metastases less than 1 mm in depth shows non-SN involvement [22]. We found depth of invasion of the SN metastatic deposit to be the strongest independent predictor of positive non-SNs. Irrespective of Breslow thickness, patients with an SN depth of invasion <0.18 mm never had positive non-SNs. The best combination of features was however SN depth of invasion and Breslow thickness of the primary tumor.

Further, none of the patients with a Breslow thickness <2.3 mm and a SN tumor load <0.2 mm^2 had positive non-SN (Fig. 2). This confirms in a completely independent set of patients from multiple institutions the results of a previous study by Vuylsteke et

al. [16], using a combination of Breslow thickness and SN tumor volume to predict non-SN involvement in melanoma patients, with almost identical thresholds.

Reeves et al. have devised a size/ulceration classification (SU score) based on the extent of the metastasis within the SN and the ulceration status of the primary melanoma. For patients with non-ulcerated primary tumors and less than 2 mm of tumor in the sentinel node, there was a zero probability of non-SN metastases [23]. However, Vuylsteke et al. could not reproduce the predictive value of this classification in their study [17]. Further, in the present study, 14 of 18 (78%) of the non-SN positive patients had an SN metastasis of less than 2 mm in maximum dimension. This concurs with earlier work by Carlson et al., suggesting that size of the SN metastasis alone is not a reliable enough predictor of non-SN involvement [34].

The present study also categorized metastases according to their microanatomic location within the SN following the method proposed by Dewar and colleagues [19]. These authors argue that the proposed microanatomic classification predicts non-SN involvement more accurately than size and depth alone because, in several patients, small deposits (some consisting of only a few cells) were associated with non-SN metastases, but none was confined to a subcapsular location. In contrast, no patient with only a subcapsular deposit was found to have tumor in non-SNs. However, some have argued that the size (or the depth) of the SN tumor burden is considerably different for the different locations of metastases [18]. In line with this argument, we could not detect a statistically significant correlation between microanatomic location and the probability of non-SN involvement.

In the study by Scolyer et al. [35] neither subcapsular nor parenchymal deposits of the metastases were a significant predictive factor for additional non-SN positivity. These investigators could demonstrate, however, that the group of patients with extensive involvement had a higher rate of non-SN metastases compared with the patients with non-extensive involvement. SN tumor burden was an independent prognostic factor for additional non-SN positivity in the study of Vuylsteke et al. [16], as no additional non-SN involvement was seen in patients with a SN metastatic area of <0.3 mm^2. However, Carlson et al. [34] did not find SN tumor burden to be a significant predictor of non-SN involvement in their patient population. In the present study, no additional non-SN positivity in the group of patients with minimal SN tumor burden (<0.18 mm) was demonstrated.

Thus, in the present study the invasion depth and size of SN tumor burden seem to be the essential factor and not so much the microanatomic location of SN metastases. This idea is supported by the study of Dewar et al. itself, because the locations with very small SN tumor burden have an excellent prognosis in contrast to the locations with larger sizes of SN tumor burden [19].

As already noted, Starz et al. have used a rather complicated classification based on the number of tumor-involved step sections and centripetal depth of tumor in the SN to predict non-SN positivity [14, 27]. This system is complex and probably not well reproducible in routine pathology practice. The methodology described herein has several important advantages. First, it is easy to use and is based on a primary tumor already routinely reported (Breslow thickness) and an SN feature (depth of

melanoma cell invasion or largest metastatic diameter) that can easily be measured by pathologists. Secondly, it independently predicts non-SN status. Thirdly, it varies proportionally with the risk of non-SN positivity. In addition, the proposed technique has an anatomo-physiologic basis, with metastatic melanoma presumably entering a lymph node via the afferent lymphatics into the subcapsular sinus, before passing into the paracortex, and ultimately replacing large parts of the node. This is reflected in the steadily increasing mean depth of metastatic deposits, and increased frequency of non-SN metastases, on progression through the SN. Given the contradictory data surrounding the influence of Breslow thickness on non-SN status, it is also advantageous that this method does not depend only on histologic characteristics of the primary melanoma, but also the SN.

Clearly, the results of this study need to be validated in other large prospective data series. Additionally, it is important to keep in mind that this study shows that this method can identify patients that are at low risk for having tumor in non-SNs detectable by H&E staining. On the other hand, it is certainly conceivable that H&E-negative non-SNs might actually contain residual tumor not detectable by H&E [36]. However, our methodology is able to stratify patients according to risk of non-SN positivity using the methods that are currently standard for tumor detection in non-SNs.

This study was not designed to investigate differences in survival between different depths of invasion of SN metastases or SN tumor load. Whether it is indeed safe not to perform CLND in patients with SN metastases with penetrative depth <0.1mm

(i.e., confined to the subcapsular zone) or low SN tumor load would need to be addressed in a properly designed (ideally: randomized) trial. Nevertheless, it is recommended, in the meantime, that the depth of invasion and, consequently, S-classification of the metastases becomes a routine part of the histopathologic reporting of positive SN.

Similar attempts to predict absence of non-SN metastases have been made in breast cancer with much less success than in melanoma [37], indicating that the biology of both cancers is apparently different in this respect.

In conclusion, SN depth of invasion of the metastatic deposit or SN tumor load may, in combination with the Breslow thickness of the primary tumor, be used to predict the absence of positive non-SNs. The combination of these factors makes it possible to formulate a microstaging model, so that CLND can be avoided in about 30% of patients, reducing costs and morbidity. The methodology described herein is easier to learn and use than the more complex micromorphometric techniques [16, 19, 23, 27], and involves no additional cost. Nevertheless, to answer these questions, a prospective trial in which these low risk SN-positive patients are randomized between CLND or watchful waiting may have to be performed.

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AUTHORS' DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

Table 1. Tumor characteristics of 70 SN positive patients with cutaneous melanoma who underwent completion lymph node dissection.

Characteristic	**N**	**%**
Total (n)	70	100%
Gender		
Female	32	45.7%
Male	38	54.3%
Type of melanoma		
Superficial spreading	47	67.1%
Nodular	19	27.1%
Acral lentiginous	2	2.9%
Spitzoid	2	2.9%
Clark level of invasion		
II	1	1.4%
III	12	17.1%
IV	49	70.0%
V	2	2.9%
Unclassified	6	8.6%
Breslow thickness		
0.5-1.0 mm	5	7.1%
1.01-2.0 mm	18	25.7%
2.01-4.0 mm	35	50.0%
>4.0 mm	12	17.1%
Site of primary melanoma		
Trunk	39	55.8%
Upper extremities	8	11.4%
Lower extremities	22	31.4%
Head and neck	1	1.4%
Ulceration, n (%)		
Present	18	25.7%
Absent	52	74.3%
Lymphatic invasion, n (%)		
Present	8	11.4%
Absent	62	88.6%

Table 2. Sentinel node (SN) characteristics of 70 SN positive patients with cutaneous melanoma who underwent completion lymph node dissection.

Characteristic	**N**	**%**
Sentinel node basin		
Axilla	33	47.1%
Groin	33	47.1%
Neck	3	4.3%
Other	1	1.4%
Number of positive SN		
One	60	85.7%
Two or more	10	14.3%
Starz (S)-classification (old)[1]		
S1	32	45.7%
S2	16	22.9%
S3	22	31.4%
Starz (S)-classification (new)[2]		
SI	20	28.6%
SII	28	40.0%
SIII	22	31.4%
Microanatomic location[3]		
Subcapsular	34	48.6%
Combined	16	22.9%
Parenchymal	10	14.3%
Multifocal	3	4.3%
Extensive	7	10.0%

[1] According to Starz et al. [14]]

[2] According to Starz et al. [27]

[3] According to Dewar et al. [19]

Table 3. Comparison of primary tumor and SN features of SN positive cutaneous melanoma patients (n= 70) (all undergoing completion lymph node dissection) with metastasis confined to the SN and patients with additional positive non-SN.

Variable	**Only SN involved**	**Additional positive lymph nodes**	***P* value**
Total, n (%)	52 (74)	18 (26)	
Age (mean)	49	54	
Breslow thickness, mm (mean)	2.52	3.08	NS [a]
Ulceration n (%)	11 (79)	3 (21)	NS [b]
SN depth of invasion, mm (mean)	0.99	3.3	0.023 [a]
SN metastatic area, mm^2 (mean)	2.3	38.5	0.014 [a]
SN diameter of largest tumor deposit, mm (mean)	0.998	2.857	0.021 [a]

SN, sentinel lymph node; LN, lymph node; NS, not significant.
[a] Mann-Whitney U-test.
[b] Chi-square test.

Fig. 1 Scatter plot of total sentinel lymph node (SN) metastasis depth of invasion and Breslow thickness for a group of cutaneous melanoma patients with (18 patients; asterisks) or without (52 patients; circles) additional (non-SN) lymph node metastases. No positive additional lymph nodes were found in patients with both a Breslow thickness of the primary tumor <2.3 mm and SN penetrative depth of invasion <0.6 mm.

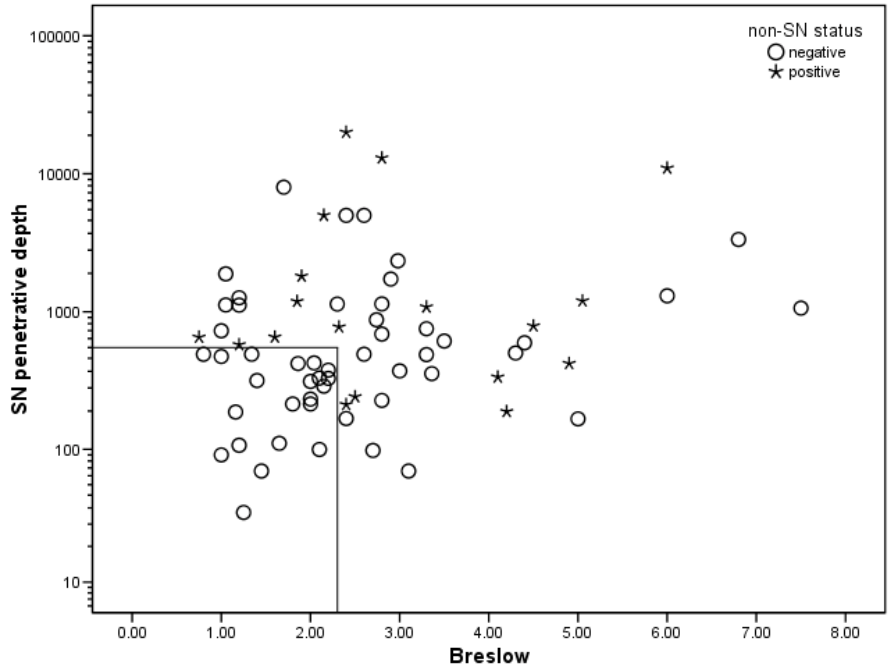


Fig. 2 Scatter plot of total sentinel lymph node (SN) metastatic area and Breslow thickness for a group of cutaneous melanoma patients with (18 patients; asterisks) or without (52 patients; circles) additional lymph node metastases. No positive additional lymph nodes were found in patients with both a Breslow thickness of the primary tumor <2.3 mm and an SN metastatic area <0.2 mm^2.

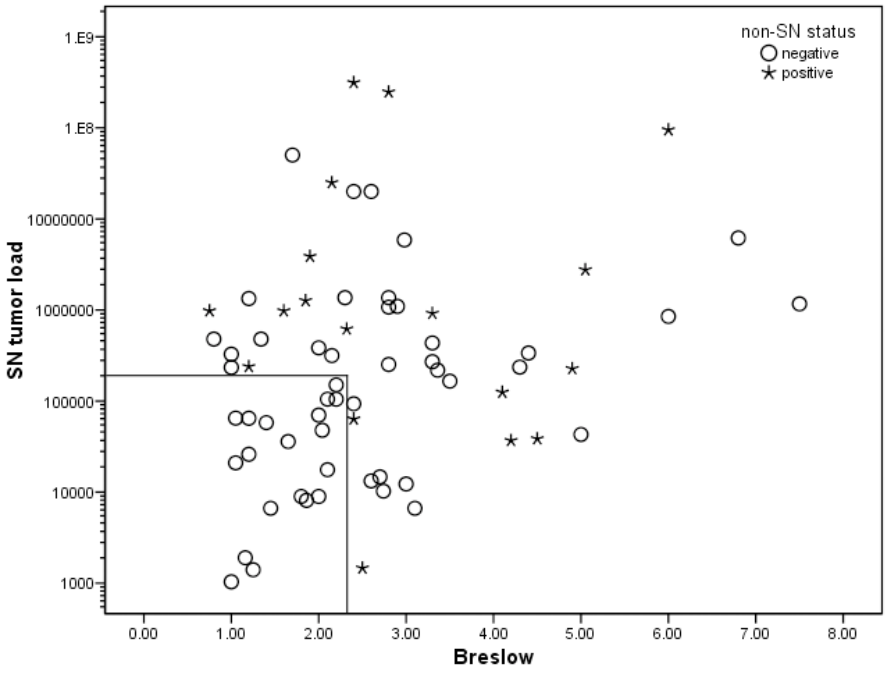
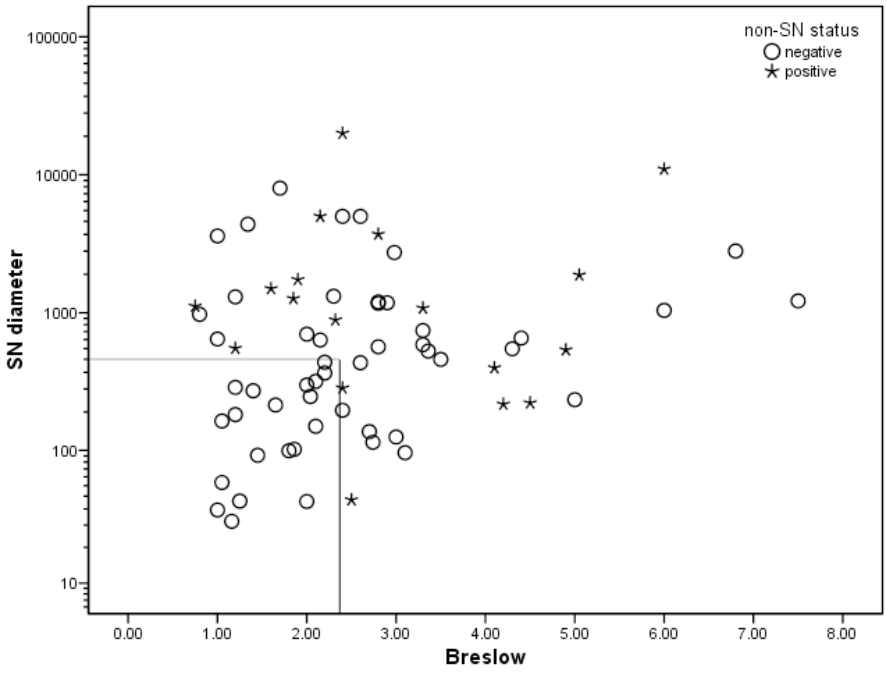


Fig. 3 Scatter plot of total sentinel lymph node (SN) metastatic tumor diameter and Breslow thickness for a group of cutaneous melanoma patients with (18 patients; asterisks) or without (52 patients; circles) additional lymph node metastases. No positive additional lymph nodes were found in patients with both a Breslow thickness of the primary tumor <2.3 mm and a largest SN tumor deposit < 500 μm.



REFERENCES

1. Cascinelli N, Belli F, Santinami M, *et al.* Sentinel lymph node biopsy in cutaneous melanoma: the WHO Melanoma Program experience. *Ann Surg. Oncol.* 2000 Jul;**7**(6):469-474.

2. Houghton A, Coit D, Bloomer W, *et al.* NCCN melanoma practice guidelines. National Comprehensive Cancer Network. *Oncology (Williston Park)* 1998 Jul;**12**(7A):153-177.

3. Scolyer RA, Murali R, Satzger I, Thompson JF. The detection and significance of melanoma micrometastases in sentinel nodes. *Surg. Oncol.* 2008 Sep;**17**(3):165-174.

4. Morton DL, Wen DR, Wong JH, *et al.* Technical details of intraoperative lymphatic mapping for early stage melanoma. *Arch Surg.* 1992 Apr;**127**(4):392-399.

5. McMasters KM, Reintgen DS, Ross MI, *et al.* Sentinel lymph node biopsy for melanoma: controversy despite widespread agreement. *J Clin Oncol.* 2001 Jun 1;**19**(11):2851-2855.

6. Balch CM, Soong S, Ross MI, *et al.* Long-term results of a multi-institutional randomized trial comparing prognostic factors and surgical results for intermediate thickness melanomas (1.0 to 4.0 mm). Intergroup Melanoma Surgical Trial. *Ann Surg. Oncol.* 2000 Mar;**7**(2):87-97.

7. Cascinelli N, Morabito A, Santinami M, MacKie RM, Belli F. Immediate or delayed dissection of regional nodes in patients with melanoma of the trunk: a randomised trial. WHO Melanoma Programme. *Lancet* 1998 Mar 14;**351**(9105):793-796.

8. Sabel MS, Gibbs JF, Cheney R, McKinley BP, Lee JS, Kraybill WG. Evolution of sentinel lymph node biopsy for melanoma at a National Cancer Institute-designated cancer center. *Surgery* 2000 Oct;**128**(4):556-563.

9. Wagner JD, Gordon MS, Chuang TY, *et al.* Predicting sentinel and residual lymph node basin disease after sentinel lymph node biopsy for melanoma. *Cancer* 2000 Jul 15;**89**(2):453-462.

10. Gershenwald JE, Thompson W, Mansfield PF, *et al.* Multi-institutional melanoma lymphatic mapping experience: the prognostic value of sentinel lymph node status in 612 stage I or II melanoma patients. *J Clin Oncol.* 1999 Mar;**17**(3):976-983.

11. McMasters KM, Wong SL, Edwards MJ, *et al.* Frequency of nonsentinel lymph node metastasis in melanoma. *Ann Surg. Oncol.* 2002 Mar;**9**(2):137-141.

12. Krag DN, Meijer SJ, Weaver DL, *et al.* Minimal-access surgery for staging of malignant melanoma. *Arch Surg.* 1995 Jun;**130**(6):654-658.

13. Thompson JF, McCarthy WH, Bosch CM, *et al.* Sentinel lymph node status as an indicator of the presence of metastatic melanoma in regional lymph nodes. *Melanoma Res* 1995 Aug;**5**(4):255-260.

14. Starz H, Balda BR, Kramer KU, Buchels H, Wang H. A micromorphometry-based concept for routine classification of sentinel lymph node metastases and its clinical relevance for patients with melanoma. *Cancer* 2001 Jun 1;**91**(11):2110-2121.

15. Gershenwald JE, Andtbacka RH, Prieto VG, *et al.* Microscopic tumor burden in sentinel lymph nodes predicts synchronous nonsentinel lymph node involvement in patients with melanoma. *J Clin Oncol.* 2008 Sep 10;**26**(26):4296-4303.

16. Vuylsteke RJ, Borgstein PJ, van Leeuwen PA, *et al.* Sentinel lymph node tumor load: an independent predictor of additional lymph node involvement and survival in melanoma. *Ann Surg. Oncol.* 2005 Jun;**12**(6):440-448.

17. Vuylsteke RJ, van Leeuwen PA, Statius Muller MG, Gietema HA, Kragt DR, Meijer S. Clinical outcome of stage I/II melanoma patients after selective sentinel lymph node dissection: long-term follow-up results. *J Clin Oncol.* 2003 Mar 15;**21**(6):1057-1065.

18. van Akkooi AC, de Wilt JH, Verhoef C, *et al.* High positive sentinel node identification rate by EORTC melanoma group protocol. Prognostic indicators of metastatic patterns after sentinel node biopsy in melanoma. *Eur J Cancer* 2006 Feb;**42**(3):372-380.

19. Dewar DJ, Newell B, Green MA, Topping AP, Powell BW, Cook MG. The microanatomic location of metastatic melanoma in sentinel lymph nodes predicts nonsentinel lymph node involvement. *J Clin Oncol.* 2004 Aug 15;**22**(16):3345-3349.

20. Lee JH, Essner R, Torisu-Itakura H, Wanek L, Wang H, Morton DL. Factors predictive of tumor-positive nonsentinel lymph nodes after tumor-positive sentinel lymph node dissection for melanoma. *J Clin Oncol.* 2004 Sep 15;**22**(18):3677-3684.

21. Wagner JD, Schauwecker D, Davidson D, *et al.* Prospective study of fluorodeoxyglucose-positron emission tomography imaging of lymph node

basins in melanoma patients undergoing sentinel node biopsy. *J Clin Oncol.* 1999 May;**17**(5):1508-1515.

22. Starz H. Pathology of the sentinel lymph node in melanoma. *Semin. Oncol.* 2004 Jun;**31**(3):357-362.

23. Reeves ME, Delgado R, Busam KJ, Brady MS, Coit DG. Prediction of nonsentinel lymph node status in melanoma. *Ann Surg. Oncol.* 2003 Jan;**10**(1):27-31.

24. Cochran AJ, Wen DR, Huang RR, Wang HJ, Elashoff R, Morton DL. Prediction of metastatic melanoma in nonsentinel nodes and clinical outcome based on the primary melanoma and the sentinel node. *Mod. Pathol.* 2004 Jul;**17**(7):747-755.

25. Balch CM, Buzaid AC, Soong SJ, *et al.* Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol.* 2001 Aug 15;**19**(16):3635-3648.

26. van Diest PJ, Torrenga H, Meijer S, Meijer CJ. Pathologic analysis of sentinel lymph nodes. *Semin. Surg. Oncol.* 2001 Apr;**20**(3):238-245.

27. Starz H, Siedlecki K, Balda BR. Sentinel lymphonodectomy and s-classification: a successful strategy for better prediction and improvement of outcome of melanoma. *Ann Surg. Oncol.* 2004 Mar;**11**(3 Suppl):162S-168S.

28. Gershenwald JE, Colome MI, Lee JE, *et al.* Patterns of recurrence following a negative sentinel lymph node biopsy in 243 patients with stage I or II melanoma. *J Clin Oncol.* 1998 Jun;**16**(6):2253-2260.

29. Statius Muller MG, van Leeuwen PA, de Lange-De Klerk ES, *et al.* The sentinel lymph node status is an important factor for predicting clinical outcome in patients with Stage I or II cutaneous melanoma. *Cancer* 2001 Jun 15;**91**(12):2401-2408.

30. Cochran AJ, Huang RR, Guo J, Wen DR. Current practice and future directions in pathology and laboratory evaluation of the sentinel node. *Ann Surg. Oncol.* 2001 Oct;**8**(9 Suppl):13S-17S.

31. Morton DL, Cochran AJ, Thompson JF, *et al.* Sentinel node biopsy for early-stage melanoma: accuracy and morbidity in MSLT-I, an international multicenter trial. *Ann Surg.* 2005 Sep;**242**(3):302-311.

32. Cascinelli N, Clemente C, Bifulco C, *et al.* Do patients with tumor-positive sentinel nodes constitute a homogeneous group? *Ann Surg. Oncol.* 2001 Oct;**8**(9 Suppl):35S-37S.

33. Wagner JD, Davidson D, Coleman JJ, III, *et al.* Lymph node tumor volumes in patients undergoing sentinel lymph node biopsy for cutaneous melanoma. *Ann Surg. Oncol.* 1999 Jun;**6**(4):398-404.

34. Carlson GW, Murray DR, Lyles RH, Staley CA, Hestley A, Cohen C. The amount of metastatic melanoma in a sentinel lymph node: does it have prognostic significance? *Ann Surg. Oncol.* 2003 Jun;**10**(5):575-581.

35. Scolyer RA, Li LX, McCarthy SW, *et al.* Micromorphometric features of positive sentinel lymph nodes predict involvement of nonsentinel nodes in patients with melanoma. *Am J Clin Pathol.* 2004 Oct;**122**(4):532-539.

36. Wrightson WR, Wong SL, Edwards MJ, *et al.* Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of nonsentinel nodes following completion lymphadenectomy for melanoma. *J Surg. Res* 2001 Jun 1;**98**(1):47-51.

37. van Deurzen CH, de BM, Monninkhof EM, *et al.* Non-sentinel lymph node metastases associated with isolated breast cancer cells in the sentinel node. *J Natl. Cancer Inst.* 2008 Nov 19;**100**(22):1574-1580.

Melanoma patients with low volume sentinel node metastases and low Breslow thickness have no further lymph node metastases and excellent prognosis

Manuscript

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Melanoma patients with low volume sentinel node metastases and low Breslow thickness have no further lymph node metastases and excellent prognosis

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Background: The sentinel node (SN) procedure has become a widely used staging procedure for stage I/II cutaneous melanoma and there is now general agreement that absence of metastases in the SN obliviates the need for further lymph node dissection and indicates excellent prognosis. The objective of this study was to investigate whether a similarly favorable subgroup can be identified among melanoma patients with SN metastases.

Methods: We reviewed a series of 70 SN–positive patients with primary cutaneous melanoma treated with completion lymph node dissection (CLND). SN tumor load (total tumor area, penetrative depth of metastatic cells, diameter of largest deposit) was assessed by morphometry. Various measures of tumor burden and traditional melanoma prognostic indicators were studied in survival analysis.

Results: Patients with a Breslow thickness <2.0 mm and SN metastatic area < 0.2 mm^2 (p=0.010) or an SN tumor penetrative depth <0.6 mm (p=0.060) or largest diameter of the SN metastasis (p=0.010) had no positive non-SN and showed excellent DFS (n=12, 17% of patients). Dewar classification of SN metastases location (extensive vs non-extensive) was also significant (p<0.001) although no subgroup with excellent survival could be identified. The Rotterdam size classification system (< 0.1 vs ≥ 0.1 mm, p=0.201 and original and new Starz-classification lacked prognostic value.

Conclusions: Microstaging by morphometry of the SN tumor burden significantly predicts prognosis in cutaneous melanoma. Patients with low volume SN metastases (most easily assessed by measuring the diameter of the largest tumor deposit) and low Breslow have no further lymph node metastases and excellent prognosis.

INTRODUCTION

Sentinel lymph node (SN) biopsy, commonly followed by completion lymph node dissection (CLND) in case of SN metastases, has become the standard for management of intermediate and high risk cutaneous melanoma with clinically negative lymph nodes (i.e., American Joint Committee on Cancer (AJCC) stage I/II) [1, 2]. SN biopsy provides a reliable, low-morbidity approach to accurate nodal staging with low false-negative rates (reviewed in [3]). Moreover, it yields important prognostic information, it can identify patients that might benefit from early nodal dissection [4], and it can define prognostically more homogeneous patient populations for entry into clinical trials [5, 6].

We have recently reported the results of a multicenter study predicting the absence of non-SN metastases in patients with a positive SN based on primary melanoma and SN features (Bogenrieder et al, data submitted for publication, [7]). That initial report, based on results of 70 SN-positive melanoma patients demonstrated that a combination of limited Breslow thickness and low SN tumor load (i.e. total tumor load, penerative depth into the SN or diameter of the largest tumor deposit) predicts absence of non-SN metastases in melanoma patients with a positive SN with 100% accuracy. We therefore proposed that this subgroup that comprises about 30% of patients may be spared CLND.

Several studies have suggested that small melanoma metastases to the SN may confer no adverse prognostic importance. However, there is no agreement about the cut-off values and optimal way of SN metastasis size assessment [8-11]. The objective

of the present study was therefore to further investigate the relationships between SN tumor burden and survival in patients with clinically localized cutaneous melanoma staged by SN biopsy and CLND for identification of occult nodal metastases, comparing different prognostic algorithms previously shown to detect SN-positive subgroups at low risk for non-SN metastases or excellent survival. These included morphometry as proposed by us [7, 12] the original and simplified classification of SN-metastasis according to Starz [13, 14], a microanatomic score of the sentinel tumor deposit as proposed by Dewar [15], and the Rotterdam criteria of SN tumor burden [10, 11].

MATERIAL AND METHODS

Pathologic examination of SN and non-SN

SN biopsy was performed according to standard procedures as previously described [9, 12] in patients presenting with clinical stage I or II malignant melanoma, as defined by the AJCC [16]. SNs were identified using a preoperative dynamic lymphoscintigraphy with radiolabeled colloid, followed by intraoperative injection of vital blue dye and use of a hand-held gamma probe (triple technique). SN specimens were processed as previously reported [12, 17]. In brief, after the SN procedure, harvested SN(s) were fixed in neutral buffered formaldehyde, lamellated according to size, and completely embedded in paraffin. SNs smaller than 0.5 cm were processed and paraffin-embedded intact. Those between 0.5 and 1.0 cm were halved, and SNs larger than 1.0 cm were lamellated into slices of approximately 0.5 cm thickness. One initial 4-μm-thick H&E-stained section was made per block. When negative, four additional step ribbons were cut at 250-μm intervals. Of each ribbon, one section was stained with H&E, and two were used for S100 and HMB-45 immunohistochemistry. Non-SNs in the CLND specimen were routinely evaluated by H&E staining of the halved lymph nodes and, occasionally, sections immunostained for S100 protein and HMB-45 when suspicious cells were seen. For the purposes of this study, all SNs were re-evaluated by a pathologist experienced in SNs and a dermatopathologist (PJvD, MvD). If necessary, further sections were cut

and stained so that all patients were assessed using the same sectioning and processing protocol.

Morphometry

The total SN metastatic area was assessed as described previously [14], using an interactive video morphometry system (Q-PRODIT; Leica, Cambridge, UK). In brief, the SN section containing the largest visible amount of metastatic tumor cells was selected. The surface areas of the individual tumor deposits in this single section were measured, and the SN metastatic area was calculated by summing up these areas. In addition, the maximum penetrative depth of metastatic deposits from the capsule into the SN and the largest diameter of SN tumor deposits were measured, In case of two positive SNs, the one with the largest tumor load was used. The intraobserver and interobserver reproducibility of this method was good as previously shown [7].

Microanatomic classification

In every SN found to contain metastatic tumor cells, the configuration of the metastatic cells, the number of foci, and their distribution within the lymph node were examined. The metastatic deposit within each sentinel node was classified as subcapsular, combined subcapsular and parenchymal, parenchymal, multifocal, or extensive as described by Dewar et al. [15]. When more than one positive SN had

been removed from a single lymph node basin, the most extensive and/or deepest metastatic deposit overall was used to categorize that basin.

S-Staging

Based on SN characteristics, patients were divided into 3 different categories according the S classification (old and new versions) by Starz and co-workers. The original S classification consists of three categories (S1, S2 and S3) based on the number of positive sections (*n*) and the maximum distance from the interior margin of the biggest metastatic group to the capsule of the SN (*d*). Criteria for these respective categories are as follows: $n \leq 1$ and d ≤1 mm for S1, $n > 2$ and d ≤1 mm for S2 and $n > 2$ and d > 1 mm for S3 [13]. The new S classification exhibits simpler criteria: d ≤0.3 mm for SI, d > 0.3 mm and ≤1 mm for SII and d >1 mm for SIII [14].

Rotterdam criteria

The size of the SN tumor burden according to van Akkooi (Rotterdam criteria) [18] was also recorded: sub-micrometastases (clusters of more than 10 cells, but <0.1 mm), tumor burden 0.1 mm–1 mm and tumor burden >1 mm. If multiple lesions were present within a SN, the largest lesion was recorded.

Statistical Analysis

Statistical analysis was performed as described previously using SPSS for Windows (SPSS Inc., Chicago, IL) [9] with special emphasis on variables predicting absence of

non-SN metastases [7]. Briefly, disease free (DFS) and overall survival (OS) Kaplan Meijer curves were plotted for the different subgroups, and differences between the curves were analyzed by logrank statistics. Two-sided *P* values <0.05 were considered significant.

RESULTS

Patient, tumor, and SN characteristics

Between 1999 and 2006, 350 consecutive patients with cutaneous melanoma underwent a SN biopsy for melanoma at the 4 participating institutions. Of these, 70 (20%) patients whose SNs contained metastatic malignant melanoma and subsequently underwent CLND: 18 from the University Medical Center Utrecht, 21 from the Radboud University Nijmegen Medical Centre, 6 from the St. Antonius Hospital Nieuwegein, The Netherlands, and 25 from the Städtisches Krankenhaus München-Schwabing, Germany.

Baseline characteristics of all reviewed patients are depicted in Table 1. The average age was 51 years (range 19–79 years). The mean Breslow thickness was 2.66 mm (range 0.7–6.8 mm). More than half of the tumors were on the trunk and 18/70 (26%) were ulcerated.

On average, 1.6 (range 1-6) SNs were removed, and 12 lymph nodes (range 1-56) were removed at CLND. Sixty percent of patients had one identifiable SN, 29% two SNs, and 11% three or more SNs. Eighty-six percent of patients showed one positive SN, 13% two positive SNs, and 1% three positive SNs. Eighteen patients (26%) had positive non-SN at CLND. The distribution of SN characteristics, S classification [13, 14], location according to Dewar [15] and the Rotterdam criteria [10, 11] are shown in Table 2.

Recurrent disease

After a median follow-up of 27 months, recurrent disease was seen in 29 (41%) of the 70 patients (Table 3). Three patients with no sign of progression developed a second melanoma. Locoregional cutaneous metastasis was the first recurrence to develop in 21 patients (15 in group 1 and 6 in group 2), whereas 7 patients (3 in group 1 and 4 in group 2) developed distant metastases as the first site of recurrence.

Prognostic value of bivariate grouping using Breslow and SN tumor load morphometry

Building on our previous work [7, 12], patients were divided into 2 subgroups based on Breslow thickness (cut-off of reset to 2.0 mm to better reflect the AJCC melanoma staging system) on the one hand and various morphometric measures of SN tumor load on the other, the low/low group (group A) not being associated with non-SN metastases while the remaining group (group B) did show frequent non-SN metastases [7]. The thresholds for low SN tumor load were < 0.2 mm^2 for SN metastatic area, < 600 μm for SN penetrative depth, and < 500 μm for diameter of the largest SN deposit. Hence, subgroup A comprised patients with a Breslow thickness <2.0 mm and an SN metastatic area < 0.2mm^2 (n=12); or Breslow thickness <2.0 mm and SN penetrative depth <600 μm (n=12); or a Breslow thickness <2.0 mm and diameter of the largest SN deposit <500 μm (n=12).

Figure 1 shows the survival curves for the Breslow/total SN metastatic area grouping. Subgroup A had a significantly better survival (DFS p=0.010, OS p=0.065) compared to group B. Figure 2 shows the survival curves for the Breslow/SN penetrative depth

grouping. Subgroup A had a better survival (DFS p=0.060, OS p=0.351) compared to group B. Figure 3 shows the survival curves for the Breslow/SN largest tumor deposit diameter grouping. Subgroup A had a better survival (DFS p=0.010, OS p=0.065) compared to group B.

Prognostic value of other algorithms

Figures 4 and 5 show the survival curves for the original and simplified S-classification, respectively, revealing no prognostic value. Figure 6 shows the survival curves for the different microanatomic location categories by Dewar. Especially a grouping into extensive versus non-extensive (Figure 7) revealed two subgroups with different prognosis (DFS p<0.001, OS p=0.097), but the non-extensive suggroup had only a 50% 5 years survival.

Figure 8 depicts the survival curves for the three different groups of the Rotterdam SN tumor burden criteria, bearing no significance (DFS p=0.369, OS p=0.385). Also when cases were grouped as < 0.1 mm versus ≥ 0.1 mm, no significance was reached (DFS p=0.201, OS p=0.333).

DISCUSSION

Even though there is mounting evidence that small cutaneous melanoma metastases to the SN do not bear predictive value for non-SN involvement or prognostic value, there is at present no staging system that confers clinical consequences to the tumor burden found in melanoma SNs: for therapeutic decision making they are simply considered to be positive or negative. In this multicenter study we therefore aimed to look at the prognostic importance of SN tumor burden, by evaluating the outcome of 70 SN positive melanoma patients using different algorithms.

The present study confirms that melanoma patients with small SN metastases have excellent DFS and OS, in the order of SN-negative patients (90-94%) as reported in the Multicenter Selective Lymphadenopathy Trial I [19] and a host of other SN studies [11, 12, 20-23]. There is however no consensus on the optimal way to assess SN tumor burden in melanoma patients. The easiest way to determine size is by measuring the diameter of the largest tumor deposit. Some authors have proposed a cut-off value of 0.2 mm to discern sub-micrometastases (or "isolated tumor cells") from micrometastases, similar to the cut-off in breast cancer SNs [12, 23-27]. We, too, have therefore considered this scenario; however, the additional non-SN positivity rate as well as the recurrence rate in < 0.2 mm metastases was considerably higher than in < 0.1 mm metastases (3% versus 0% and 4% versus 0%, respectively). This is in line with the findings from a recent study by Scheri and co-workers [23] where melanoma patients with < 0.2 mm metastases had a worse prognosis than SN-negative patients. Hence we are in agreement with van Akkooi [11] that the cut-off

value for melanoma should more stringently set at 0.1 mm, although the survival of the quite small favorable subgroup of patients (in the present study 6/70, 8.6%) was still not significantly better than the remaining patients.

Better results were obtained when combining diameter of the largest tumor deposit with Breslow thickness. Breslow thickness was identified in studies by Lee and co-workers [28] as well as Sabel et al. [29] to be indicative for additional non-SN positivity. Increasing the threshold for diameter of the largest SN tumor deposit to 0.5 mm and setting the Breslow threshold to 2 mm resulted in a larger subgroup of 12/70 patients (17%) which not only had no non-SN metastases as previously shown [7] but also a 100% disease free and overall survival. This is completely in line with the previous results by Vuylsteke et al. [12]. We therefore strongly believe Breslow thickness adds clinical utility. This notion is underlined by the fact that in a large recent multi-center trial assessing the Rotterdam criteria, 4 patients in the sub-micrometastases group (< 0.1 mm) developed a recurrence with typical melanoma recurrence patterns [11]. Identical results were obtained using the total SN metastatic load (threshold 0.2 mm^2) instead of diameter of the largest SN tumor deposit. Assessment of total SN metastatic load is however more complicated and time consuming than relatively simple diameter measurements. Penetrative depth of the SN metastases yielded in combination with Breslow thickness no significant results. We have also evaluated the algorithms by Dewar [15] and Starz (both the original and simplified criteria) [13, 14] in our cohort. The micro-anatomic classification by Dewar and co-workers predicts non-SN involvement [15], and in the present study

we detect a significantly better survival for patients with non-extensive metastases. However, the 5 year disease free survival of this subgroup was still only 50%. We further failed to show a correlation between prognosis and the original and revised classifications by Starz et al. [13, 14]. Even if one takes into account the much larger size of the patient cohort in their original publication, the 1 mm threshold for penetrative depth of tumor in the SN is a cut-off value considerably higher compared to proposed by us and other groups [12, 23-26], considering that the largest diameter of the metastasis will often be higher than the penetrative depth. What is more, our findings regarding the lack of prognostic significance of both the Dewar and Starz algorithms corroborate those by Van Akkooi et al [10].

So, melanoma patients with low volume sentinel node metastases (most easily measured by assessing the diameter of the largest tumor deposit) and low Breslow thickness have no further lymph node metastases [7] and excellent prognosis similar to SN-negative patients as shown the present study, confirming the results of Vuylsteke et al. The question now arises whether patients meeting these criteria can now safely spared a CLND which seems to have a curative effect in many SN positive melanoma patients [19], thereby avoiding its morbidities such as wound infections and limb edema [3, 11]. A prospective multi-center trial randomizing these low risk patients over CLND-or-not is probably needed to firmly establish this.

In conclusion, low SN tumor burden (measured by total SN tumor area, penetrative depth into the SN or largest SN tumor deposit diameter) in combination with a Breslow thickness < 2.0 mm is not only associated with absence of non-SN metastases

as previously shown but also with excellent prognosis. We believe these data support the idea that these patients may safely be spared a CLND, and we propose a prospective trial randomizing these patients over CLND-or-not.

Table 1. Tumor characteristics of 70 SN positive patients with cutaneous melanoma who underwent completion lymph node dissection.

Characteristic	**N**	**%**
Total (n)	70	100%
Gender		
Female	32	45.7%
Male	38	54.3%
Type of melanoma		
Superficial spreading	47	67.1%
Nodular	19	27.1%
Acral lentiginous	2	2.9%
Spitzoid	2	2.9%
Clark level of invasion		
II	1	1.4%
III	12	17.1%
IV	49	70.0%
V	2	2.9%
Unclassified	6	8.6%
Breslow thickness		
0.5-1.0 mm	5	7.1%
1.01-2.0 mm	18	25.7%
2.01-4.0 mm	35	50.0%
>4.0 mm	12	17.1%
Site of primary melanoma		
Trunk	39	55.8%
Upper extremities	8	11.4%
Lower extremities	22	31.4%
Head and neck	1	1.4%
Ulceration, n (%)		
Present	18	25.7%
Absent	52	74.3%
Lymphatic invasion, n (%)		
Present	8	11.4%
Absent	62	88.6%

Table 2. Sentinel node (SN) characteristics of 70 SN positive patients with cutaneous melanoma who underwent completion lymph node dissection.

Characteristic	**N**	**%**
Sentinel node basin		
Axilla	33	47.1%
Groin	33	47.1%
Neck	3	4.3%
Other	1	1.4%
Number of positive SN		
One	60	85.7%
Two or more	10	14.3%
Starz (S)-classification (old)[1]		
S1	32	45.7%
S2	16	22.9%
S3	22	31.4%
Starz (S)-classification (new)[2]		
SI	20	28.6%
SII	28	40.0%
SIII	22	31.4%
Microanatomic location[3]		
Subcapsular (1)	34	48.6%
Combined (2)	16	22.9%
Parenchymal (3)	10	14.3%
Multifocal (4)	3	4.3%
Extensive (5)	7	10.0%
SN tumor burden (Rotterdam criteria)[4]		
<0.1 mm	6	8.6%
0.1-1.0 mm	42	60.0%
>1.0 mm	22	31.4%

[1] According to Starz et al. [13]]
[2] According to Starz et al. [14]
[3] According to Dewar et al. [15]
[4] Accoring to van Akkooi et al. [10]

Figure 1. Disease-free (left) and overall (right) survival curves for cutaneous melanoma patients with a Breslow thickness < 2.0 mm and an SN metastatic area < 0.2 mm^2 (group A, n=12) versus the remaining patients (group B, n=58).

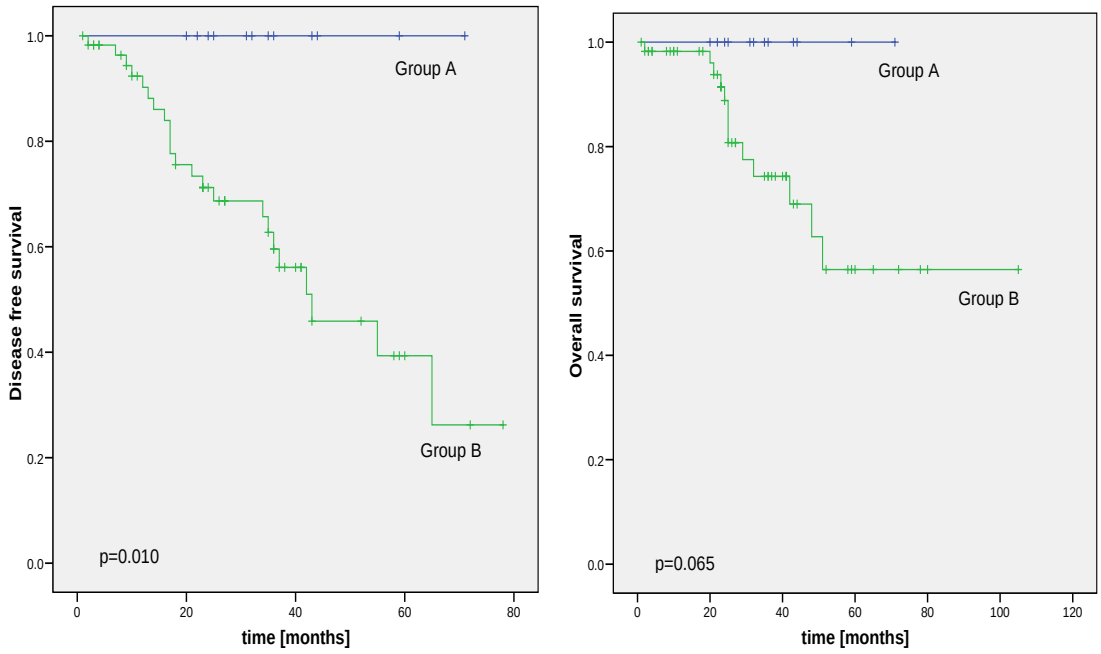


Figure 2. Disease-free (left) and overall survival curves (right) for cutaneous melanoma patients with a Breslow thickness < 2.0 mm and an SN penetrative depth <600 μm (group A, n=12) versus the remaining patients (group B, n=58).

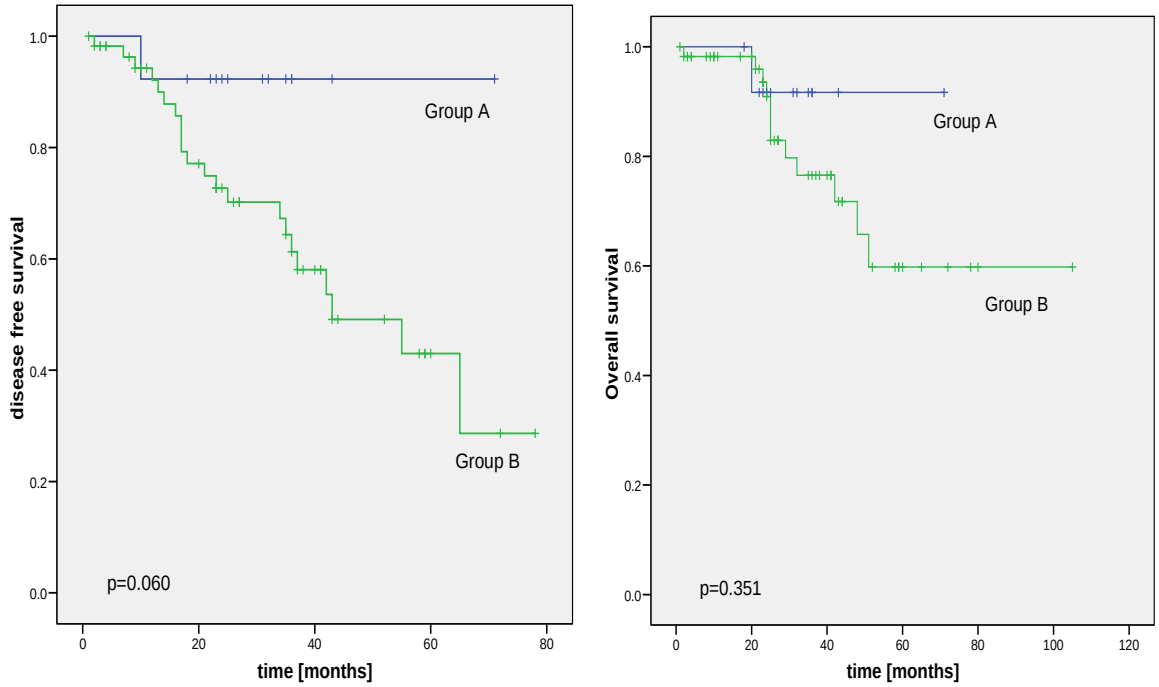


Figure 3. Disease-free (left) and overall survival curves (right) for cutaneous melanoma patients with a Breslow thickness < 2.0 mm and an SN diameter of the largest tumor deposit <500 µm (group A, n=12) versus the remaining patients (group B, n=58).

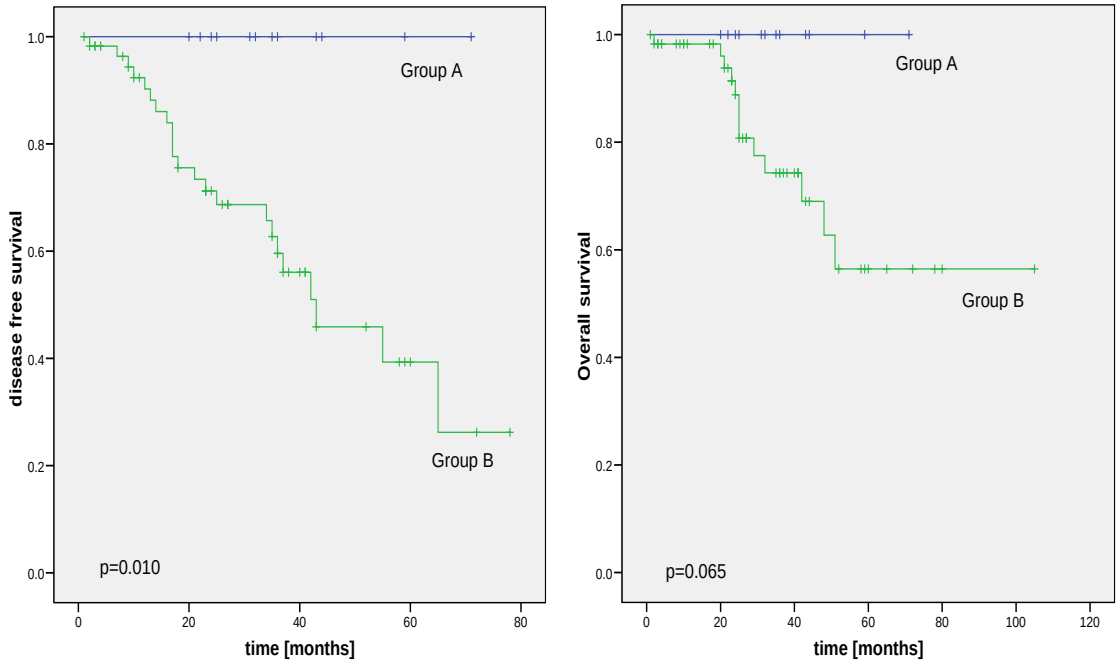


Figure 4. Kaplan-Meier estimated disease-free (left) and overall survival (right) according to the original S-classification by Starz for melanoma metastases to the SN.

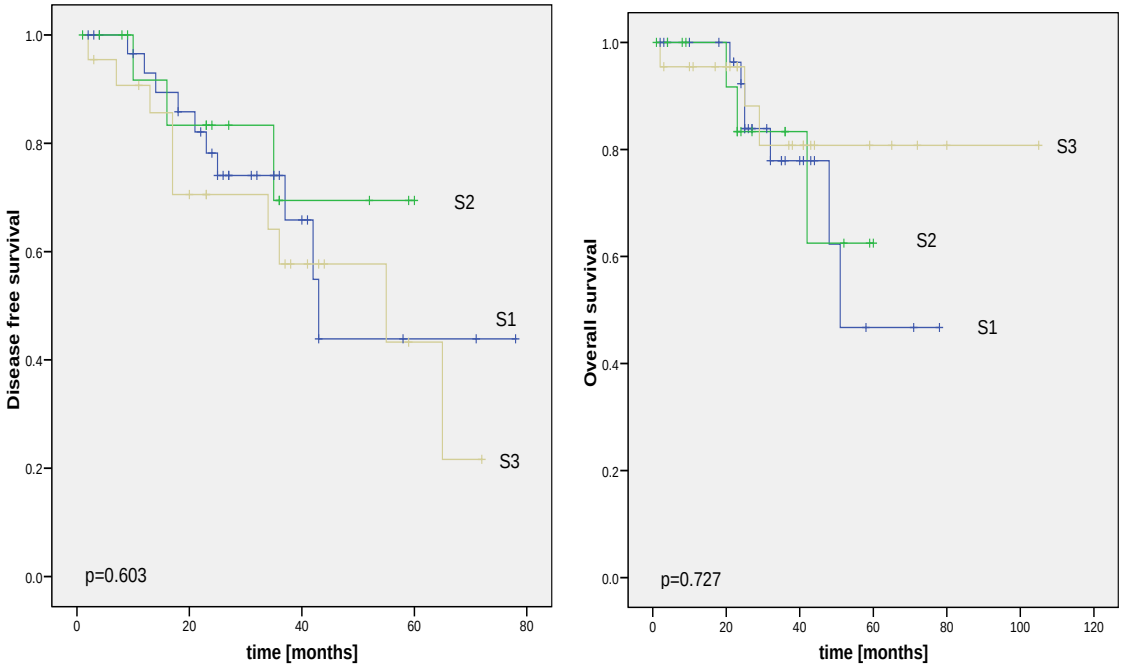


Figure 5. Kaplan-Meier estimated disease free (left) and overall survival (right) according to the simplified S-classification by Starz for melanoma metastases to the SN.

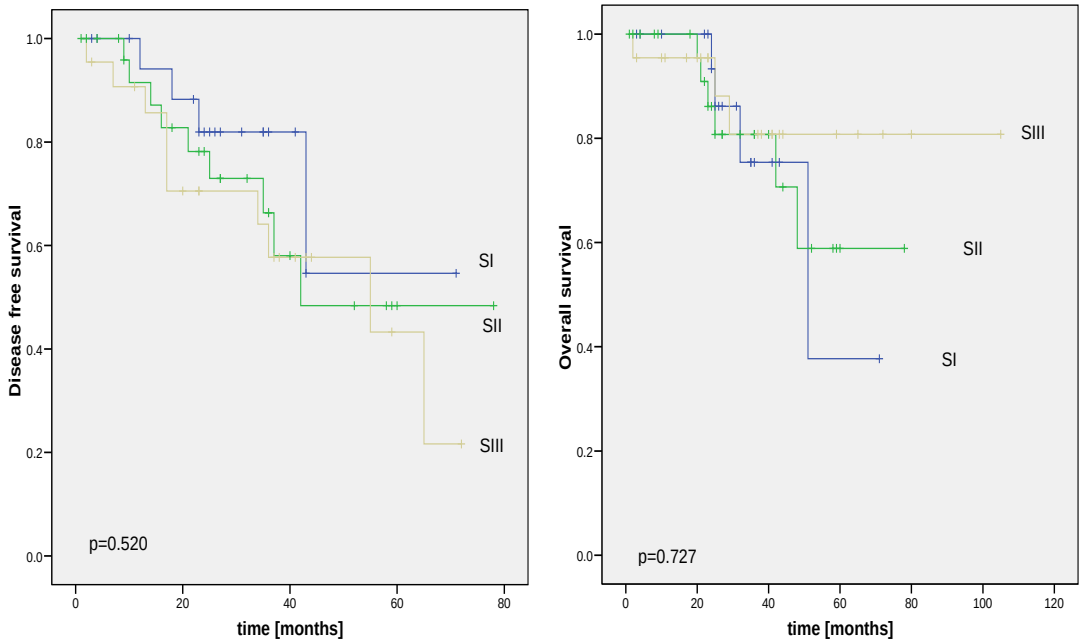


Figure 6. Kaplan-Meier estimated disease-free (left) and overall survival (right) according to the SN metastatic tumor deposit location categories by Dewar SN for cutaneous melanoma patients.

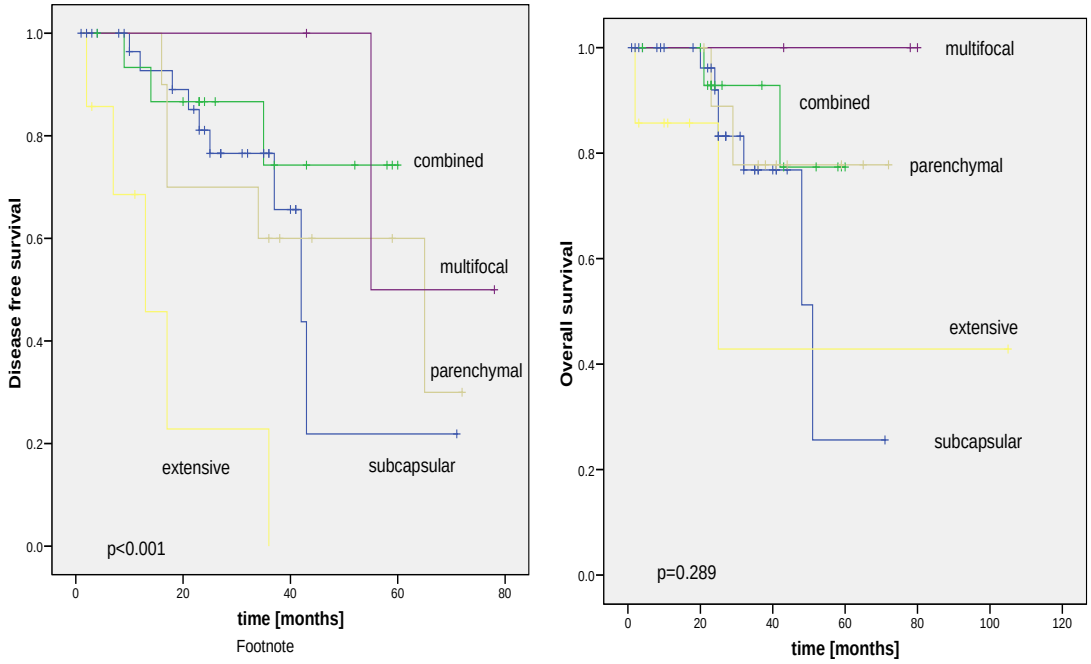


Figure 7. Kaplan-Meier estimated disease-free (left) and overall survival (right) according to the SN metastatic tumor deposit location categories by Dewar, now lumped as extensive versus non-extensive.

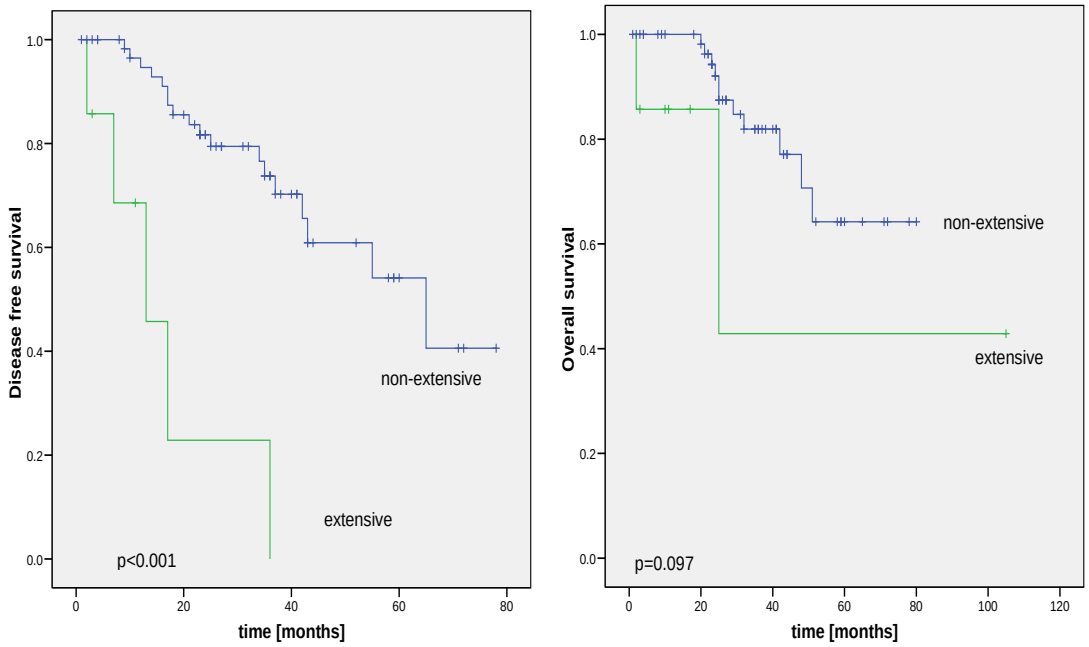
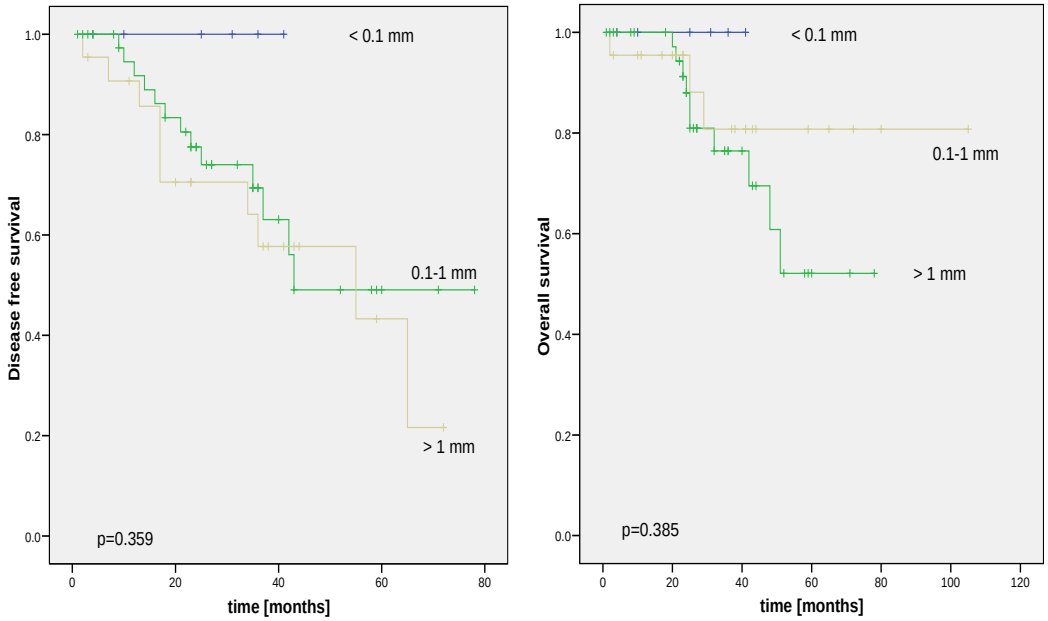


Figure 8. Kaplan-Maier estimated disease-free (left) and overall survival (right) according to the Rotterdam classification of melanoma SN tumor burden (*P*= 0.36).



REFERENCES

1. Cascinelli N, Belli F, Santinami M et al. Sentinel lymph node biopsy in cutaneous melanoma: the WHO Melanoma Program experience. Ann Surg Oncol 2000; 7: 469-474.

2. Houghton A, Coit D, Bloomer W et al. NCCN melanoma practice guidelines. National Comprehensive Cancer Network. Oncology (Williston Park) 1998; 12: 153-177.

3. Scolyer RA, Murali R, Satzger I et al. The detection and significance of melanoma micrometastases in sentinel nodes. Surg Oncol 2008; 17: 165-174.

4. Morton DL, Wen DR, Wong JH et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg 1992; 127: 392-399.

5. Reeves ME, Coit DG. Melanoma. A multidisciplinary approach for the general surgeon. Surg Clin North Am 2000; 80: 581-601.

6. McMasters KM, Reintgen DS, Ross MI et al. Sentinel lymph node biopsy for melanoma: controversy despite widespread agreement. J Clin Oncol 2001; 19: 2851-2855.

7. Bogenrieder T, Canninga van Dijk M, Blokx W et al. Absence of second echelon involvement in melanoma patients with limited Breslow thickness and low sentinel tumor load. J Clin Oncol 2008; 26 May 20 suppl: (Abstr 9060).

8. Thomas JM. Prognostic false-positivity of the sentinel node in melanoma. Nat Clin Pract Oncol 2008; 5: 18-23.

9. Vuylsteke RJ, van Leeuwen PA, Statius Muller MG et al. Clinical outcome of stage I/II melanoma patients after selective sentinel lymph node dissection: long-term follow-up results. J Clin Oncol 2003; 21: 1057-1065.

10. van Akkooi AC, de Wilt JH, Verhoef C et al. Clinical relevance of melanoma micrometastases (<0.1 mm) in sentinel nodes: are these nodes to be considered negative? Ann Oncol 2006; 17: 1578-1585.

11. van Akkooi AC, Nowecki ZI, Voit C et al. Sentinel node tumor burden according to the Rotterdam criteria is the most important prognostic factor for survival in melanoma patients: a multicenter study in 388 patients with positive sentinel nodes. Ann Surg 2008; 248: 949-955.

12. Vuylsteke RJ, Borgstein PJ, van Leeuwen PA et al. Sentinel lymph node tumor load: an independent predictor of additional lymph node involvement and survival in melanoma. Ann Surg Oncol 2005; 12: 440-448.

13. Starz H, Balda BR, Kramer KU et al. A micromorphometry-based concept for routine classification of sentinel lymph node metastases and its clinical relevance for patients with melanoma. Cancer 2001; 91: 2110-2121.

14. Starz H, Siedlecki K, Balda BR. Sentinel lymphonodectomy and s-classification: a successful strategy for better prediction and improvement of outcome of melanoma. Ann Surg Oncol 2004; 11: 162S-168S.

15. Dewar DJ, Newell B, Green MA et al. The microanatomic location of metastatic melanoma in sentinel lymph nodes predicts nonsentinel lymph node involvement. J Clin Oncol 2004; 22: 3345-3349.

16. Balch CM, Buzaid AC, Soong SJ et al. Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. J Clin Oncol 2001; 19: 3635-3648.

17. van Diest PJ, Torrenga H, Meijer S et al. Pathologic analysis of sentinel lymph nodes. Semin Surg Oncol 2001; 20: 238-245.

18. van Akkooi AC, de Wilt JH, Verhoef C et al. High positive sentinel node identification rate by EORTC melanoma group protocol. Prognostic indicators of metastatic patterns after sentinel node biopsy in melanoma. Eur J Cancer 2006; 42: 372-380.

19. Morton DL, Thompson JF, Cochran AJ et al. Sentinel-node biopsy or nodal observation in melanoma. N Engl J Med 2006; 355: 1307-1317.

20. Carlson GW, Murray DR, Lyles RH et al. The amount of metastatic melanoma in a sentinel lymph node: does it have prognostic significance? Ann Surg Oncol 2003; 10: 575-581.

21. Estourgie SH, Nieweg OE, Valdes Olmos RA et al. Review and evaluation of sentinel node procedures in 250 melanoma patients with a median follow-up of 6 years. Ann Surg Oncol 2003; 10: 681-688.

22. Kretschmer L, Beckmann I, Thoms KM et al. Sentinel lymphonodectomy does not increase the risk of loco-regional cutaneous metastases of malignant melanomas. Eur J Cancer 2005; 41: 531-538.

23. Scheri RP, Essner R, Turner RR et al. Isolated tumor cells in the sentinel node affect long-term prognosis of patients with melanoma. Ann Surg Oncol 2007; 14: 2861-2866.

24. Fournier K, Schiller A, Perry RR et al. Micrometastasis in the sentinel lymph node of breast cancer does not mandate completion axillary dissection. Ann Surg 2004; 239: 859-863.

25. Govindarajan A, Ghazarian DM, McCready DR et al. Histological features of melanoma sentinel lymph node metastases associated with status of the completion lymphadenectomy and rate of subsequent relapse. Ann Surg Oncol 2007; 14: 906-912.

26. Rutgers EJ. Sentinel node micrometastasis in breast cancer. Br J Surg 2004; 91: 1241-1242.

27. de Boer M., van Deurzen CH, van Dijck JA et al. Micrometastases or isolated tumor cells and the outcome of breast cancer. N Engl J Med 2009; 361: 653-663.

28. Lee JH, Essner R, Torisu-Itakura H et al. Factors predictive of tumor-positive nonsentinel lymph nodes after tumor-positive sentinel lymph node dissection for melanoma. J Clin Oncol 2004; 22: 3677-3684.

29. Sabel MS, Griffith K, Sondak VK et al. Predictors of nonsentinel lymph node positivity in patients with a positive sentinel node for melanoma. J Am Coll Surg 2005; 201: 37-47.

General Discussion and Summary

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General Discussion and Summary

General Discussion

Cutaneous melanoma has long been identified as one of the most important of all skin diseases and inscrutable of all cancers. DeCholnoky (1), in a 1941 review concluded: ‘‘Radical surgery is the treatment of choice, and should consist of wide local excision... followed by regional lymph node dissection....’’ While the recommended extent of surgery may have decreased over the last 7 decades, it is remarkable that our increased knowledge of the basic molecular and cellular biology of melanoma has not yet resulted in significant changes in treatment. Indeed, metastatic melanoma remains one of the malignancies most resistant to treatment. In 1930, the noted pathologist James Ewing (2) wrote: ‘‘The problems of melanoma maintain their position as the most interesting and complex of any department of oncology.... Possibly there are other important data lying within easy reach of the alert observer.’’ While one must grudgingly admit that the ‘‘problems of melanoma’’ sadly persist, this thesis has aimed to demonstrate the significant progress and challenges that characterize our understanding of melanoma in the 21st century.

And yet there is hope. Within the scope of this thesis, some of the recent molecular and clinicopathological advances in our understanding of the biology of melanoma are featured. For example, impressive advances have been made in our knowledge of the commonest genetic and epigenetic alterations found in sporadic cutaneous melanomas. Valuable recent contributions came from analyses of gene copy number by comparative genome hybridization, and from large-scale gene expression profiling. All of the commonest affected genes encode regulatory components. Loci with established importance in melanoma, like CDKN2A, BRAF and PTEN, have been joined by some less familiar genes including transcription factor sequences TBX2 and STK11 (LKB). This knowledge is complemented by an increased understanding of the cellular signaling pathways affected by these molecules, their biological outcomes, and the implications as to what changes are required overall to generate a melanoma.

Sentinel lymph node biopsy is currently a matter of heated debate. It is a basic tenet of the sentinel lymph node biopsy procedure that all positive sentinel lymph nodes will inevitably progress to palpable nodal recurrence if not removed. Comparison of survival is, therefore, considered permissible among patients with positive sentinel lymph nodes who undergo early lymphadenectomy with that among patients who have delayed lymphadenectomy for palpable regional node metastasis, providing that survival is calculated from the date of wide local excision of the primary tumor. However, that fundamental assumption has been contested by some authors including ourselves and evidence is presented to show that a positive sentinel lymph node might have no adverse prognostic relevance in up to one-third of patients. Furthermore, in the same patients, progression to palpable nodal disease might not have occurred even if the positive sentinel node had not been removed. Thus, the term prognostic false-positivity is used to describe this phenomenon. Such patients are incorrectly up-staged, are given inaccurate prognostic information and can undergo unnecessary completion lymphadenectomy and unnecessary adjuvant therapy.

Cancer and melanoma research over the past decades has been largely focused on events occurring within the boundaries of the plasma membrane of the malignant cell. However, the predominant paradigm, wherein multiple genetic lesions, e.g. of the cyclinD/cdk4-p16^{INK4A}-pRb-pathway (3;4), provide both the impetus for and the possible Achilles heel of cancer (5), is not quite sufficient to understand melanoma as a disease process. Furthermore, some of the genetic lesions frequently encountered in other solid tumors, e.g., alterations of the p53 tumor suppressor gene product, are apparently not as relevant in the evolution of melanoma (6;7). Hence, rather than asking how normal progenitor cells (melanocytes) become cancer (melanoma) cells, one might ask how a multicellular tissue such as the skin is transformed into a highly aggressive neoplasma? Biological events are now beginning to be understood in terms of specific molecules affecting not only the lesional cell itself, but also the surrounding micoenviroenment, cell–cell contact, tumor cell-stroma crosstalk, cell adhesion and migration.

A myriad of data support a model in which the genesis of melanoma requires changes that (1) initiate clonal expansion, (2) overcome cell senescence, and (3) reduce apoptosis. For instance, it is now becoming increasingly clear that fibroblast or endothelial cell recruitment and subversion for the benefit of the malignant cell is a dynamic and intricate process (8;9). It is also apparent that bone marrow stroma-derived “mesenchymal” stem cells play a hitherto under-appreciated role in this scenario and their contributions to neoplasia are coming into focus. These observations may have profound repercussions on the future treatment of invasive and metastasizing melanoma. Consequently, it is possible that the stroma may be successfully targeted for melanoma therapy (“stromal therapy”) (10). Another case in point: since apoptotic programs can experimentally be manipulated to produce massive changes in cell death, the genes and proteins controlling apoptosis are attractive, yet still speculative, drug targets. However, voluminous evidence suggests that such strategies are feasible (for review, see refs (11;12)).
Genetically, biochemically, histopathologically and clinically, cutaneous melanoma is a highly heterogeneous neoplasm. Melanoma can be seen as a spectrum of different etiopathological entities and we believe that it will in the future be classified according to the underlying causative molecular event(s), as is already possible for hematologic malignancies. We now understand that MC1R is a genetic link to melanoma and non-melanoma skin cancer; and it is genetic assessment, not skin color, that is the best indicator of skin cancer risk. People with darker skin who do not burn easily cannot assume that they are *per se* protected from the sun’s damaging effects (13).
Additionally, “master” genes or switches, such as HOX genes, AP-2, MITF have been identified that sit on top of regulatory pyramids and regulate whole batteries of crucial downstream targets.

The abnormal stroma associated with tumors provides a permissive environment for growth, survival and invasion. There is a strong argument that therapeutically targeting the stroma could reverse the altered phenotype of the tumor. If this were possible, the cancer would be less likely to locally invade and metastasize, reducing the tumor to a more benign state. Even more promisingly, the stroma represents a very early target of tumor progression, and therapy could be applied at multiple stages during the evolution of the tumor; perhaps even as a chemopreventative measure.
Currently, antistromal therapy is in its infancy and it is likely that newer targets will emerge as more is learnt about the host–tumor interaction. It is expected that a

greater range of inhibitors for growth factor receptors will become available that will allow the targeting of the melanoma stroma to be validated as a therapeutic option. Currently, there is sufficient preliminary and proof-of-principle data to suggest that when the correct pathway(s) are blocked, the tumor will be brought back under control of the tissue microenvironment, reverting it to a more benign state. As the tumor microenvironment is an important regulator of local invasion, it is hoped that targeting the stroma may suppress metastasis. As melanoma metastases are responsible for nearly all of the associated morbidity and mortality, this would be a most favorable outcome.

There is now a host of evidence that many major pathways of tumorigenesis and regulatory circuits, programs and networks (“gatekeepers”, “caretakers” and “landscapers” (14;15)) -the “hallmark capabilities” (16) - suffer deregulation in melanoma, albeit through different mechano-molecular strategies (intra- versus epigenetic, structural versus functional), at various frequencies and in variable order. Some of these strategies may be redundant, some complementary, and some mutually exclusive. Recently, powerful models have been emerging, such as artificial skin reconstructs and orthotopic skin grafts (17), which will allow us to dissect more accurately these pathways and events leading to melanoma. High throughput technology such as cDNA-microarray chips and the rapidly emerging field of proteomics will allow us to faster and more efficiently analyse large quantities of samples for biochemical alterations and thus be a “quantum leap” towards a molecular pathology and epidemiology. Although there is much more to learn, we anticipate that, in the near future, this information will produce new, rational strategies to exploit melanoma pathobiology for prophylactic, diagnostic and therapeutic benefit.

Summary

Cutaneous melanoma is the most aggressive skin cancer, even one of the most deleterious of all neoplasms, for which complete surgical excision at an early stage remains the only curative treatment option. The dismal progress in the therapy of metastatic melanoma in the last 30 years indicates that none of the previous strategies in chemotherapy or immunotherapy has succeeded in eliminating all malignant cells.
We now view melanoma as a complex tissue resulting from disrupted skin homeostasis, rather than focusing on the melanoma cell, and the genes within it, alone. Normal skin homeostasis is maintained by dynamic interactions between the melanocytes and their microenvironment, such as keratinocytes, fibroblasts, endothelial and immunocompetent cells, and the extracellular matrix.
Similarly, during transformation and progression of melanocytes to melanoma cells, there are, however deregulated, reciprocal and conspirational interactions between the neoplastic cells and the adjacent stromal cells. Thus, “normal” appearing skin cells within the cancerous tissue are not idle bystanders, but rather, after having been recruited and subverted by the melanoma cells, they are active participants that shape the aggressive features of this neoplasm. The molecular events of melanoma development and progression are now being deciphered (Figure 2 of General Introduction).

One of the most powerful prognostic factors for survival in melanoma patients is the presence of (micro-) metastatic disease in the regional lymph nodes. The optimal surgical management of clinically occult lymph node metastases, often termed “micrometastases” because they only consist of a limited number of cells, has been hotly debated for many years and continues to do so unabatedly.
Basically, there are two treatment options: one is elective lymph node dissection and the other is the so-called “wait-and-watch” approach. A serious disadvantage of the elective lymph node dissection is its invasiveness and hence associated morbidity. Roughly 80% of patients with no sign of metastatic disease are subjected to an operation with no apparent benefit for the patient.

Conceptually a good way forward, then, is the removal of only the regional lymph node(s) which directly drain the primary tumor, the so-called sentinel lymph node(s). The sentinel lymph node concept is based on the idea of an “orderly” progression of metastatic cells through the lymphatic system. It assumes that early lymphatic metastases, if present, are always encountered first within the first draining lymph node, i.e. the sentinel node. A sentinel lymph node free of tumor would thus predict the absence of metastatic disease in the rest of the draining lymph node basin and hence negate the necessity of a completion or elective lymph node dissection.

In this thesis we described molecular, cellular and clinicopathological aspects of melanoma. Furthermore, we re-evaluated and expanded a model to predict non-sentinel node involvement in a multicenter study.

Normal skin architecture and melanocyte function is maintained by a dynamic interplay between the melanocytes themselves, the epithelial cells between which they are interspersed, and their microenvironment. The microenvironment consists of the extracellular matrix, fibroblasts, migratory immune cells, and neural elements supported by a vascular network, all within a milieu of cytokines, growth factors, and

bioactive peptides as well as proteolytic enzymes. In **Chapter 3**, our current knowledge of the role of cell surface peptidases in melanoma is summarised and discussed. Cell-surface (membrane) peptidases are a multi-functional group of ectoenzymes that have been implicated in the control of growth and differentiation of many cellular systems.

On the basis of recent findings we propose in **Chapter 4** that involvement of fibroblasts in melanoma-stromagenesis occurs through different stages: recruitment, activation, and conversion to myofibroblasts, or differentiation to fibrocytes. We reason that this involvement is topographically linked to different areas in and around the tumor, and hypothesise that stromal activation, as seen in tumor ulceration or immunological regression in melanoma, stimulates tumor progression.

As outlined in **Chapter 5**, recent studies have identified defects at multiple levels of the apoptosis program in melanoma, which provided new clues to drug resistance of this highly aggressive neoplasm. The process of apoptosis provides a conceptual framework to link melanoma genetics with the outcome of melanoma therapy. Thus, the genes and proteins that control apoptosis provide exciting new targets for rationally designed anti-melanoma therapeutic strategies. Hence, there is evidence that melanoma is no longer a “molecular black box” and hope that our increased knowledge of the apoptotic progress will also lead to new therapeutic avenues.

In **Chapter 6** we describe our analyses of the mutation and expression of the low affinity neurotrophin receptor $p75^{NTR}$ in melanoma. For this purpose, we examined cell lines established from normal human melanocytes and metastatic melanomas for expression of $p75^{NTR}$ mRNA and protein.
The results showed that, compared with normal melanocytes, levels of $p75^{NTR}$ specific protein were high in seven melanoma cell lines, markedly decreased in two melanoma cell lines and comparable in two melanoma cell lines. The conserved transmembrane domain of $p75^{NTR}$ was analysed for point mutations by single strand conformation polymorphism analysis and direct DNA sequencing. We report here for the first time the detection of point mutations in the transmembrane domain of $p75^{NTR}$ in human melanomas. Identical point mutations were detected in the transmembrane domain of $p75^{NTR}$ in the two melanoma lines with reduced $p75^{NTR}$ protein expression. These mutations occurred in a conserved region near the transmembrane domain at codon 802. The effect resulting from this mutation is unclear. It is possible that a conformational change in the structure of the transmembrane secondary to substitution of an uncharged amino acid (Gly) for a negatively charged one (Asp) could alter the affinity of $p75^{NTR}$ for its ligand, resulting in an inhibition of its apoptotic function. Alternatively, a mutated cytoplasmatic domain could result in aberrant signal transduction via sphyngomyelin and ceramide. Since $p75^{NTR}$ binds to all neurotrophins, a potential role may be to provide additional specificity to the interactions with individual trk family members. Moreover, a defect in $p75^{NTR}$ could potentially affect the biosynthesis of this receptor. Since some melanomas produce neurotrophins, it is conceivable that these stimulate the growth of melanoma cells via an autocrine loop involving the p75 receptor. Further functional studies are required to define the effects of mutations on $p75^{NTR}$ function and the biology of melanomas.

The induction of melanocytic skin lesions in adult cancer patients undergoing chemotherapy for metastatic disease as described in **Chapter 7** has not yet been

related previously. Our clinicopathologic observation is of importance for two reasons:

1. it underlines the existence of a histological simulator of lentigo maligna, as there has been found for in situ superficially spreading melanoma after experimental ultraviolet irradiation of melanocytic naevi;
2. an adult patient who develops eruptive melanocytic skin lesions while undergoing chemotherapy for metastatic cancer with 5-FU has hitherto not been described .

Thus, we believe that relevant lessons can be learned for the pathogenesis of melanoma from this report as it adds further evidence to the link between systemic (iatrogenic or intrinsic) immunosuppression and the induction of melanocyte proliferation and transformation.

As mentioned previously, 70-90% of melanoma patients with a positive sentinel lymph node have no positive additional lymph nodes. Yet, they are usually all indiscriminately subjected to an additional, completion lymph node dissection with associated morbidity and maybe over-treatment with potentially harmful adjuvant therapy. In **Chapter 8**, we have re-evaluated and refined a model in an attempt to predict the absence of additional lymph node metastases in patients with a positive sentinel lymph node.
For this purpose, we employed features of the primary melanoma and sentinel tumor load. Tumor load of the sentinel lymph node was assessed by measuring the total surface area of metastasis in the affected lymph node by computerized morphometry. In a similar fashion, we also determined the penetrative depth of metastatic cells into the sentinel node and the diameter of largest tumor cell deposit.
We analyzed 70 sentinel lymph node positive patients, of whom 52 had metastases limited to the sentinel node, and 19 of whom had one or more positive additional lymph nodes. None of the 19 patients (27%) with a Breslow thickness <2.3 mm and a sentinel node tumor load <0.2 mm^2 had non-sentinel node metastases. Similarly, none of the 21 patients (30%) with a Breslow thickness <2.3 mm and sentinel node penetrative depth <600 □m had non-SN metastases. Lastly, none of the 19 patients (27%) with a Breslow thickness <2.3 mm and a diameter of the largest sentinel node deposit <500 □m had non-sentinel node metastases. Logistic regression identified sentinel node penetrative depth to be the strongest single predictor of non-sentinel node metastases. Consequently, a combination of limited Breslow thickness and low sentinel node tumor load predicts absence of non-sentinel node metastases in melanoma patients with a positive sentinel node with high accuracy.

The methodology described in Chapter 7 has several important advantages:

1) It is easy to use in daily routine and is based on a primary tumor already routinely reported (Breslow thickness) and an sentinel node feature (depth of melanoma cell invasion or largest metastatic diameter) that can easily be measured by pathologists.
2) It independently predicts non-sentinel node status.
3) It varies proportionally with the risk of non-sentinel node positivity.
4) The proposed technique has an anatomo-physiologic basis, with metastatic melanoma presumably entering a lymph node via the afferent lymphatics into the subcapsular sinus, before passing into the paracortex, and ultimately replacing large parts of the node.
5) Given the contradictory data surrounding the influence of Breslow thickness on non-sentinel node status, it is also advantageous that this method does not

depend on histologic characteristics of the primary melanoma only, but also on the sentinel node.

Using this model, we should now able to divide sentinel lymph node-positive melanoma patients, some of whom do not benefit from an additional (completion) lymph node dissection, because they are very un likely to have additional lymph node involvement. We propose that this subgroup, which comprises about 30% of patients, may be spared completion lymph node dissection. Clearly, larger prospective confirmatory trials are needed to test this, by which these “low risk” sentinel node-positive patients are randomized between upfront completion lymph node dissection or watchful waiting with secondary completion lymph node dissection once palpable lymph node metastases occur.

In **Chapter 9**, we took this approach one step further and evaluated whether these “low risk” subgroups had better disease-free as well as overall survival. We found that SN tumor burden (morphometrically measured by SN total tumor area, penetrative depth of invasion into the SN or diameter of the largest SN tumor deposit), in combination with a Breslow thickness < 2.0 mm can predict the prognosis in melanoma patients. Using a threshold for diameter of the largest SN tumor deposit of 0.5 mm and a Breslow threshold of 2 mm resulted in a subgroup of 17% of patients (which not only had no non-SN metastases as previously shown in chapter 8 but also a 100% disease free and overall survival. This is completely in line with the previous results from the VUmc. Identical results were obtained using the total SN metastatic load (threshold 0.2 mm^2) instead of diameter of the largest SN tumor deposit. Assessment of total SN metastatic load is however more complicated and time consuming than relatively simple diameter measurements. Penetrative depth of the SN metastases yielded in combination with Breslow thickness no significant results although a similar trend was seen as for total tumour area and size. Using just size as proposed by the Rotterdam group yielded no prognostic significance, as was the case for the old and new Starz classification. Microanatomic location according to Dewar did indicate significantly better survival for patients with non-extensive metastases, but the 5 year disease free survival of this subgroup was still only 50%.

<u>Conclusions: the sentinel lymph node procedure</u>
In terms of overall survival, the actual therapeutic benefit of the sentinel lymph node procedure remains yet to be determined. Whether examination of the sentinel lymph node with selective lymphadenectomy in clinically node-negative patients and adjuvant medical treatment only in sentinel lymph node–positive patients is more beneficial than clinical follow-up with therapeutic dissection at the clinical onset of nodal disease is still under intense discussion. Some data suggest that an early removal of positive lymph nodes may improve survival (21;22). The Intergoup Melanoma Surgical Trial suggested that the benefit of elective lymph node dissection may be meaningful in patients whose tumors are non-ulcerated, with extremity primaries, with a Breslow thickness from 1 to 2 mm, and who are less than 60 years of age (21-24).
The sentinel node biopsy represents a minimally invasive procedure for identifying patients who have subclinical stage III disease, with a high risk of developing occult distant metastases. Ongoing clinical trials attempt to determine the efficacy of selective lymph node dissection guided by sentinel lymph node mapping. In 2006 the final results of the Multicenter Selective Lymphadenectomy Trial I (MSLT-I) were published. This trial compared selective sentinel lymph node dissection and selective

complete lymph node dissection with observation (18). Results of the Multicenter Selective Lymphadenectomy Trial II (MSLT-II) are eagerly awaited.

Irrespective of the outcome of these trials, the sentinel lymph node procedure has some major advantages: First and foremost, the detection of micrometastatic disease using the sentinel lymph node procedure allows a more accurate staging. The sentinel lymph node status is a strong and independent prognostic factor. Secondly, the sentinel lymph node procedure potentially spares node-negative patients further lymph node dissection, which is often associated with significant morbidity. Thirdly, it enables a more consistent interpretation of clinical trials through the provision of the "true" pathological (*versus* clinical) stage. Fourthly, a more accurate knowledge of lymphatic drainage patterns is provided by the sentinel lymph node procedure. Fifthly, there is a highly important psychological benefit for patients whose sentinel lymph node does not reveal metastases. Lastly, it provides a tool to select patients for adjuvant therapy trials. In our opinion, therefore, the sentinel lymph node biopsy should always be performed in melanoma patients.

Overall conclusions

Overall conclusions we can draw from the research described in this thesis:

- Melanoma is a complex tissue resulting from disrupted skin homeostasis, rather than putting the focus on the melanoma cell, and the genes within it, alone.

- During transformation and progression of melanocytes to melanoma cells, there are reciprocal and conspirational interactions between the neoplastic cells and the adjacent stromal cells. Skin cells within the cancerous tissue are not idle bystanders, but rather active participants that shape the aggressive features of this neoplasm.

- Careful clinical observations and what they teach us about the biology of melanoma still have a place in modern medicine, even in the molecular age.

- Patients with positive sentinel lymph nodes are a heterogeneous group; not all sentinel node-positive patients have necessarily a poor prognosis.

- Sentinel lymph node tumor load, i.e. depth of invasive penetration, or metastatic size, can be used to predict the absence of additional metastatic lymph nodes in melanoma patients.

- Based on sentinel tumor load and Breslow thickness of the primary tumor, subgroups of patients with a positive sentinel node can be identified who will not significantly benefit from completion lymph node dissection and have excellent prognosis.

- Selective lymph node dissection is potentially curative for a subset of patients

- Even if selective sentinel lymph node dissection in and by itself should not improve survival, it is of great prognostic value and should be continued until another, less invasive staging procedure with similar predictive value is available.

- Randomised clinical trials are essential to answer the remaining questions, especially in terms of overall survival benefit of the sentinel lymph node procedure.

References

(1) de Cholnoky T. Ann Surg 1941;113:392-410.

(2) Ewing J. The Problems of melanoma. Br J Med 1930;11:852-6.

(3) Sherr CJ. The Pezcoller lecture: cancer cell cycles revisited. Cancer Res 2000 Jul 15;60(14):3689-95.

(4) Piepkorn M. Melanoma genetics: an update with focus on the CDKN2A(p16)/ARF tumor suppressors. J Am Acad Dermatol 2000 May;42(5 Pt 1):705-22.

(5) Park CC, Bissell MJ, Barcellos-Hoff MH. The influence of the microenvironment on the malignant phenotype. Mol Med Today 2000 Aug;6(8):324-9.

(6) Castellano M, Parmiani G. Genes involved in melanoma: an overview of INK4a and other loci. Melanoma Res 1999 Oct;9(5):421-32.

(7) Albino AP, Vidal MJ, McNutt NS, Shea CR, Prieto VG, Nanus DM, et al. Mutation and expression of the p53 gene in human malignant melanoma. Melanoma Res 1994 Feb;4(1):35-45.

(8) Ruiter D, Bogenrieder T, Elder D, Herlyn M. Melanoma-stroma interactions: structural and functional aspects. Lancet Oncol 2002 Jan;3(1):35-43.

(9) Elenbaas B, Weinberg RA. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. Exp Cell Res 2001 Mar 10;264(1):169-84.

(10) Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. Nature 2001 May 17;411(6835):375-9.

(11) Schmitt CA, Lowe SW. Apoptosis and therapy. J Pathol 1999 Jan;187(1):127-37.

(12) Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis 2000 Mar;21(3):485-95.

(13) Sturm RA, Teasdale RD, Box NF. Human pigmentation genes: identification, structure and consequences of polymorphic variation. Gene 2001 Oct 17;277(1-2):49-62.

(14) Kinzler KW, Vogelstein B. Cancer-susceptibility genes. Gatekeepers and caretakers. Nature 1997 Apr 24;386(6627):761, 763.

(15) Kinzler KW, Vogelstein B. Landscaping the cancer terrain. Science 1998 May 15;280(5366):1036-7.

(16) Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000 Jan 7;100(1):57-70.

(17) Satyamoorthy K, Meier F, Hsu MY, Berking C, Herlyn M. Human xenografts, human skin and skin reconstructs for studies in melanoma development and progression. Cancer Metastasis Rev 1999;18(3):401-5.

(18) Morton DL, Thompson JF, Cochran AJ, Mozzillo N, Elashoff R, Essner R, et al. Sentinel-node biopsy or nodal observation in melanoma. N Engl J Med 2006 Sep 28;355(13):1307-17.

(19) van Akkooi AC, de Wilt JH, Verhoef C, Schmitz PI, van Geel AN, Eggermont AM, et al. Clinical relevance of melanoma micrometastases (<0.1 mm) in sentinel nodes: are these nodes to be considered negative? Ann Oncol 2006 Oct;17(10):1578-85.

(20) van Akkooi AC, Nowecki ZI, Voit C, Schafer-Hesterberg G, Michej W, de Wilt JH, et al. Sentinel node tumor burden according to the Rotterdam criteria is the most important prognostic factor for survival in melanoma patients: a multicenter study in 388 patients with positive sentinel nodes. Ann Surg 2008 Dec;248(6):949-55.

(21) Cascinelli N, Morabito A, Santinami M, MacKie RM, Belli F. Immediate or delayed dissection of regional nodes in patients with melanoma of the trunk: a randomised trial. WHO Melanoma Programme. Lancet 1998 Mar 14;351(9105):793-6.

(22) Kretschmer L, Hilgers R, Mohrle M, Balda BR, Breuninger H, Konz B, et al. Patients with lymphatic metastasis of cutaneous malignant melanoma benefit from sentinel lymphonodectomy and early excision of their nodal disease. Eur J Cancer 2004 Jan;40(2):212-8.

(23) Balch CM, Soong SJ, Bartolucci AA, Urist MM, Karakousis CP, Smith TJ, et al. Efficacy of an elective regional lymph node dissection of 1 to 4 mm thick melanomas for patients 60 years of age and younger. Ann Surg 1996 Sep;224(3):255-63.

(24) Balch CM, Soong S, Ross MI, Urist MM, Karakousis CP, Temple WJ, et al. Long-term results of a multi-institutional randomized trial comparing prognostic factors and surgical results for intermediate thickness melanomas (1.0 to 4.0 mm). Intergroup Melanoma Surgical Trial. Ann Surg Oncol 2000 Mar;7(2):87-97.

Future Perspectives

11

Future Perspectives

The sentinel node procedure

The benefit of the sentinel lymph node biopsy, especially with regard to recurrences, is currently hotly debated. Some groups argue that the sentinel lymph node increases the risk of local and in-transit metastases in melanoma and, therefore, should be abandoned (1-4). We, together with others, beg to differ with this conclusion, since in our opinion a careful examination of the evidence does not support it (5-7). Cohorts of patients are not compared correctly, or the data of compared cohorts are incompatible. Patients who had undergone a wide local excision only were compared either with sentinel lymph node-negative patients or with sentinel lymph node-positive patients. Since the sentinel lymph node status of patients who had undergone a wide local excision is unknown, this group of patients is merely comparable to the total sentinel lymph node biopsy group. What's more, some trials concerning wide local excision only excluded patients with a Breslow thickness exceeding 2 mm, whereas these trials were compared to studies concerning sentinel lymph node biopsy that also included patients with a Breslow thickness of 2 mm and above. Since Breslow thickness is a strong independent prognostic factor, this makes these data incomparable. The phase III Multicenter Selective Lymphadenectomy Trial I has failed to settle this dispute definitively. Thus, until more data from randomised trials emerge, the use of the sentinel lymph node procedure, the most accurate staging procedure for early-stage melanoma, should continue.

Sentinel lymph node patients are a heterogeneous group. As we and others have shown, not all these patients have additional positive lymph node involvement (8-12). Therefore, we do not believe that all sentinel lymph-node positive patients should automatically undergo an additional lymph node dissection. We, and others, have shown that not all sentinel lymph node-positive patients have a poor prognosis (13-15). Indeed, some subgroups have an excellent five- year survival of around 100%. Using models to predict additional lymph node involvement could select subpopulations to undergo an additional (completion) lymph node dissection-or-not (13,16-19). However, the question of the "correct" cut-off point for melanoma sub-micrometastases and the most reliable algorithm to predict disease recurrence remains.

Clearly, to settle these outstanding issues, prospective studies, in which sentinel lymph node positive patients are randomized between additional lymph node dissection and no additional lymph node dissection, need to be performed.

For this reason, a sequel to the Multicenter Selctive Lymphadenectomy Trial, i.e. MSLT-II, has started. Possibly, the MSLT-II will answer this question. MSTL-II, directed by Morton and colleagues, is investigating whether SN biopsy followed by CLND is superior to SN biopsy alone in patients with evidence of metastases in the SNs by histopathological or molecular techniques. In this study, sentinel lymph node-positive patients are stratified and randomized for additional lymph node dissection or observation (20).

Melanoma research: were do we go from here?

The dismal progress in the treatment of disseminated melanoma over the past decades clearly indicates that none of the previous strategies in chemotherapy or immunotherapy have succeeded in eliminating all malignant cells and that our understanding of melanoma biology has only just begun.

Based on the work presented herein, it becomes evident that future melanoma research will have to address several pivotal issues:

1) how melanoma stem cells or tumor-initiating cells may play a critical role in the pathogenesis of melanoma and its resistance to conventional therapy;
2) the need to explore the role of UV light in the pathogenesis of melanoma;
3) how animal models can advance our understanding of both tumor initiation and metastasis;
4) the continuous need for more powerful models such as artificial skin reconstructs and orthotopic skin grafts, which will allow us to dissect more accurately the pathways and events leading to melanoma;
5) the availability of high throughput technology such as cDNA-microarray chips and the rapidly emerging field of proteomics;
6) based on the aforementioned methodological progress: an increased understanding of the molecular genetics and biology of melanoma;
7) to identify and evaluate more reliable prognostic and predictive (bio-)markers;
8) to elucidate melanoma–stroma interactions and if they could be therapeutically exploited;
9) to further our understanding of melanoma immunology and, consequently, strategies for using the immune system to prevent or treat life-threatening metastatic disease;
10) how all of the above may lead to new specific (targeted) therapeutic interventions, especially drug candidates (small molecules, antibodies and vaccines);
11) to get a greater number of innovative compounds more rapidly into the clinic ("from bench to bedside");
12) to involve more patients in well-designed (ideally: randomized) clinical trials;
13) to make efficacious and safe drugs, with added benefit, available for as many patients as possible, at an acceptable cost for society.

References

1. Estourgie SH, Nieweg OE, Kroon BB. High incidence of in-transit metastases after sentinel node biopsy in patients with melanoma. Br J Surg. 2004; 91:1370-1371.

2. Thomas JM, Clark MA. Selective lymphadenectomy in sentinel node-positive patients may increase the risk of local/in-transit recurrence in malignant melanoma. Eur J Surg Oncol. 2004; 30:686-691.

3. Thomas JM, Giblin AV. Dermatologists should exert a greater influence over sentinel lymph node biopsy for melanoma in the U.K. Br J Dermatol. 2005; 152:176-177.

4. Medalie N, Ackerman AB. Sentinel node biopsy has no benefit for patients whose primary cutaneous melanoma has metastasized to a lymph node and therefore should be abandoned now. Br J Dermatol. 2004; 151:298-307.

5. Kang JC, Wanek LA, Essner R et al. Sentinel lymphadenectomy does not increase the incidence of in-transit metastases in primary melanoma. J Clin Oncol. 2005; 23:4764-4770.

6. Kretschmer L, Beckmann I, Thoms KM et al. Sentinel lymphonodectomy does not increase the risk of loco-regional cutaneous metastases of malignant melanomas. Eur J Cancer. 2005; 41:531-538.

7. Molenkamp BG, Statius Muller MG, Vuylsteke RJ et al. Selective lymphadenectomy in sentinel node-positive patients may increase the risk of local/in-transit recurrence in malignant melanoma. Eur J Surg Oncol. 2005; 31:211-212.

8. Thompson JF, Scolyer RA, Kefford RF. Cutaneous melanoma. Lancet. 2005; 365:687-701.

9. Wagner JD, Gordon MS, Chuang TY et al. Predicting sentinel and residual lymph node basin disease after sentinel lymph node biopsy for melanoma. Cancer. 2000; 89:453-462.

10. Gershenwald JE, Thompson W, Mansfield PF et al. Multi-institutional melanoma lymphatic mapping experience: the prognostic value of sentinel lymph node status in 612 stage I or II melanoma patients. J Clin Oncol. 1999; 17:976-983.

11. Statius Muller MG, van Leeuwen PA, de Lange-De Klerk ES et al. The sentinel lymph node status is an important factor for predicting clinical outcome in patients with Stage I or II cutaneous melanoma. Cancer. 2001; 91:2401-2408.

12. Cochran AJ, Huang RR, Guo J et al. Current practice and future directions in pathology and laboratory evaluation of the sentinel node. Ann Surg Oncol. 2001; 8:13S-17S.

13. Vuylsteke RJ, Borgstein PJ, van Leeuwen PA et al. Sentinel lymph node tumor load: an independent predictor of additional lymph node involvement and survival in melanoma. Ann Surg Oncol. 2005; 12:440-448.

14. Vuylsteke RJ, van Leeuwen PA, Statius Muller MG et al. Clinical outcome of stage I/II melanoma patients after selective sentinel lymph node dissection: long-term follow-up results. J Clin Oncol. 2003; 21:1057-1065.

15. van Akkooi AC, Nowecki ZI, Voit C et al. Sentinel node tumor burden according to the Rotterdam criteria is the most important prognostic factor for survival in melanoma patients: a multicenter study in 388 patients with positive sentinel nodes. Ann Surg. 2008; 248:949-955.

16. Starz H, Balda BR, Kramer KU et al. A micromorphometry-based concept for routine classification of sentinel lymph node metastases and its clinical relevance for patients with melanoma. Cancer. 2001; 91:2110-2121.

17. Starz H, De DA, Balda BR. The Augsburg experience: histological aspects and patient outcomes. Ann Surg Oncol. 2001; 8:48S-51S.

18. Starz H. Pathology of the sentinel lymph node in melanoma. Semin Oncol. 2004; 31:357-362.

19. Cochran AJ, Wen DR, Huang RR et al. Prediction of metastatic melanoma in nonsentinel nodes and clinical outcome based on the primary melanoma and the sentinel node. Mod Pathol. 2004; 17:747-755.

20. Morton DL, Cochran AJ. The case for lymphatic mapping and sentinel lymphadenectomy in the management of primary melanoma. Br J Dermatol. 2004; 151:308-319.

Glossary

Apaf-1, apoptotic protease activating factor-1; **APN**, aminopeptidase N; **ARF**, alternative reading frame; **bFGF**, basic fibroblast growth factor; **CAM**, cell adhesion molecule; **CDK**, cyclin-dependent kinase; **CKI**; cyclin-dependent kinase inhibitor; **CLND**, completion lymph node dissection; **DFS**, disease-free survival; **DPPIV**, dipeptidyl peptidase IV; **FAMMM**, familial atypical multiple mole/melanoma; **IAP**, inhibitor of apoptosis protein; **IGF-1**, insulin-like growth factor 1; **IL**, interleukin; **INK4**, inhibitor of the cyclin-dependent kinase 4; **LOH**, loss of heterozygosity; **MAPK**, mitogen-activated protein kinase; **MC1R**, melanocortin receptor; **MEK**, mitogen-activated protein kinase kinase; **MMP**, matrix metalloproteinase; **MSH**, melanocyte-stimulating hormone; **MSLT**, Multicenter Selective Lymphadenectomy Trial; **MT1-MMP**, membrane-type matrix metalloproteinase; **NEP**, neutral endopeptidase; **NGFR**, nerve growth fctor recptor; **OS**, overall survival; **PAX3**, paired-box gene 3; **PDGF**, platelet derived growth factor; **PI3K**, phosphoinositide 3'-kinase; **PKB**, protein kinase B; **PTEN**, phosphatase and tensin homolog deleted on chromsome 10; **RGP**, radial growth phase; **SCF**, stem cell factor; **SN**, sentinel (lymph) node; **TGF-β**, transforming growth factor-β; **TIMP**, tissue inhibitor of matrix metalloproteinases; **UV**, ultraviolet; **VEGF**, vascular endothelial growth factor; **VGP**, vertical growth phase.

List of publications

Original Articles and Reviews

1. Hoffman AD, Engelstein D, **Bogenrieder T**, Papandreou C, Steckelman E, Motzer RJ, Dmitrovsky E, Albino AP, Nanus DM. Expression of retinoid acid receptor beta correlates with sensitivity to the antiproliferative effects of retinoic acid in human renal cell carcinomas. *Clin Cancer Res* 1996;2:1077-1082.

2. Papandreou CN, **Bogenrieder T**, Scher HI, Albino AP, Nanus DM. Expression and sequence analysis of the SDI1/WAF1/CIP1/p21 tumor suppressor gene in prostate cancer cell lines. *Int J Oncol* 1996;8:1237-1241.

3. Papandreou C, **Bogenrieder T**, Loganzo F, Chao MV, Nanus DM, Albino AP. Mutation and expression of the low affinity nerve growth factor receptor in human malignant melanoma. *Melanoma Res* 1996;6:373-378.

4. Hardy DO, Scher HI, **Bogenrieder T**, Sabbatini P, Zhang Z-F, Nanus DM, Catterall JF. Androgen receptor CAG repeat lengths in prostate cancer: Correlation with age of onset. *J Clin Endo Metab* 1996;81:4400-4405.

5. Papandreou CN, **Bogenrieder T**, Loganzo F, Albino AP, Nanus DM. Expression and sequence analysis of the p21(WAF1/CIP1) gene in renal cancers. *Urology* 1997;41:481-486.

6. **Bogenrieder T**, Finstad CL, Freeman R, Reuter VE, Papandreou C, Scher HI, Albino AP, Nanus DM. Expression and localization of aminopeptidase A, aminopeptidase N, and dipeptidyl peptidase IV in human benign and malignant prostate tissue. *Prostate* 1997;33:225-232.

7. **Bogenrieder T**, Griese DP, Schiffner R, Buettner R, Riegger G, Hohenleutner U, Landthaler M. Wells' syndrome in combination with hypereosinophilic syndrome. *Br J Dermatol* 1997;137:978-982.

8. Papandreou CN, Usmani B, Geng Y, **Bogenrieder T**, Freeman R, Wilk S, Finstad CL, Reuter VE, Powell CT, Scheinberg D, Magill C, Scher HI, Albino AP, Nanus DM. Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. *Nature Med* 1998;4:50-57.

9. **Bogenrieder T**, Landthaler M, Stolz W. $p16^{INK4A}$/CDKN2A/MTS1 - das „Melanomgen″? Stand der Forschung und Ausblick. *Hautarzt* 1998;49:91-100.

10. **Bogenrieder T**, Landthaler M, Szeimies R-M. Succesful Treatment of granuloma annulare with a topically applied vitamin E emulsion: a retrospective study. *J Dermatol Treat* 1998,9:169-173

11. Allert MH, **Bogenrieder T**, Hosemann W. Chondrodermatitis multinodularis bilateralis treated by a modified Stentström approach. *Otolaryngol Nova* 1998;7:217-219

12. Nanus DM, **Bogenrieder T**, Papandreou CN, Finstad CL, Lee A, Vlamis V, Motzer RJ, Bander NH, Albino AP, Reuter VE. Characterization of aminopeptidase A expression and enzymatic activity in primary human renal cancers. *Int J Oncol* 1998, 13:261-267

13. Kroiss M, Bosserhoff AK, Vogt T, Buettner R, **Bogenrieder T**, Landthaler M, Stolz W. Loss of expression or mutations of the p73 tumor suppressor gene are not involved in the pathogenesis of malignant melanoma. *Melanoma Res* 1998, 8:504-509

14. *Papandreou CN, ***Bogenrieder T**, Finstad CL, Freeman RH, Chao MV, Albino AP, Scher HI, Reuter VE, Nanus DM. Reversal of the low affinity neurotrophin receptor stromal-epithelial expression pattern between benign and malignant human prostate. *Urologic Oncol* 1998, 4:210-217 (*equal contribution)

15. Lohmann CP, Kroher G, **Bogenrieder T**, Spiegel D, Preuner J. Severe loss of vision during adjuvant interferon alfa-2b treatment for malignant melanoma. *Lancet* 1999, 353:1326

16. Vogt T, Kroiss M, McClelland M, Gruss C, Becker B, Bosserhoff AK, Rumpler G, **Bogenrieder T**, Landthaler, Stolz W. Deficiency of a novel retinoblastoma binding protein 2-homolog is a consistent feature of sporadic human melanoma skin cancer. *Lab Invest* 1999, 79:1615-1627

17. Bosserhoff AK, Hauschild A, Hein R, Schadendorf D, Stockflesh E, **Bogenrieder T**, Landthaler M, Stolz W. Elevated MIA serum levels are of relevance for management for metastasized malignant melanomas: results of a German multicenter study. *J Invest Dermatol* 2000, 114:395-396

18. **Bogenrieder T**, Landthaler M, Stolz W. Airborne contact dermatitis due to chloroacetamide in wall paint. *Contact Dermatitis* 2001, 45:55

19. Satyamoorthy K, **Bogenrieder T**, Herlyn M. No longer a molecular black box: new clues to apoptosis and drug resistance in melanoma. *Trends Mol Med* 2001, 7:191-194

20. Gabler B, Kroher G, **Bogenrieder T**, Spiegel D, Preuner J, Lohmann CP. Schwerer beidseitiger Sehverlust bei malignem Melanom der Haut. *Ophthalmologe* 2001, 98:672-673

21. Vogt T, McClelland M, Jung B, Popova S, **Bogenrieder T**, Becker B, Rumpler G, Landthaler M, Stolz W. Progression and NSAID-induced apoptosis in malignant melanomas are independent of cyclooxygenase II. *Melanoma Res* 2001, 11:587-599

22. Burgdorff T, Douwes KE, **Bogenrieder T**, Szeimies RM, Hohenleutner U, Landthaler M, Stolz W. Superior vena cava syndrome: an important differential diagnosis in patients with facial edema. *Acta Derm Venereol* 2001, 81:205-206

23. Ruiter D, **Bogenrieder T**, Elder D, Herlyn M. Melanoma-stroma interactions: structural and functional aspects. *Lancet Oncology* 2002, 3:35-43

24. **Bogenrieder T**, Herlyn M. Cell-surface proteolysis, growth factor activation and intercellular communication in the progression of melanoma. *Crit Rev Hem Onc* 2002, 44:1-15

25. **Bogenrieder T**, Weitzel C, Schölmerich J, Landthaler M Stolz W. Eruptive multiple lentigo maligna-like lesions in a patient undergoing chemotherapy with an oral 5-FU-prodrug for metastasising colorectal carcinoma: a lesson for the pathogenesis of malignant melanoma? *Dermatology* 2002, 205:174-175

26. **Bogenrieder T**, Kuske M, Landthaler M, Stolz W. Benign Degos' disease developing during pregnancy and followed for 10 years. *Acta Derm Venereol* 2002, 82:284-287

27. **Bogenrieder T**, Allert MH, Landthaler M, Stolz W. Multiple, bilateral and painful ear nodules of the antihelices: a variant of chondrodermatitis nodularis? *Eur J Dermatol* 2002, 12:482-484

28. Girlich C, **Bogenrieder T**, Palitzsch KD, Schölmerich J, Lock G. Orofacial granulomatosis as initial manifestation of Crohn's disease: a report of two cases. *Eur J Gastroenterol Hepatol* 2002, 14:873-876

29. Schaider H, Oka M, **Bogenrieder T**, Nesbit M, Satyamoorthy K, Berking C, Matsushima K, Herlyn M. Biphasic response of primary melanomas to neutrophils attracted by IL-8. *Int J Cancer* 2003, 103:335-343

30. Gruss CJ, Satyamoorthy K, Berking C, Lininger J, Nesbit M, Schaider H, Liu ZJ, Oka M, Hsu MY, Shirakawa T, Li G, **Bogenrieder T**, Carmeliet P, El-Deiry WS, Eck SL, Rao JS, Baker AH, Bennett J, Crombleholme TM, Karmacharya J, Margolis DJ, Wilson JM, Detmar M, Skobe M, Robbins PD, Johnson C, Buck C, Herlyn M. Re-modeling of human skin architecture in vivo by adenovirus-mediated gene transfer of growth factors and proteolytic enzymes. *J Invest Dermatol* 2003, 120:683-692

31. **Bogenrieder T**, Stolz W. Aus der Neuen Welt: Louis A. Duhring und die Dermatitis herpetiformis. *Hautarzt* 2003, 54:167-172

32. **Bogenrieder T**, Landthaler M, Stolz W. Bullous congenital ichthyosiform erythroderma: safe and effective topical treatment with calcipotriol ointment in a child. *Acta Derm Venereol* 2003, 83:52-54

33. Li G, Satyamoorthy K, Meier F, Berking C, **Bogenrieder T**, Herlyn M. Function and regulation of melanoma –stromal fibroblast interactions: when seeds meet soil. *Oncogene* 2003, 22:3162-3171

34. **Bogenrieder T**, Rogler, G, Vogt T, Landthaler M, Stolz W. Orofacial granulomatosis as the initial presentation of Crohn's disease in an adolescent. *Dermatology* 2003, 206:273-278

35. **Bogenrieder T**, Landthaler M, Stolz W. Treatment of old world cutaneous Leishmaniasis with intralesionally injected meglumine antimoniate using a Dermojet device. *Dermatology* 2003, 206:269-272

36. **Bogenrieder T**, Herlyn M. Axis of evil: molecular mechanisms of metastasis. *Oncogene* 2003, 22:6524-6536.

37. Li G, Kalabis J, Xu X, Maier F, **Bogenrieder T**, Herlyn M. Reciprocal regulation of MelCAM and AKT in human melanoma. *Oncogene* 2003, 22: 6891-6899.

38. Klebl FH, Gelbmann C, Lammert I, **Bogenrieder T,** Stolz W, Schölmerich J, Schlottmann K. Palpatorische und sonographische Detektion von Lymphknotenmetastasen beim lokal fortgeschrittenen malignen Melanom. *Med Klin* 2003, 98: 783-787.

39. Ackermann S, Thiel F, Beckmann MW, **Bogenrieder T**. Topotecan in cervical cancer. *Int J Gynecol Oncol* 2007, 17: 1215-1223.

40. Koensgen D, Stengel D, Belau A, Klare P, Oskay-Oezcelik G, Steck T, Camara O, Mustea A, Sommer H, Coumbos A, **Bogenrieder T**, Lichtenegger W, Sehouli J, on behalf of the NOGGO Study Group Ovarian Cancer. Topotecan and carboplatin in patients with platinum-sensitive recurrent ovarian cancer: results of a multicenter NOGGO- phase I/II study. *Cancer Chemother Pharmacol* 2008, 62: 393-400.

Book Chapters

Bogenrieder T, Elder DE, Herlyn M. Molecular and Cellular Biology. *In:* CM Balch, AN Houghton, AJ Sober, SJ Soong (eds.), Cutaneous Melanoma, 4th edition, 2003, pp. 713-751

Curriculum vitae

Thomas Bogenrieder werd geboren in Tuttlingen (Baden-Württemberg) op 12 juli 1965. Hij groeide op in Rottweil, alwaar hij in (jaartal) het diploma behaalde aan het Albertus-Magnus-Gymnasium.

In 1985 begon hij met de studie geneeskunde aan de Universität des Saarlands te Homburg/Saar. Na 4 jaar geneeskunde te hebben gestudeerd aan deze Universiteit, startte hij in 1989 met dezelfde studie aan de Université Louis Pasteur, Strasbourg (Frankrijk). Het doctoraal diploma geneeskunde werd behaald in mei 1992 aan de Albert-Ludwigs-Universität te Freiburg im Breisgau.

Daarnaast startte hij in 1993 met zijn eerste laboratorium onderzoek naar melanoma en prostaat kanker bij Dr. Anthony P. Albino en Dr. David M Nanus in het Laboratory of Mammalian Cell Transformation van het Memorial Sloan-Kettering Cancer Center te (New York, NY).

Na het behalen van zijn artsexamen begon hij in juni 1995 met de (voor)opleiding dermatologie aan de Universität van Regensburg (hoofd Prof. dr. Michael Landthaler). In August 2000 besloot hij om bijna drie jaar fulltime onderzoek te gaan doen naar melanomen bij Dr. Meenhard Herlyn in het Wistar Institute te Philadelphia, PA.

Daarna werkte hij van 2003 tot 2004 in een privé dermatologie praktijk, in een deeltijdbaan met zijn echtgenote, na de geboorte van hun tweede kind. Na het behalen van zijn specialisatie Dermatologie ging hij in januari 2005 werken bij GlaxoSmithKline in München, Duitsland. Sinds juli 2008 is hij Director Oncology bij GlaxoSmithKline te Londen.

Thomas is gehuwd met Dr. med. Marion Kuske (eveneens dermatoog) en zij hebben twee zoons, Vincent (geboren in 1998) en Henri (geboren in 2003).